THE UNIVERSITY OF HULL

Enzymology of dicarboxylic acid metabolism in *Corynebacterium* sp. strain 7E1C

being a Thesis submitted for the

Degree of Doctor of Philosophy

in the University of Hull

by

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October 1990

" I should see the garden far better" said Alice to herself, " if I could get to the top of that hill: and there's a path that leads straight to it - at least, no it doesn't do that "....." but I suppose it will at last - but how curiously it twists."

Lewis Caroll
Through the Looking Glass

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Summary of Thesis submitted for PhD degree

by Neil Michael Broadway

on

Enzymology of dicarboxylic acid metabolism in

Corynebacterium sp. strain 7E1C

1. Dicarboxylic acid metabolism was investigated in the alkane-utilizing bacterium *Corynebacterium* 7E1C

2. The best yields of dicarboxylic acid were obtained during growth on dodecane although significant amounts of tetradecanedioic acid were produced during growth on methyl myristate. No dicarboxylic acid was produced during growth on hexadecane, methyl palmitate or 16-hydroxypalmitate.

3. *Corynebacterium* 7E1C possesses constitutive NAD^+-dependent and $NADP^+$-dependent alcohol dehydrogenases active with mono-ol, α,ω-diol and ω-hydroxyfatty acids. Additional $NADP^+$-dependent octanol dehydrogenase activity was detected after growth on alkyl-containing substrates.

4. *Corynebacterium* 7E1C possesses a constitutive acyl-CoA thioesterase active with monocarboxyl-, ω-hydroxymonocarboxyl- and dicarboxyl-CoA esters. Long chain-length acyl-CoAs are the preferred substrates.

5. *Corynebacterium* 7E1C possesses constitutive acyl-CoA synthetase(s) active with monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids.

6. *Corynebacterium* 7E1C possesses a constitutive β-oxidation system. Long chain monocarboxyl-CoA esters and 16-hydroxypalmitoyl-CoA are good β-oxidation substrates whereas short chain monocarboxyl-CoAs, long chain dicarboxyl-CoAs and 12-hydroxylauroyl-CoA are poor β-oxidation substrates.

Significant accumulation of saturated β-oxidation intermediates occurred during the β-oxidation of palmitate by cell-free extracts. When hydroxyacyl-CoA dehydrogenase was inhibited, hexadecenoyl-CoA and 3-hydroxypalmitoyl-CoA accumulated.

7. The specificities of these enzyme systems are consistent with the range of dicarboxylic acids accumulated by this organism.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. F. Mark Dickinson and Professor Colin Ratledge for their invaluable help and advice throughout the course of my PhD studies. Thanks are also due to Dr.M. Bahn and Dr. F. Meussdorfer of Henkel KGaA, Düsseldorf for useful discusions of the project. The Commission of the European Communities and Henkel KGaA, Düsseldorf are thanked for financial support through the CEC Biotechnology Action Program (BAP). Many thanks are also due to Carol Gillyon for drawing all the figures.

I would also like to thank the many people who have helped and encouraged me throughout my time in Hull.

ABBREVIATIONS

Except for those defined below, all abbreviations used in this Thesis are the standard abbreviations accepted by the Biochemical Journal (Policy of the Journal and instructions to authors: Biochem. J. (1985) 225, 1 - 26).

The following non-standard abbreviations are used in this thesis.

ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
CoASH	Reduced coenzyme A
DC_n	α,ω-Dicarboxylic acid with n carbon atoms
DCPIP	2,6-dichlorophenolindophenol
DMSO	Dimethyl sulphoxide
DNAase	Deoxyribonuclease
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
F.P.L.C.	Fast protein liquid chromatography
G.L.C.	Gas liquid chromatography
H.P.L.C.	High pressure liquid chromatography
Lauroyl-CoA	Dodecanoyl-CoA
Myristoyl-CoA	Tetradecanoyl-CoA
Palmitoyl-CoA	Hexadecanoyl-CoA
PEI	Polyethylene imine
PMS	Phenazine methosulphate
TMSH	Trimethylsulphonium hydroxide

1. INTRODUCTION

1.1. GENERAL BACKGROUND

Long-chain α,ω-dicarboxylic acids are important industrial chemicals and are used in the synthesis of perfumes, plasticizers, lubricants, polyurethanes and polyamides (Buhler and Schindler 1984). Long-chain dicarboxylic acids are currently produced industrially by a multi-step chemical synthesis. Hence there is much interest in the commercial exploitation of microbial dicarboxylic acid production from alkanes as an alternative source of these dicarboxylic acids. Over the last 20 years many industrial concerns have isolated and patented dicarboxylic acid-producing yeast and bacteria eg: *Corynebacterium* sp. 27744 to 21747 (du Pont, US Pat.3773 621 (1973)), *Corynebacterium dioxydans* (Hasegawa Co., DE 216 426 (1972)), *Torulopsis bombicola* (Philips Petroleum Co., US Pat. 3 769 621 (1974)), *Candida tropicalis* (Nippon Mining Co. Ltd., US Pat. 4 339 536 (1982))(see Buhler and Schindler (1984) for a comprehensive list of patents). However, despite the great interest shown in microbial dicarboxylic acid production, the biochemistry of microbial dicarboxylic acid production is still poorly understood. Much of the work has concentrated simply on increasing dicarboxylic acid yields by a combination of mutation, to isolate mutants producing increased amounts of dicarboxylic acid, and optimization of reaction conditions. Little or no attention has been paid to the underlying biochemistry. Thus, this is an area ripe for biochemical investigation.

1.2. PHYSIOLOGY OF ALKANE-UTILIZING MICROBES

Since the growth of microbes on hydrocarbons was first reported (Miyoshi 1895) a large literature has been accumulated concerning the physiology and biochemistry of microbial growth on hydrocarbons. This subject has been reviewed in detail by a number of authors (Rehm and Reiff 1981;

Buhler and Schindler 1984; Boulton and Ratledge 1984). Many genera of bacteria, yeast and fungi can utilize alkanes as sole carbon and energy source (see Buhler and Schindler 1984). Most work has been carried-out with microbes grown on C_{10} to C_{18} hydrocarbons, since growth is generally better on these than on the shorter chain-length compounds. This is thought to be a consequence of the toxicity of shorter chain-length hydrocarbons due to their greater solubility in aqueous media (Gill and Ratledge 1973).

The insolubility of alkanes in aqueous media presents special problems for the uptake of these compounds into microbial cells. Three uptake mechanisms have been proposed: (i) direct contact through attachment to large alkane droplets, (ii) direct contact through accomodation of sub-micron "pseudosolubilized" alkane emulsions to the cell surface, (iii) uptake of hydrocarbon in true solution in the aqueous phase.

he solubility of long-chain alkanes in water is very low and so uptake of dissolved alkane is only likely to be significant during growth on shorter chain-length alkanes (Yoshida *et al* 1971; Yoshida and Yamane 1971).

In many alkane-utilizing microbes the production of emulsifying agents is induced by alkanes. Many different types of biosufactant have been described (see Cooper and Zajic 1980). Examples are sophorolipid production by *Torulopsis candida* (Einsele *et al* 1975), the high molecular weight glycolipid (M_r = 980 kDalton) produced by *Acinetobacter calcoaceticus* (Rosenberg *et al* 1979a,b; Zukerberg *et al* 1979; Rubinovitz *et al* 1982) and the suface active agent synthesized by *Corynebacterium hydrocarboclastus* during growth on linear hydrocarbons. This compound contained carbohydrate, lipid and protein (Zajic *et al* 1977a,b).

In addition to the above mentioned reports of biosurfactant production there have been a number of reports of direct adherence of microbial

cells to alkane droplets. Adherence of *Acinetobacter calcoaceticus* RAG-1 to alkane droplets is associated with fimbriae (Rosenberg *et al* 1982). Shearing of these fimbrae by vigorous agitation results in decreased adherence to alkane (Rosenberg 1984). Thus both direct adherence to alkane droplets and emulsification of the alkane appear important in microbial hydrocarbon uptake.

In this connexion it should be noted that intracellular hydrocarbon inclusions have been observed in a number of alkane-grown bacteria. These inclusions have been studied mainly in hexadecane-grown *Acinetobacter calcoaceticus* HO1-N. The hydrocarbon inclusions, which consist mainly of unchanged hexadecane, are bounded by a monolayer membrane (Kennedy and Finnerty 1975; Scott *et al* 1976; Scott and Finnerty 1976b). However, since the menbrame structure is different from the cytoplasmic membrane these authors concluded that these membrane-bound inclusions were not formed by pinocytosis of the cytoplasmic membrane. Similar hydrocarbon inclusions have been reported in hexadecane-grown species of *Pseudomonas*, *Arthrobacter*, *Mycobacterium*, *Nocardia* and *Corynebacterium* (Scott and Finnerty 1976a). Thus it appears that uptake of alkane is associated with intracellular accumulation of the alkane, but the reason for this is unclear.

Alkanes can be degraded via 3 routes: (i) monoterminal oxidation via alcohol and aldehyde to fatty acid which is then degraded via β-oxidation, (ii) diterminal oxidation to form the α,ω-dicarboxylic acid, (iii) subterminal oxidation.

The pathways of monoterminal and diterminal oxidation are shown in Fig. 1.1. and are discussed in sections 1.4., 1.5. and 1.6..

In subterminal oxidation one or more methylene groups are oxidized (via the secondary alcohol) to the ketone. The various subterminal pathways are shown in Fig. 1.2.

Figure 1.1: Pathways of dicarboxylic acid formation from alkanes

[ ] = compound has never been isolated

1 = ω-hydroxylase

2 = alcohol dehydrogenase

3 = aldehyde dehydrogenase

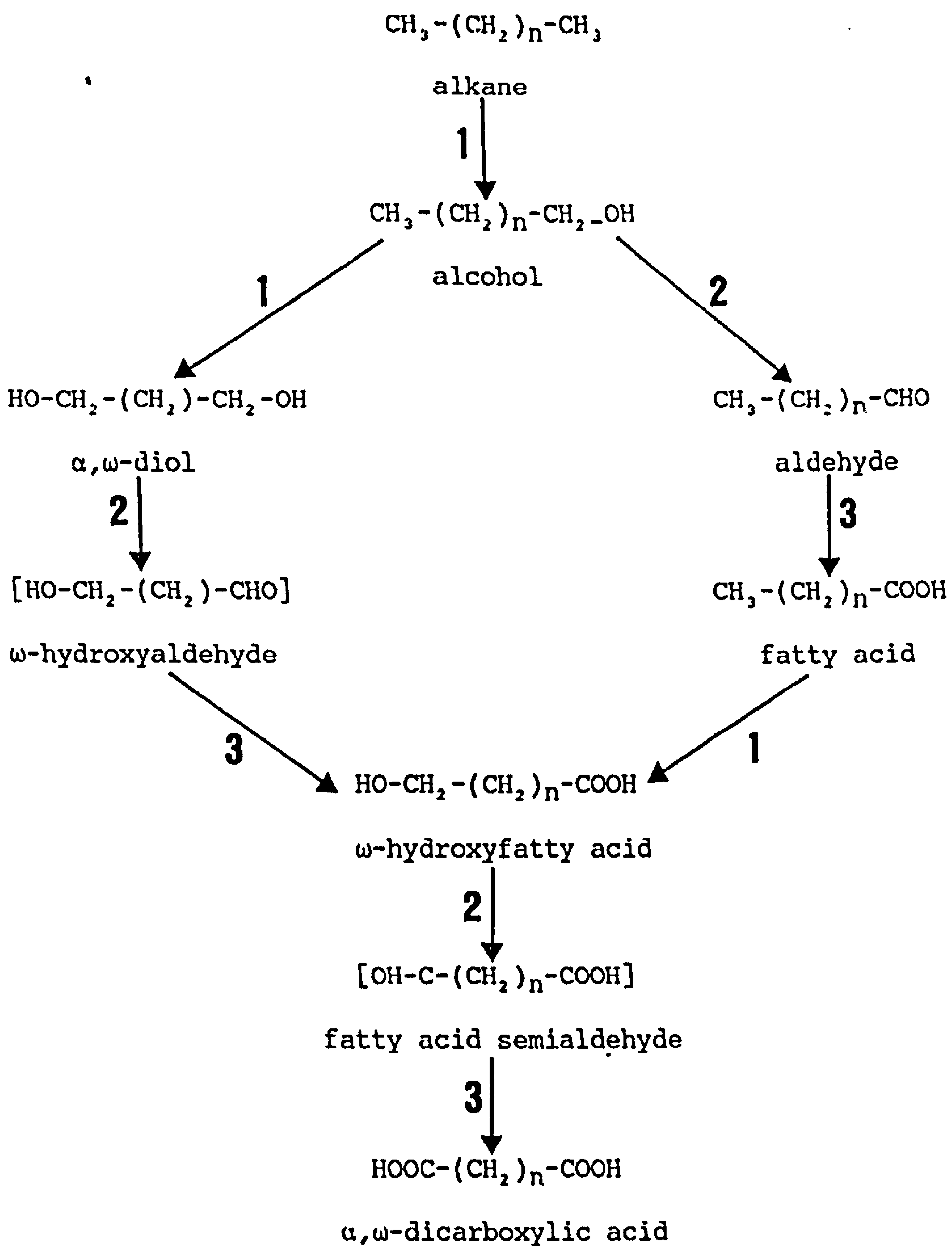

CH3-(CH2)n-CH3
alkane
1
CH3-(CH2)n-CH2-OH
alcohol
1
2
HO-CH2-(CH2)-CH2-OH
α,ω-diol
2
[HO-CH2-(CH2)-CHO]
ω-hydroxyaldehyde
CH3-(CH2)n-CHO
aldehyde
3
CH3-(CH2)n-COOH
fatty acid
3
1
HO-CH2-(CH2)n-COOH
ω-hydroxyfatty acid
2
[OH-C-(CH2)n-COOH]
fatty acid semialdehyde
3
HOOC-(CH2)n-COOH
α,ω-dicarboxylic acid

Figure 1.2: Subterminal pathways of alkane oxidation

After Buhler and Schindler 1984

Figure 1.2: Subterminal pathways of alkane oxidation

After Buhler and Schindler 1984

$H_3C-(CH_2)_n-CH_2-(CH_2)_n-CH_2-(CH_2)_n-CH_3$

$\frac{1}{2}\ O_2$

$H_3C-(CH_2)_n-CH_2-(CH_2)_n-CH(OH)-(CH_2)_n-CH_3$

$\frac{1}{2}\ O_2$ $[H_2]$

$H_3C-(CH_2)_n-CH(OH)-(CH_2)_n-CH(OH)-(CH_2)_n-CH_3$

$H_3C-(CH_2)_n-CH_2-(CH_2)_n-C(=O)-(CH_2)_n-CH_3$

$[H_2]$ $\frac{1}{2}\ O_2$

$H_3C-(CH_2)_n-CH(OH)-(CH_2)_n-C(=O)-(CH_2)_n-CH_3$

$R-C(=O)-O-(CH_2)_n-CH_3$

$[H_2]$ $\frac{1}{2}\ O_2$ H_2O

$H_3C-(CH_2)_n-C(=O)-(CH_2)_n-C(=O)-(CH_2)_n-CH_3$

$R-C(=O)-O-(CH_2)_n-CH_3$

$R-C(=O)-OH$ $HO-(CH_2)_n-CH_3$

Further degradation of acid and alcohol

one Baeyer-Villiger reaction

$\frac{1}{2}\ O_2$

two Baeyer-Villiger reactions

$\frac{2}{2}\ O_2$

H_2O

$R-C(=O)-OH$ $HO-(CH_2)_n-CH_3$

Further degradation

$H_3C-(CH_2)_n-O-C(=O)-(CH_2)_n-C(=O)-(CH_2)_n-CH_3$

Further degradation

$H_3C-(CH_2)_n-O-C(=O)-(CH_2)_n-C(=O)-O-(CH_2)_n-CH_3$

Further degradation

1.3. RANGE OF ORGANISMS PRODUCING DICARBOXYLIC ACIDS

Dicarboxylic acids are produced by a wide range of organisms; bacteria, yeasts, plants and animals. Many strains of bacteria and yeast produce long-chain dicarboxylic acids during growth on alkanes or fatty acids (see Table 1.1). In plants long-chain dicarboxylic acids are components of the protective polymer suberin (Kolattukudy and Agrewal 1974; Kolattukudy *et al* 1975). In mammals C_6 to C_{10} dicarboxylic acids are excreted in the urine. Increased dicarboxylic acid excretion (dicarboxylic aciduria) is observed in cases of impaired mitochondrial β-oxidation caused by defects of the β-oxidation enzymes (Turnbull *et al* 1988; Bhuiyan *et al* 1987), or by hypoglycin poisoning (Veitch *et al* 1987). Increased lipid metabolism as in diabetes or starvation also results in increased dicarboxylic acid excretion (Van Hoof *et al* 1988).

1.4. PATHWAYS OF DICARBOXYLIC ACID METABOLISM

Starting from alkane, dicarboxylic acids can be formed either via the α,ω-diol and/or via the fatty acid (Fig. 1.1.). Thus, microbes growing on alkane (or "resting" cells presented with alkane) can potentially use both pathways to form dicarboxylic acid. In bacteria the pathways operating in dicarboxylic acid formation have not been investigated. However, a strain of *corynebacterium* was shown to accumulate 1,10-decanediol during growth on decane (Bacchin *et al* 1974) indicating that this genus can potentially form dicarboxylic acid via the α,ω-diol pathway. Yi and Rehm (1982a,b,c) demonstrated that in the yeast *candida tropicalis* the formation of DC_{12} and DC_{13}, from dodecane and tridecane respectively, proceeds via both the α,ω-diol and via the fatty acid. It is important to note that it is not known whether, at the level of the fatty acid and the ω-hydroxyfatty acid, if it is the free acid and/or

the acyl-CoA ester that is the substrate for the subsequent reactions. In plants and animals the starting point for dicarboxylic acid formation is the ω-hydroxylation of fatty acid so in these organisms the α,ω-diol pathway will be inoperative.

1.5. PHYSIOLOGY OF DICARBOXYLIC ACID METABOLISM

Although dicarboxylic acid production has been noted in a number of bacteria only in *Corynebacterium* 7E1C has this production been examined in any detail. When grown on decane, dodecane or tetradecane *Corynebacterium* 7E1C produces DC_{10}, DC_{12} and DC_{14} respectively (Kester and Foster 1963). During growth on decane, decanoic acid, 10-hydroxydecanoic acid and DC_{10} were detected; the time-course of appearence being consistent with DC_{10} being produced via the route decane ——> decanoate ——> 10-hydroxydecanoate ——> DC_{10}. During growth on dodecane 3-hydroxydodecanoate was also detected. These workers made no attempt to determine if 1,10-decanediol was produced. In a later paper (Bacchin *et al* 1974) the products of decane oxidation by *Corynebacterium* 7E1C and *Corynebacterium* 269 were compared. No DC production was detected with *Corynebacterium* 269 but, interestingly, significant accummulation of 1,10-decanediol occurred demonstrating diterminal oxidation at the level of the decanol. No diol was detected with *Corynebacterium* 7E1C but significant accummulation of DC_{10} was reported. Most of the DC_{10} was present as the free acid but a significant, though small amount was diterminally esterified to decanol.

The degradation of dicarboxylic acids has been investigated in *Pseudomonas aeruginosa* and *Moraxella lwoffi* 8250 (*Acinetobacter calcoaceticus* 8250)(Chapman and Duggleby 1967). *Pseudomonas aeruginosa* grown on DC_6 oxidized DC_4 to DC_7 without a lag whilst higher homologues were not oxidized although this organism grew on DC_3 to DC_{10} indicating

that the ability to oxidize the longer homologues was induced by growth on them. Similarly, *A. calcoaceticus* grown on DC_5 only oxidized DC_4 and DC_5 without a lag whilst DC_7-grown cells readily oxidized DC_4 to DC_{12}, again indicating induction of long-chain dicarboxylic acid degradative capacity by growth on these compounds. In a recent paper (Modrzakowski and Finnerty 1989) demonstrated that *Acinetobacter calcoaceticus* HO1-N grown on dialkyl ethers (which are degraded via dicarboxylic acids, see Modrzakowski *et al* 1977; Modrzakowski and Finnerty 1980) could oxidize DC_4 to DC_{10} more rapidly than hexadecane-grown cells. These authors also demonstrated that growth on dialkyl ethers induced dicarboxyl-CoA synthetase activity with levels of up to 36.9 nmol/min/mg (DC_8) in cells grown on didecyl ether (which would be degraded via DC_8), as compared to approx. 5 nmol/min/mg in hexadecane-grown cells.

The physiological significance of dicarboxylic acid production is uncertain. However, degradation of dicarboxylic acids results in succinate formation and it has been suggested that this may provide an extra anaplerotic sequence (Chapman and Duggleby 1967; Wada *et al* 1971).

The physiology of dicarboxylic acid production has been investigated in some detail in yeast. The pathways of dicarboxylic acid formation were elucidated using the yeast *Candida tropicalis* (Yi and Rehm 1982a,b,c). Using both "resting" cell suspensions and growing cells these authors demonstrated the production of dicarboxylic acid from alkane, alcohol, α,ω-diol, fatty acid and ω-hydroxyfatty acid. Their results demonstrated the precursor-product relationship between the various intermediates. Yi and Rehm (1988) also demonstrated the production of the unsaturated dicarboxylic acid *cis*-Δ^9-octadecenedioic acid from oleate by resting cells of *Candida tropicalis*.

Due to the industrial interest in microbial dicarboxylic acid production many studies have concentrated on the isolation of mutants producing dicarboxylic acids in high yield. These mutants are selected on the

basis of their inability to grow on alkane and/or dicarboxylic acid and are presumably blocked in β-oxidation (Xiuzhen 1988; Hill *et al* I1986; Shiio and Uchio 1971; Uchio and Shiio 1972a,b,c). In a classic series of papers Uchio and Shiio (Shiio and Uchio 1971; Uchio and Shiio 1971a,b,c,) investigated the production of long-chain dicarboxylic acids by *Candida cloacae*. They initially selected a strain of *C. cloacae* on the basis of its ability to produce significant amounts of DCS12S from dodecane (Shiio and Uchio 1971). Using this organism various factors (eg: pH, rate of aeration, temperature, media composition) affecting dicarboxylic acid production were investigated. From the wild-type yeast a mutant (M-1) (produced by N-methyl-N-nitro-N'-nitrosoguanidine mutagenesis) was isolated that was unable to assimilate DC_{10} and produced dicarboxylic acids in increased yield compared to the wild-type; using resting cells the wild-type produced 0.9g/l DC_{12} whilst M-1 produced 4.9g/l DC_{12} from dodecane (Uchio and Shiio 1972a). Interestingly, the range of dicarboxylic acids produced by the wild-type and by M-1 were markedly different. With the wild-type the best yields of dicarboxylic acid (DC_{12}) were obtained fron dodecane whilst with M-1 the best yields of dicarboxylic acid (DC_{16}, 29.3g/l which corresponds to a 60% yield) were obtained from hexadecane. From M-1 another mutant (MR-12)was isolated that was unable to grow on alkane or dicarboxylic acid and which produced increased amounts of dicarboxylic acid (up to 42.7 g/l DC_{16} by resting cells)(Uchio and Shiio 1972b). Comparison of the rate of degradation of dicarboxylic acids by resting cells demonstrated that the rate of degradation increased with increasing chain-length. The relative rate of degradation of DC_{16} by the wild-type, M-1 and MR-12 were 100%, 40% and 10% respectively (Uchio and Shiio 1972b). Unfortunately, these workers did not investigate the enzymology of the various mutants and so the factors resulting in the change of chain-length specificity of dicarboxylic acid production remain unknown.

However, it seems that the rate of degradation of the dicarboxylic acid is important in determining both the amount and chain-length-specificity of accummulation. Mutant MR-12 was unable to grow on alkane so the production of dicarboxylic acid by cells grown on acetate, in he presence of hexadecane, was investigated and up to 61.5g/l DC_{16} was produced under optimal conditions (Uchio and Shiio 1972c).

here is only one study where investigations of DC production by whole cells has been accompanied by enzymological investigations. Blasig *et al* (1988) looked at the products obtained when hexadecane was incubated with membrane preparations from the yeast *Candida maltosa*. The main products detected were those of the monoterminal pathway ie: 1-hexadecanol and palmitic acid. Small amounts of intermediates (1,16-hexadecanediol, 16-hydroxypalmitate) and the product of the diterminal pathway (DC_{16}) were also detected. The diterminal oxidation products corresponded to approx. 10% of the total oxidation products, indicating that diterminal oxidation is a minor pathway in this organism. This correlated well with the whole cell studies which also indicated diterminal oxidation to be a minor pathway (Blasig *et al* 1984).

The degradation of dicarboxylic acids has also been investigated in rats. Rats supplied with DC_{12} intravenously excreted DC_6, DC_8 and DC_{10} in the urine. Clofibrate treatment decreased dicarboxylic acid excretion (4.4% of the initial dose excreted) whilst riboflavin deficiency increased dicarboxylic acid excretion (75.6% of initial dose excreted) as compared to control rats which excreted 28.6% of the initial dose (Van Hoof *et al* 1988; Veitch *et al* 1988). These authors concluded that long-chain dicarboxylic acids are degraded both in the mitochondria and in the peroxisomes, the chain-shortened dicarboxylates in the urine deriving from incomplete peroxisomal β-oxidation. This is in agreement with the observation that isolated mitochondrial and peroxisomal

fractions can both degrade long-chain dicarboxylic acids, the peroxisomal degradation being incomplete (Kolvraa and Gregarson 1986).

1.6. ENZYMOLOGY OF ALKANE, MONOCARBOXYLIC ACID AND DICARBOXYLIC ACID METABOLISM

The various enzyme systems involved in the scheme of dicarboxylic acid metabolism depicted in Fig. 1.1 are discussed below.

1.6.1. ω-HYDROXYLASE

Three mechanisms have been proposed for the initial oxidation of alkanes: (1) hydroxylation by a mixed function oxidase, often referred to as a ω-hydroxylase, that catalyses the incorporation of molecular oxygen onto the alkane to yield the corresponding n-alkanol; (2) dehydrogenation by a NAD^+-dependent alkane dehydrogenase to give the 1-alkene which is susequently hydrated to yield an alcohol; (3) direct reaction with oxygen to form the corresponding n-alkyl hydroperoxide.
Evidence for the formation of the alkyl hydroperoxide is indirect. *Acinetobacter calcoaceticus* HO1-N oxidizes n-alkyl hydroperoxides and these compounds have been isolated from hexadecane-grown cells of this organism (Finnerty *et al* 1962). Boyer *et al* (1971) demonstrated that the rubredoxin component of the *Pseudomonas oleovorans* ω-hydroxylase catalysed the reduction of alkyl-hydroperoxides to the corresponding n-alcohol.
Although it was initially thought that the yeast *candida tropicalis* oxidized alkanes via an alkane dehydrogenase (Lebault *et al* 1970a) it has subsequently been shown that this was an artifact caused by a contaminant in the alkane; alkane oxidation being catalysed by a cytochrome P-450 dependent ω-hydroxylase (Lebault *et al* 1971; Gallo *et*

al 1973). It seems that oxidation by a hydroxylase to the corresponding n-alcohol is the most likely mechanism by which microbes catalyse the initial attack on alkanes.

Two types of hydroxylase system have been described. In one the electron carrier is cytochrome P-450 (see Ortiz de Montellano 1986) whilst in the other the non-haem iron protein rubredoxin (Peterson *et al* 1966; Peterson *et al* 1967) acts as electron carrier. Both cyt. P-450- and rubredoxin-dependent hydroxylase systems have been reported in bacteria. *Corynebacterium* 7E1C posseses a cytochrome P-450-dependent hydroxylase system (Cardini and Jurtshuk 1968,1970; Jurtshuk and Cardini 1971) which uses NADH as electron donor. Crude extracts of octane-grown cells catalysed the oxidation of octane to octanol and octanoic acid. Interestingly, 8-hydroxyoctanoate or DC_8 were detected indicating that this organism is unable to diterminally oxidise short-chain alkanes. *Pseudomonas oleovorans* posseses a rubredoxin-dependent hydroxylase system capable of ω-hydroxylating octanoate, decanoate and laurate; both the ω-hydroxyfatty acid and the dicarboxylic acid being formed by crude extracts (Kusunose *et al* 1964a,b; Peterson *et al* 1966, 1967; Peterson and Coon 1968). There are both cytochrome P-450 and rubredoxin producing strains of *Acinetobacter calcoaceticus* (Asperger *et al* 1981; Kleber *et al* 1983). Although alkane hydroxylation by a cell-free system has only been demonstrated in *A. calcoaceticus* 69-V (Aurich *et al* 1977), a rubredoxin containing strain, the induction of cytochrome P-450 in other strains correlates extremely well with exposure to alkane and is presumed to be involved in the hydroxylation of alkane (Asperger *et al* 1984). All yeast ω-hydroxylase systems that have been investigated appear to be cytochrome P-450-dependent, eg: *Sacharomycopsis lipolytica* (Marchal *et al* 1982; *Lodddcromyces elongiosporus* (Honck *et al* 1982); *Endomycopsis lipolytica* (Delaisse and Nyns 1974); *Candida guilliermondia* (Schunk *et al* 1978).

Cytochrome P-450 hydroxylase systems catalysing the ω-hydroxylation of fatty acids have also been reported in rat liver microsomes (Robbins 1961; Preiss and Bloch 1964; Robbins 1968; Bjorkhem and Danielson 1970a,b).

1.6.2. ALCOHOL DEHYDROGENASE/OXIDASE

Long-chain alcohol dehydrogenases have been reported in a number of bacteria. Two types of enzyme have been reported, $NAD(P)^+$-dependent and $NAD(P)^+$-independent. The latter enzymes are assayed by following the reduction of a suitable dye such as DCPIP.

Prior to this investigation there had been no investigation of the alcohol dehydrogenases of *Corynebacterium* 7E1C, although when investigating the ω-hydroxylation of octane by a cell-free extract of *Corynebacterium* 7E1C it was found that the reaction products were octanol and octanoic acid thus demonstrating the presence of NAD^+-dependent octanol and octanal dehydrogenases (Cardini and Jurtshuk 1968, 1970). *Pseudomonas aeruginosa* (strain 196) posseses 2 NAD^+-dependent and 2 $NADP^+$-dependent alcohol dehydrogenases and a $NAD(P)^+$-independent alcohol dehydrogenase (Tassin and Vandecasteele 1972; Tassin *et al* 1973; Vandecasteele 1983). The $NAD(P)^+$-dependent enzymes are constitutive and soluble whereas the $NAD(P)^+$-independent enzyme is particulate and is induced by growth on hexadecane and, to a lesser extent, by heptane or 1,10-decanediol. The dye-linked enzyme is optimally active with long-chain alcohols and is presumably the enzyme involved in alkanol dissimilation. The constitutive enzymes were active with both mono-ols and α,ω-diols. In accord with these results van der Linden (1967) and van der Linden and Huybregtse (1969) reported a constitutive $NADP^+$-dependent and 2 $NAD(P)^+$-independent alcohol dehydrogenases in *Pseudomonas aeruginosa* (strain 473). The

$NADP^+$-dependent enzyme was active with α,ω-diols (C_6 optimal) and mono-ols (C_5 optimal) although the authors considered this activity not to be important in α,ω-diol dissimilation. The $NAD(P)^+$-independent enzyme was active with both mono-ols and α,ω-diols. Interestingly this enzyme was poorly active with 8-hydroxyoctanoate but displayed good activity (approx. 10-fold greater) with the corresponding methyl ester. Soluble, constitutive NAD^+-dependent alcohol dehydrogenase has also been reported in *Pseudomonas aerugiosa* sol 20 (Azoulay and Heydeman 1963). Cell-free extracts of *Pseudomonas oleovorans* possess $NADP^+$-dependent ω-hydroxyfatty acid dehydrogenase activity (Kusunose *et al* 1964. *Pseudomonas putida* posseses both a soluble, constitutve, chromasomally encoded NAD^+-dependent alcohol dehydrogenase and a particulate, inducible, $NAD(P)^+$-independent plasmid encoded alcohol dehydrogenase (Grund *et al* 1975; Benson and Shapiro 1976; Shapiro *et al* 1979). Multiple alcohol dehydrogenses have also been reported in various strains of *Acinetobacter calcoaceticus*. *A. calcoaceticus* 8250 posssesses an alkane-inducible NADR+R-dependent primary alcohol dehydrogenase (Fewson 1966). Similarly, *A. calcoaceticus* HO1-N posseses a hexadecane- and hexadecanol-inducible NAD^+-dependent hexadecanol dehydrogenase which appears to have both particulate and soluble locations (Singer and Finnerty 1985b). *A. calcoaceticus* also possesses a constitutive $NADP^+$-dependent alcohol dehydrogenase with broad chain-length specificity (Singer and Finnerty 1985b; Fixter and Nagi 1984; Jirausch *et al* 1986). Jirausch *et al* (1986) also reported a $NAD(P)^+$-independent alcohol dehydrogenase in this organism. Beardmore-Gray and Anthony (1983), investigating 4 strains of *A. calcoaceticus* detected an ethanol-induced NAD^+-dependent ethanol dehydrogenase and a constitutive NAD^+-dependent enzyme active with longer chain alcohols (C_8 was the longest tested).

Alcohol dehydrogenase activity has been investigated in a number of

alkane-utilizing yeasts and moulds. However, it has recently been demonstrated (Kemp *et al* 1988; G.D. Kemp personal communication) that all yeasts and moulds tested possesed a long chain alcohol oxidase rather than a dehydrogenase as previously thought. Thus although earlier papers describe alcohol dehydrogenases they were, it would seem, unwittingly assaying an oxidase as a dehydrogenase due to the presence of a NAD^+-dependent aldehyde dehydrogenase. The enzyme fron *Candida tropicalis* is active with mono-ols, α,ω-diols and ω-hydroxyfatty acids (Lebault *et al* 1971a,b; Kemp *et al* 1988; Kemp 1988). Interestingly, the the yeast *Candida* (*Torulopsis*) *bombicola* which produces ω-hydroxyfatty acids (as components of sophorolipids) possesses an alcohol oxidase active with mono-ols and α,ω-diols but not with ω-hydroxyfaty acids (Hommel and Ratledge 1990). The yeast alcohol oxidases are induced by growth on alkane (Kemp *et al* 1988; Hommel and Ratledge 1990).

ω-Hydroxyfatty acid dehydrogenase has been investigated in some detail in animals. A medium-chain specific ω-hydroxyfatty acid dehydrogenase has been partially purified from hog liver (Mitz and Heinrikson 1961) and from rabbit liver (Kamei *et al* 1964). These enzymes were inactive with long-chain (C_{18}) substrates which are oxidized by alcohol (ethanol) dehydrogenase (Bjorkhem 1972b) and by a microsomal alcohol dehydrogenase (Bjorkhem 1972a).

The best studied ω-hydroxyfatty acid dehydrogenase is the wound-induced enzyme of potato tuber. This enzyme has been purified and shown to be distinct from ethanol dehydrogenase. The enzyme is $NADP^+$-dependent and is optimally active with 16-hydroxypalmitate and is involved in the formation of the long-chain dicarboxylic acids that are components of the protective polymer suberin (Agrewal and Koluttukudy 1978a,b; Kolattukudy and Agrewal 1978).

1.6.3. ALDEHYDE DEHYDROGENASE

Long-chain specific aldehyde dehydrogenases have been investigated in a number of alkane-utilizing bacteria. *Pseudomonas aeruginosa* sol 20 possesses $NAD(P)^+$–dependent heptanal dehydrogenase activity induced by growth on higher alcohols, aldehydes, fatty acids or alkanes (Heydeman and Azoulay 1963). In the same paper they reported, in a different strain of *P. aeruginosa,* a particulate NAD^+–dependent dodecanal dehydrogenase.

Psedomonas aeruginosa 196 (Vandecasteele *et al* 1983) possesses 3 aldehyde dehydrogenases; 2 NAD^+–dependent and 1 $NADP^+$–dependent. The $NADP^+$–dependent and one of the NAD^+–dependent enzymes are both constitutive and soluble. The other NAD^+–dependent enzyme is particulate and is induced 18-fold and 50-fold by growth on heptane and hexadecane respectively (as compared to succinate-grown cells). Multiple aldehyde dehydrogenases have also beeen reported in *Acinetobacter calcoaceticus* HO1-N (Singer and Finnerty 1985a). This organism possesses a membrane-bound $NADP^+$–dependent aldehyde dehydrogenase inducible by growth on hexadecane, dodecanal or hexadecanol and a constitutive NAD^+–dependendent enzyme.

Although aldehyde dehydrogenase activity towards ω-oxofatty acids (a prerequisite for dicarboxylic acid formation) has not been investigated in bacteria the formation of DC_{12} from laurate by cell-free extracts of *Pseudomonas oleovorans* (Kusunose *et al* 1964a) demonstrates that cell-free extracts do posses such an activity.

NAD^+–dependent aldehyde dehydrogenase activity has been reported in a number of alkane-utilizing yeasts; *Candida tropicalis* (Lebault *et al* 1970a,b,c), *Candida intermedia* (Lui and Johnson 1971), *Candida famata* (Il'chenko and Tsfasman 1987). The formation of DC_{16} from hexadecane by membrane preparations of *Candida maltosa* (Blasig *et al* 1988) implies the

presence of an aldehyde dehydrogenase active with ω-oxopalmitate although the presence of such an activity has not been investigated in its own right. Mammalian (rat liver) microsomes have also been shown to posses z-oxofatty acid dehydrogenase (NAD^+-dependent), involved in the formation of dicarboxylic acids (Bjorkhem 1972a). Agrewal and Koluttukudy (1978a) demonstrated the presence of a constitutive NAD^+-dependent ω-oxopalmitate dehydrogenase in potato tubers; a tissue which contains DC_{16} as a component of the protective polymer suberin.

1.6.4. ACYL-CoA SYNTHETASE

Acyl-CoA synthetase occupies a central role in fatty acid metabolism; this enzyme provides the acyl-CoA substrates for degradation (β-oxidation) and for incorporation into complex lipids (via sn-glycerol-3-phosphate acyltransferase). Additionally, acyl-CoA synthetase appears to be involved in fatty acid uptake in the bacterium *E. coli* (Klein *et al* 1971).

Acyl-CoA synthetase has been investigated in a number of bacteria, of which *E. coli* has beeen studied in most detail. In *E. coli* acyl-CoA synthetase is induced by growth on long-chain fatty acids (Overath *et al* 1969; Weeks *et al* 1969; Samuel and Ailhaud 1969). Although acyl-CoA synthetase is induced by fatty acids with at least 12 carbon atoms the enzyme is active with fatty acids of chain-length C_6 or C_8 up to C_{18} (Overath *et al* 1969; Samuel *et al* 1970; Klein *et al* 1971; Kameda and Nunn 1981). The low levels of activity observed during growth on acetate or amino acids are repressed by glucose. This catabolite repression is not overcome by the addition of long-chain fatty acid to the growth medium (Weeks *et al* 1969; Klein *et al* 1971). This catabolite repression is, however, overcome by the addition of cAMP (Pauli *et al* 1974). The acyl-CoA synthetase is part of the *fad* regulon which also encodes the

genes for the β-oxidation enzymes (see Nunn 1986). The regulon is under the control of the *fad* R gene product, a diffusible repressor protein (Overath *et al* 1969; Simmons *et al* 1980a,b; Di Russo and Nunn 1985). The number of acyl-CoA synthetases present in *E. coli* is uncertain. On the basis of genetic evidence Overath *et al* (1969) proposed the presence of 1 enzyme with broad substrate specificity. Samuel *et al* (1970) demonstrated a complex of a medium and a long-chain specific enzyme. In contrast Kameda and Nunn (1981) purified (350-fold) an acyl-CoA synthetase active with C_8 to C_{18} fatty acids, although the ratio of specific activities with decanoate and oleate changed from 2 to 5 during the course of the purification which may indicate the presence of a second enzyme lost during the purification.

In contrast to the highly inducible acyl-CoA synthetase of *E. coli* the acyl-CoA synthetase activity of a number of other bacteria is less dependent on growth substrate. The Gram negative bacterium *Caulobacter crescentus* posses high constitutive levels of acyl-CoA synthetase activity in succinate-grown cells that are induced further by long-chain fatty acid. Only mild catabolite repression (2-3-fold) by glucose is observed and is abolished by cAMP (O'Connoll *et al* 1986). The Gram positive bacterium *Nocardia asteroides* possesses a constitutive acyl-CoA synthetase active with C_8 to C_{22} fatty acids. The specific activity of this enzyme varied no more than 2-fold between cells grown on palmitate, amino acids or glucose (Calmes and Deal 1973). *Bacillus megaterium* posseses a constitutive long-chain acyl-CoA synthetase (Lennarz 1963; Massaro and Lennarz 1965). Trust and Millis (1971) demonstrated the activation of C_2 to C_{19} monocarboxylic acids by palmitate-grown *Pseudomonas* strains. From one strain they purified an acetate kinase, a medium-chain specific acyl-CoA synthetase and a long-chain specific acyl-CoA synthetase. Crude extracts of *Mycobacterium tuberculosis avium* display acyl-CoA synthetase activity with DC_8, DC_9 and DC_{10} (longer

dicarboxylic acids were not tested)(Kimura and Sasakawa 1956). Modrzakowski and Finnerty (1990) reported dicarboxyl-CoA synthetase in *Acinetobacter calcoaceticus* HO1-N active with C_3 to C_{10} dicarboxylic acids. Low constitutive activities (5 nmol/min/mg) were detected in hexadecane-grown cells. In cells grown on didecyl ether (which is degraded via DC_8) activities of up to 36.8 nmol/min/mg were observed (= 7.4-fold induction).

The best studied microbial acyl-CoA synthetases are those of the alkane-utilizing yeast *Candida lipolytica* which possesses 2 long-chain specific acyl-CoA synthetases. Acyl-CoA synthetase I is constitutive, being present in both glucose-grown and oleate-grown cells, and is found in microsomes, peroxisomes and mitochondria (Mishina *et al* 1978b; Hosaka *et al* 1981). Acyl-CoA synthetase II is induced by growth on oleate and is restricted to the peroxisomal fraction (Mishina *et al* 1978b; Hosaka *et al* 1981). Acyl-CoA synthetase I which provides acyl-CoA exclusively for incorporation into cellular lipid (Kamiryo *et al* 1977) is active only with monocarboxylic acids (Hosaka *et al* 1979) whereas acyl-CoA synthetase II, which provides acyl-CoA exclusively for β-oxidation in the peroxisomes, is active with monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids (Mishina *et al* 1978a). The wild-type organism incorporates exogenous fatty acids directly into cellular lipids whereas mutants lacking acyl-CoA synthetase I, in effect, synthesize fatty acids *de novo* from acetyl-CoA from the β-oxidation of exogenous fatty acid (Kamiyro *et al* 1977). A dicarboxylic acid-producing strain of *Candida tropicalis* has been shown to posses a constitutive dicarboxyl-CoA synthetase active with C_4 to C_{16} dicarboxylic acids. The specific activity of dicarboxyl-CoA synthetase was approx. 2-fold lower in a mutant producing dicarboxylic acid in increased yield (Zhihua and Xiuzhen 1986). Shimizu *et al* (1980) found that microbes could be divided into 2 groups on the basis of induction of acyl-CoA synthetase. One

group displayed acyl-CoA synthetase activity only after growth on palmitate whilst the other group displayed similar specific activity whether grown on glucose or palmitate.

Mammalian (rat) liver posses a microsomal dicarboxyl-CoA synthetase (Vamecq *et al* 1985) optimally active with DC_{12}. Rat liver also possesses long-chain acyl-CoA synthetase located in microsomes, peroxisomes and mitochondria. The purified microsomal and mitochondrial enzymes are active with monocarboxylic acids and 16-hydroxypalmitate but not with dicarboxylic acids (Tanaka *et al* 1979) indicating that the microsomal dicarboxyl-CoA synthetase is a separate enzyme. Antibodies raised against the purified microsomal enzyme cross-react, indicating identity, with the peroxisomal and mitochondrial enzymes (Miyazawa *et al* 1985). Using a linked assay (ie: acyl-CoA generated *in situ*) Vamecq and Draye (1987) demonstrated that rat rat liver homogenates posses acyl-CoA oxidase activity towards monocarboxylates (C_8 to C_{18}), ω-hydroxymonocarboxylates (C_{10}, C_{12} and C_{16}) and dicarboxylates (C_6 to C_{16}), thus demonstrating good acyl-CoA synthetase activity with all these substrates. Rat liver mitochondria also posses a GTP-dependent long-chain acyl-CoA synthetase (Galzigna *et al* 1967).

1.6.5. β-OXIDATION

Bacterial β-oxidation has been investigated in a limited number of organisms.

The best studied bacterial β-oxidation system is that of *E. coli*. In this organism the β-oxidation enzymes are induced by growth on long-chain fatty acids (Overath *et al* 1967, 1969; Weeks *et al* 1969; Samuel and Ailhaud 1969). The induction of the β-oxidation enzymes is subject to catabolite repression by glucose (Overath *et al* 1967; Weeks *et al* 1969). The genes encoding the β-oxidation enzymes and acyl-CoA

synthetase form a regulon (the *fad* regulon) which is under the negative control of the *fad* R gene product, a diffusible repressor protein (Overath *et al* 1969; Simons *et al* 1980a,b; Di Russo and Nunn 1985). Catabolite repression by glucose is partially overcome by cAMP. Mutants lacking a functional cAMP receptor protein cannot be induced to express the β-oxidation enzymes (Pauli *et al* 1974). As well as being highly regulated the β-oxidation enzymes of *E. coli* also display a high degree of structural organization. Apart from acyl-CoA dehydrogenase the β-oxidation enzymes of *E. coli* are part of a multienzyme complex (Binstock and Schulz 1977; O'Brian and Frereman 1977; Pramanik *et al* 1979a,b; Pawar and Schulz 1981; Pramanik and Schulz 1983; Yang and Schulz 1983; Spratt *et al* 1984). The structural organization of the β-oxidation enzymes from a number of sources are discussed in section 1.6.5.1. Both the regulatory and structural organization of β-oxidation are rather different in the Gram-negative bacterium *Caulobacter crescentus*. In this bacterium the expression of the β-oxidation enzymes is subject to catabolite represion by glucose although the difference in specific activity between glucose-grown and oleate-grown cells is at most 3-fold (O'Connell *et al* 1986). β-Oxidation has also been demonstrated in a number of other bacteria; *Leptospira* (Hennebery and Cox 1970), *Mycobacterium* (Goldman and Geldbard 1959), *Pseudomonas* (Kunau *et al* 1988) and *Yersinia pestis* (Moncla *et al* 1983).

β-Oxidation is inducible in yeast and filamentous fungi. Kawamoto *et al* (1978) first reported a peroxisomal β-oxidation system in n-alkane-grown *Candida tropicalis* and subsequently peroxisomal β-oxidation systems have been reported in *Candida lipolytica* (Yamada *et al* 1980) and *Saccharomyces cerevisiae* (Veenhuis *et al* 1987). In all these peroxisomal β-oxidation systems the first reaction is catalysed by an H_2O_2-generating acyl-CoA oxidase. In the filamentous fungi *Neurospora crassa* (Kionka and Kunau 1985) and *Aspergillus tamri* (Kunau *et al* 1988)

the β-oxidation system is located in catalase-free microbodies and the first reaction is catalysed by an acyl-CoA dehydrogenase.

Animals possess both mitochondrial and peroxisomal β-oxidation systems. The chain-shortening of fatty acids by multiples of 2 carbons was first observed by Knoop at the beginning of this century in experiments that involved feeding ω-phenylfatty acids to dogs and humans and examiming the chain-shortened ω-phenylfatty acids excreted in the urine (see Greville and Tubbs 1968) and the enzymology first investigated in the 1950s (see Greville and Tubbs 1968). Peroxisomal β-oxidation was discovered in 1976 (Lazarow and de Duve 1976). There are a number of differences between the mitochondrial and peroxisomal systems. In mitochondria the various enzyme activities are all located on separate proteins (see Kunau *et al* 1988) and the first reaction is catalysed by a series of acyl-CoA dehydrogenases with different chain-length specificities (Frereman 1988). Peroxisomes have a bifunctional protein possesing both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity (Hashimoto 1982; Reddy *et al* 1987) and the first reaction is catalysed by an H_2O_2-generating acyl-CoA oxidase (Lazarow and de Duve 1976; Lazarow 1978; Osumi and Hashimoto 1978; Inestostra *et al* 1979; Hyrb and Hogg 1979). Mitochondrial β-oxidation appears to go to completion whereas in peroxisomes no more than 4-5 cycles of β-oxidation of palmitate have been observed (Lazarow 1978; Osmundsen 1982b) although the presence of all the saturated acyl-CoA intermediates from C_{16} down to C_2 have recently been demonstrated (Bartlett *et al* 1990) demonstrating that peroxisomes can in fact completely b-oxidize fatty acids. Entry acyl-CoA esters into peroxisomes was thought to be carnitine-independent (Lazarow and de Duve 1976; Osumi and Hashimoto 1978; Hyrb and Hogg 1979), although it now appears that *in vitro* peroxisomal β-oxidation is carnitine-independent. The apparent carnitine-independence due to isolated peroxisomes being "leaky"

(Buechler and Lowenstein 1990). It appears that intact peroxisomes are impermeable to acyl-CoA esters and that *in vivo* acyl-groups enter the peroxisomes as the corresponding acyl-carnitine (Buechler and Lowenstein 1990). Mitochondrial b-oxidation is carnitine-dependent (eg: Norum 1964; West *et al* 1971; Declerq *et al* 1987). Entry of acyl-CoA esters into the mitichondria is catalysed by carnitine palmitoyl transferase I (CPT I) and acyl-carnitine translocase (Pande 1975), the acyl-CoA being reformed within the mitochondrion by CPT II. It is not known for certain whether CPT I and CPT II are the same or different proteins (Declerq *et al* 1987). CPT I is inhibited by malonyl-CoA (the product of the first step of *de novo* fatty acid synthesis (McGarry *et al* 1978) and this may be important in regulating the rate of β-oxidation *in vivo.*

β-Oxidation has been demonstrated in *Tetrahymena* (Blum 1973), *Euglena* (Graves and Becker 1974) and a number of plants. Plants possess both peroxisomal (glyoxysomal) (Cooper and Beevers 1969; Hutton and Stumpf 1969; Masterson *et al* 1990) and mitochondrial (Wood *et al* 1986; Masterson *et al* 1990) β-oxidation systems.

It was originally thought that unsaturated fatty acids were β-oxidized via a pathway invovlving *cis* Δ^3 *trans* Δ^2 enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase (Stoffel and Caesar 1965). However, with the discovery of the enzyme 2,4-dienoyl-CoA reductase (Kunau and Dommes 1978) an alterntive pathway for the β-oxidation of unsaturated fatty acids has been proposed (see Schulz and Kunau 1987). 2,4-Dienoyl-CoA reductase activity is essential for the β-oxidation of fatty acids with a double bond on an even numbered carbon atom (Schulz and Kunau 1987; Osmundsen and Hovik 1988).

Fatty acid oxidation has recently been extensively reviewed (see Tanaka and Coates 1990).

1.6.5.1. STRUCTURAL ORGANIZATION OF β-OXIDATION

The structural organization of the β-oxidation enzymes have been investigated in a number of organisms. The mitochondrial β-oxidation enzymes are all located on separate polypeptides (Greville and Tubbs 1968; Kunau *et al* 1988). In mammalian peroxisomes enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase are located on the same multifunctional protein (Hashimoto 1982; Reddy *et al* 1987) which also possesses enoyl-CoA isomerase activity (Palosaari and Hiltunen 1990). The peroxisomes of *Saccharomyces cerevisiae*, *Candida tropicalis*, *C. lipolytica*, *Neurospora crassa* and *Aspergillus tamarii* possess a trifunctional protein with enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase activity (Kunau *et al* 1988). The *E. coli* β-oxidation complex consists of a 78kDalton protein (the α subunit, encoded by the *fad* B gene), which possesses medium chain enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase and enoyl-CoA isomerase activities, and a 42kDalton protein (the β subunit, encoded by the *fad* A gene) possesing 3-ketothiolase activity (Yang and Schulz 1983; Spratt *et al* 1984). The complex has an $\alpha_2\beta_2$ quaternary structure with a native molecular weight of approx. 260kDaltons (Pawar and Schulz 1981) although O'Brian and Frerman (1977) reported higher molecular weight complexes which appeared to be multiples of the $\alpha_2\beta_2$ structure. Kunau *et al* (1988) reported β-oxidation complexes consisting of 2 subunits and of similar molecular weight to that of *E. coli* in *Acinetobacter calcoaceticus* and in 5 strains of *Pseudomonas*. Recently it was reported that the β-oxidation activities of the bacterium *Caulobacter crescentus* are each located on a separate protein (O'Connell *et al* 1990); a situation analogous to that of the mammalian mitochondrial enzymes. Thus it is uncertain how typical of other bacteria is the high degree of structural organization

exhibited by the *E. coli* β-oxidation enzymes.

1.6.5.2. INTERMEDIATES OF β-OXIDATION

It was long thought that the β-oxidation of fatty acids proceeds without the formation of intermediates and that once a fatty acid entered the β-oxidation spiral it was completely degraded to acyl-CoA without release from the β-oxidation enzymes (see Garland and Tubbs 1968). Garland *et al* (1965) found that in intact mitochondria oxidizing palmitate virtually all the intramitochondrial CoA could be accounted for as acetyl-CoA, long-chain acyl-C-A or as free CoASH indicating the absence of, short-chain acyl-CoA intermediates. In view of the lack of intermediates it was suggested that the β-oxidation enzymes are organized in the form of a multienzyme complex (Garland and Tubbs 1968). Stewart *et al* (1973), when investigating the β-oxidation of octanoate by aqueous extracts of acetone-dried mitochondria detected (by paper chromatography of the acyl-hydroxamates) 3-hydroxyoctanoate, hexanoate, butyrate and acetate from [8 ^{14}C]octanoate. The amount of 3-hydroxyoctanoate was increased when NADH dehydrogenase was inhibited by rotenone. These authors demonstrated the preferential oxidation of the carboxyl- end of octanoate which indicated that β-oxidation proceeded via free intermediates. When these workers investigated the β-oxidation of octanoate by intact mitochondria they found far lower levels of intermediates. Stanley and Tubbs (1974, 1975) detected (by radio-GLC) C_{14} down to C_8 saturated intermediates during the β-oxidation of palmitate by intact mitochondria. 3-Hydroxy- intermediates were only detected in the presence of rotenone. It was demonstrated (Stanley and Tubbs 1974) that the intermediates they found were not on the main kinetic pathway and proposed a "leaky hosepipe" model of β-oxidation in which the intermediates represented leakage. Similar results were

obtained by Watmough *et al* (1989) who only detected myristoyl-CoA and traces of lauroyl-CoA during the β-oxidation of palmitate. The lack of intermediates is consistent with some form of structural organization of the β-oxidation enzymes. In this connexion it should be noted that an association of some β-oxidation enzymes with the inner mitochondrial membrane has been demonstrated (Sumegi and Srere 1984).

ecently, Bartlett *et al* (1990) demonstrated the presence of all the saturated acyl-CoA intermediates from C_{16} down to C_2 during the peroxisomal β-oxidation of palmitate. Thus the behaviour of the peroxisomal β-oxidation system is rather different to the mitochondrial system.

1.6.6. THIOESTERASE

Acyl-CoA thioesterases catalyse the hydrolysis of acyl-CoA esters to the corresponding fatty acid plus free CoASH. Acyl-CoA thioesterases are of seemingly ubiquitous distibution being found in bacteria (Barnes and Wakil 1968; Barnes *et al* 1970; Boyce and Lueking 1984; Seay and Lueking 1986), plants (Murphy *et al* 1985), and various animal tissues (with a variety of subcellular locations)(eg: Berge *et al* 1981; 1984; 1985; Osmundsen *et al* 1980; Cheesborough and Kolattakudy 1985). The function of thioesterases catalysing the hydrolysis of acyl-CoA esters are of uncertain physiological function, although their wide distribution indicates an important function in lipid metabolism.

The most studied bacterial thioesterases are those of *E. coli*. This organism posseses two thioesterases both of which have been purified. Thioesterase I is specific for long-chain (C_{10} to C_{18}) acyl-CoA esters. Good activity is observed with unsaturated acyl-CoA esters (Barnes and Wakil 1968; Barnes 1975). Thioesterase II has a broader substrate specificity than thioesterase I, being active with C_6 to C acyl-CoAs

and with 3-hydroxyacyl-CoAs (the D isomer being a better substrate than a DL racemate)(Barnes *et al* 1970; Barnes 1975). Bonner and Bloch (1972) purified an acyl-CoA thioesterase from *E. coli* which they termed "fatty acyl thioesterase I" which appears to be the same as thioesterase II using the nomenclature of Barnes *et al* (1970). Both thioesterases show very little activity with acyl-acyl-carrier protein (acyl-ACP), enzymically synthesized acyl-ACP being a poorer substrate than chemically synthesized acyl-ACP (Spencer *et al* 1978). Samuel and Ailhaud (1969) found that pamitoyl-CoA thioesterase activity in *E.coli* is induced by growth on oleate (specific activity up to 43 nmol/min/mg protein) although significant activity was observed in glucose-grown cells (6 nmol/min/mg protein). Thioesterases I and II were purified from non-induced cells so it is not known which (or both) thioesterase is induced by fatty acid. The induction by oleate is consistent with a role in controlling intracellular acyl-CoA concentrations as originally suggested by Barnes and Wakil (1968). Narasimheen *et al* (1986) isolated an *E. coli* mutant lacking (less than 10% of the wild-type activity) thioesterase II. No mutant phenotype could be found when grown on non-fatty acid-containing media; growth rates and phospholipid acylation were the same for both mutant and wild-type organism. Thus the function of this enzyme remains obscure. Unfortunately growth on fatty acid (ie: under inducing conditions) was not examined; such experiments may shed more light on the function of this enzyme. Interestingly, two acyl-CoA thioesterases have been purified from the photosynthetic bacterium *Rhodopseudomonas shaeroides* that are remarkably similar to the *E. coli* enzymes. Thioesterase I has a narrow substrate specificity (C_{12} to C_{18})(Boyce and Lueking 1984). Thioesterase II has a broad substrate specificity being active with C_4 to C_{18} acyl-CoA esters (Seay and Lueking 1986). Boyce and Lueking (1984) suggested that in *R. sphaeroides* thioesterase may be involved in the incorporation of exogenous fatty

acid into phospholipid. In this bacterium *sn*-glycerol-3-phosphate acyltransferase has an obligate requirement for acyl-ACP as acyl-donor (Lueking and Goldfine 1975; Cooper and Lueking 1984). These authors assumed that exogenous fatty acids were transported into the cell as their acyl-CoA esters which were then hydrolsed to the free fatty acid and then converted to acyl-ACP by acyl-ACP synthetase (Rock and Cronan 1979). Such a function for thioesterase is only conjecture.

1.6.7. AIMS OF RESEARCH PROJECT

The aims of this project were to investigate bacterial dicarboxylic acid production. To date studies of microbial dicarboxylic acid production (which have concentrated on yeasts rather than bacteria) have consisted almost exclusively of physiological studies with intact cells. However, if microbial dicarboxylic acid production is to be fully exploited commercially a thorough understanding of the biochemistry of the process is essential. To date microbial strains producing dicarboxylic acids in increased yield have been produced by random mutagenesis. If a more strategic approach to strain improvement is to be adopted (ie: the use of recombinant DNA technologies) then the enzymology of dicarboxylic acid metabolism needs to be understood in order that the appropriate enzymes can be targeted. It is important to know, for example, which enzyme systems are responsible for the range of dicarboxylic acids produced.

Therefore it was decided to investigate the physiology and the enzymology of bacterial dicarboxylic acid metabolism with the following aims:

1. Selection of a suitable dicarboxylic acid-accumulating bacterium to use as a model.

2. Determination of the range of dicarboxylic acids produced.

3. To investigate a number of enzyme systems involved in dicarboxylic acid metabolism and to see if the specifities of these enzymes could be correlated with the range of dicarboxylic acids produced.

2. METHODS

2.1. BACTERIA AND MAINTENANCE

2.1.1. BACTERIA

The following bacteria were obtained from the departmental culture collection:

Acinetobacter calcoaceticus RAG-1 (ATCC 14 987)

Acinetobacter calcoaceticus HO1-N (ATCC 31 012)

Corynebacterium sp. strain 7E1C (ATCC 19 067)

Corynebacterium dioxydans (ATCC 21 766)

The following bacteria were obtained directly from the American Type Culture Collection (ATCC), Rockville , Maryland , U.S.A.:

Corynebacterium sp. (ATCC 21 744)

Corynebacterium sp. (ATCC 21 745)

Corynebacterium sp. (ATCC 21 746)

Corynebacterium sp. (ATCC 21 747)

2.1.2. MAINTENANCE OF BACTERIA

Acinetobacter calcoaceticus RAG-1 , *Acinetobacter calcoaceticus* HO1-N , *Corynebacterium dioxydans* and *Corynebacterium* sp. strain 7E1C were maintained on nutrient agar slopes (see 2.2.1.) at 4°C after completion of growth at 30°C. *Corynebacterium* sp. (ATCC 21744 to 21747) were maintained on brain heart infusion slopes (see 2.2.2.) after completion of growth at 4°C.

All bacteria were subcultured at approx. 6 week intervals.

2.2. MEDIA

2.2.1 NUTRIENT BROTH

Nutrient broth (13 g/l) was dissolved in distilled water. For solid media agar (15 g/l) was added.

2.2.2. BRAIN HEART INFUSION

Brain heart infusion (37 g/l) was dissolved in distilled water. For solid media agar (15 g/l) was added.

2.2.3. MODIFIED JAYASURIYA (JAYS) MEDIUM

The medium was essentially the same as that described by Jayasuriya (1955) except that the phosphate buffer concentration was doubled. The modified medium contained per litre of distilled water: KH_2PO_4 2.72g, Na_2PO_4 4.26g, $(NH_4)_2SO_4$ 0.5g, $MgSO_4 \cdot 7H_2O$ 0.2g, $CaCl_2 \cdot 2H_2O$ 0.01g, $MnSO_4 \cdot 4H_2O$ 0.0025g, $Na_2MoO_4 \cdot 2H_2O$ 0.0025g, $FeSO_4 \cdot 7H_2O$ 0.005g, yeast extract 1.0g.

2.2.4. MEDIA STERILIZATION

All media were sterilized by autoclaving at 121°C for 15 minutes.

2.3. GROWTH OF BACTERIA

2.3.1. MONITORING OF GROWTH

Growth on nutrient broth, brain heart infusion and Jays/succinate medium

was followed by measuring the apparent absorbance at 600 nm of a suitably diluted sample.

It was not possible to follow growth in this way with *Corynebacterium* sp. strain 7E1C growing on alkanes due to extensive clumping of the cells.

It was also not possible to measure growth on hexadecanol or dodecanediol in this manner due to the solid substrate forming a particulate suspension.

In these cases growth was measured as described by Hug and Fiechter (1973). An aliquot (10 ml) of the culture was aseptically removed and mixed with an equal volume of methanol/butanol/chloroform (10:10:1 v/v) in a tared centrifuge tube. After 5 minutes the sample was centrifuged (48 000 g, 10 min., 20°C) to pellet the solvent-washed cells. The supernatant was decanted and the pellet resuspended in a volume of didtilled water equal to that of the original culture aliquot. The sample was then centrifuged as above, the supernatant decanted and the pellet dried to constant weight at 55°C *in vacuo* over P_2O_5.

2.3.2. GROWTH OF BACTERIA

A loopful of bacteria were transferred from a slope to nutrient broth (100ml) in a 250ml conical flask and grown for 24-36 hrs. on a rotary shaker (160 r.p.m.) at 30°C. An aliquot (10ml) of bacteria were then transferred to Jays medium (800ml), supplemented with the appropriate carbon source, in a 1 litre vortex aerated bottle. The medium was stirred at a rate such that the vortex extended to the bottom of the bottle. Cells were grown at 30°C and harvested during mid to late exponential growth.

The following carbon-sources were used: hexadecane (0.2% w/v), hexadecanol (0.2% w/v), methyl palmitate (0.2% w/v), dodecanediol (0.2%

w/v), sodium succinate (1% w/v), glucose (1% w/v)

2.4. PREPARATION OF CELL-FREE EXTRACTS

Cells were harvested by centrifugation at 25 000g for 15 mins. at 4°C. Cells were washed 3 times in potassium phosphate buffer (100mM, pH 7.5) by resuspension and centrifugation. The final pellet was resuspended in the minimum volume of Hepes buffer (20mM, pH 7.5). Cells were disrupted by 3 passages through a French pressure cell (35 MPa). After the first passage DNAase 1 (approx. 2 mg) was added to the extract which was highly viscous due to the presence of DNA. Whole cells and cell debris were removed by centrifugation at 8 000g for 15 mins. at 4°C. The supernatant fraction was designated 'crude cell-free extract' and was either used immediately or stored in small aliquots (1.5ml) in liquid N_2 until required.

2.5. DICARBOXYLIC ACID PRODUCTION

2.5.1. SCREENING FOR DICARBOXYLIC ACID-PRODUCING BACTERIA

A loop of bacteria was transferred from a slope to the corresponding liquid medium (ie: nutrient broth or brain heart infusion)(100ml) in a 250ml conical flask and incubated on a rotary shaker (160 r.p.m.) at 30°C for 24 hrs.. An aliquot (1ml) was transferred to Jays/dodecane (100ml, 2% w/v dodecane) in a 250ml conical flask. The cultures were incubated on a rotary shaker (160 r.p.m.) at 30°C for 120 hrs. then any dicarboxylic acids present were extracted (see 2.5.3..).

2.5.2. DICARBOXYLIC ACID PRODUCTION FROM DIFFERENT CARBON-SOURCES

Nutrient broth or brain heart infusion cultures were prepared as described in 2.5.1.. An aliquot (1ml) was transferred to Jays medium (100ml) in a 250ml conical flask supplemented with the appropriate carbon-source (2% w/v). The cultures were then incubated for 120 hrs. as above.

2.5.3 EXTRACTION OF DICARBOXYLIC ACIDS

Entire cultures (100ml) were acidified to pH 1-2 with conc. H_2SO_4. An internal standard (DC_{13}; 10mg) was added, then the culture was extracted twice with an equal volume of diethyl ether. The ether layers were combined and washed with distilled water (0.1 vol.). The washed ether layer was evaporated to dryness in a rotary evaporator at 30°C. The residue was dried *in vacuo* over P_2O_5 at 55°C. The dried residue was dissolved in methanol (5ml) then methylated and analysed by G.L.C. (see 2.8.2. and 2.8.3.)

2.6. WHOLE CELL EXPERIMENTS

2.6.1. DEGRADATION OF DICARBOXYLIC ACIDS BY WASHED-CELL SUSPENSIONS

Cells were grown and harvested as described in 2.3.2. and 2.4. respectively except that sterile buffer and centrifuge tubes were used through out. Whole-cell suspension (5ml; approx. 9mg dry weight/ml) was aseptically transferred to sterile potassium phosphate buffer (100mM, pH 7.0; 40ml) in a 100ml conical flask containing dicarboxylic acid (80mg). The flasks were incubated on a rotary shaker (160 r.p.m.) for 18-120 hrs.. At the end of the incubation period the suspensions were acidified

to pH 1-2 with conc. H_2SO_4. A dicarboxylic acid internal standard (10mg) was added prior to ether extraction as described in 2.5.3.. The folllowing internal standards were used. For cultures incubated with DC_{16} the internal standard was DC_{13}. For shorter chain-length dicarboxylic acids the internal standard was the dicarboxylic acid 2 carbons longer than the test dicarboxylic acid.

2.7. ENZYME ASSAYS

All assays were performed at 25°C.

2.7.1. ALCOHOL DEHYDROGENASE

2.7.1.1. $NAD(P)^+$-dependent assay

Activity was assayed by following $NAD(P)^+$ reduction fluorimetrically in a fluorimeter constructed as described by Dalziel (1962).
The standard assay contained in a final volume of 4ml: potassium phosphate buffer pH 8.0 (3.1mmol), $NAD(P)^+$ (1μmol), cell-free extract. The reaction was initiated by substrate addition. The substrate was either dodecanediol (0.27μmol), octan-1-ol (1.3μmol) or 12-hydroxylaurate (1.3μmol) introduced into the assay in 20μl of DMSO. When investing the kinetic parameters of alcohol dehydrogenase activity the volume of DMSO used to introduce the various amounts of substrate was kept constant at 20μl.

2.7.1.2. DCPIP/PMS ASSAY

This assay was exactly the same as the $NAD(P)^+$-dependent assay except that the $NAD(P)^+$ was replaced by phenazine methosulphate (PMS, 0.1mM)

and 2,6-dichlorophenolindophenol (DCPIP, 0.05mM). The decrease in absorbance at 600nm was followed. An extinction coefficient of 21 850 $l.mol^{-1}.cm^{-1}$ at pH 8 was used.

2.7.2. ALDEHYDE DEHYDROGENASE

Activity was assayed by following $NAD(P)^+$ reduction fluorimetrically. The assay contained in a final volume of 4ml: either potassium phosphate buffer pH 8.0 (3.1μmol) or glycine-NaOH buffer pH 9.5 (0.2μmol), $NAD(P)^+$ (1μmol), cell-free extract. The reaction was initiated by addition of the substrate, dodecanal (0.1μmol), introduced into the assay in 20μl of DMSO.

When investigating the kinetic parameters of aldehyde dehydrogenase activity the volume of DMSO used to introduce the substrate was kept constant at 20μl.

2.7.3. ACYL-CoA SYNTHETASE

Activity was assayed by a modification of the methods of Webster (1969) and Vamecq *et al* (1985) by following the disappearance of free CoASH, on incorporation into acyl-CoA esters. The free CoASH was reacted with DTNB.

The assay contained in a final volume of 190μl: Li_3CoASH (120nmol), Na_2ATP (750nmol), $MgCl_2 \cdot 6H_2O$ (750nmol), potassium phosphate buffer pH 8.0 (24μmol), fatty acid substrate (450nmol, introduced into the assay in 6μl of DMSO). The reaction was initiated by the addition of cell-free extract. The reaction was terminated by the addition of trichloroacetic acid (60μl, 15% w/v). Protein was precipitated by centrifugation (5 mins.) in a MSE Microcentaur Eppendorf centrifuge. An aliquot of supernatant (200μl) was removed and added to Tris-HCl buffer pH8.0, 0.8M

(0.8ml) containing DTNB (250nmol). The absorbance at 412nm was measured against an aliquot from a blank reaction in which the fatty acid substrate was omitted. A molar extinction coefficient for DTNB of 13 600 $l.\ mol^{-1}.cm.^{-1}$ was used.

2.7.4. β-OXIDATION

B-oxidation was assayed by following NAD^+ reduction by the 3-hydroxyacyl-CoA dehydrogenase or by measuring acetyl-CoA production essentially as described by Kawamoto *et al* (1978).

2.7.4.1. LINKED (FREE FATTY ACID-DEPENDENT) β-OXIDATION ASSAY

In this assay the acyl-CoA esters were generated *in situ* by the acyl-CoA synthetases present in the extract. The assay contained in a final volume of 0.9ml: potassium phosphate buffer pH 8.0 (240µmol), Na_2ATP (3.6µmol), $MgCl_2 \cdot 6H_2O$ (3.6µmol), NAD^+ (1.7µmol), CoASH (0.6mmol) cell-free extract and fatty acid substrate (0.68µmol, introduced into the assay in 7µl of DMSO). The reaction was initiated by substrate addition. Activity was routinely measured by following NAD^+ reduction at 340nm (ε_{340} = 6220 $l.mol.^{-1}cm.^{-1}$). Activity could also be measured by following acetyl-CoA prodution (see 2.7.4.3.).

2.7.4.2. DIRECT (ACYL-CoA DEPENDENT) β-OXIDATION ASSAY

The assay contined in a final volume of 0.9ml: potassium phosphate buffer pH 8.0 (240µmol), NAD^+ (1.7µmol), Li_3CoASH (72nmol), cell-free extract and acyl-CoA ester (72nmol, introduced into the assay as an aqueous solution). The reaction was initiated by acyl-CoA addition. Ativity was routinely measured by following NAD^+ reduction at 340nm.

Activity could also be measured by following acetyl-CoA production (see 2.7.4.3.).

2.7.4.3. MEASUREMENT OF ACETYL-CoA PRODUCTION IN β-OXIDATION ASSAYS

Acetyl-CoA was assayed with citrate synthase in the presence of oxaloacetate. CoASH released on condensation of acetyl-CoA with oxaloacetate was measured by reaction with DTNB.

An aliquot of reaction mixture (200µl) was mixed with trichloroacetic acid (60µl, 15% w/v) and protein precipitated by centrifugation (5 mins.) in a MSE Microcentaur Eppendorf centrifuge. An aliquot of the centrifuge supernatant (200µl) was added to Tris-HCl buffer pH 8.0, 0.8M (0.8ml) containing oxaloacetate (approx. 2mg solid) and DTNB (250nmol). The absorbance at 412nm (A_1) was read against a reagent blank. Citrate synthase (Pigeon breast muscle, 1 unit) was added and the increased absorbance at 412nm (A_2) due to release of CoASH from acetyl-CoA measured.

In the linked β-oxidation assay the total acyl-CoA formation could be determined by measuring total CoASH consumption.

$$\text{ie: } A_{1\,t_0} - A_{1\,t_x} \equiv \text{total acyl-CoA at } t=x$$

$$A_{2\,t_x} - A_{1\,t_x} \equiv \text{acetyl-CoA at } t=x$$

'long-chain' acyl-CoA = total acyl-CoA − acetyl-CoA

Note: 'long-chain' refers to acyl-CoA other than acetyl-CoA

2.7.5. THIOESTERASE (ACYL-CoA HYDROLASE)

2.7.5.1 CONTINUOUS THIOESTERASE ASSAY

Thioesterase activity was assayed essentially as described by Barnes

(1975). Release of CoASH was measured continuously by its reaction with DTNB.

The assay contained in a final volume of 0.9ml: potassium phosphate buffer pH 8.0 (240μmol), DTNB (250nmol), cell-free extract and acyl-CoA ester (72nmol). The reaction was initiated by substrate addition. Control assays in which cell-free extract was omitted were run to correct for any non-enzymic alkaline hydrolysis of the acyl-CoA substrate (negligible at pH 8.0). Activity was measured by following the increase in absorbance at 412nm (ε_{412} = 13 600 $l.^{-1}mol.^{-1}cm.^{-1}$).

2.7.5.2 DISCONTINUOUS THIOESTERASE ASSAY

When investigating the effect of CoASH on thioesterase activity it was not possible to use the continuous assay due to the reaction of CoASH with DTNB. Therefore a discontinuous assay was used in which the increase in free CoASH, after a fixed incubation period, was measured by reaction with DTNB. The reaction mixture was identical to that of the continuous assay except that DTNB was omitted. The reaction was initiated by substrate addition. After a set incubation period (such that a linear increase in absorbance with respect to time was observed) an aliquot (200μl) was removed and added to trichloroacetic acid (60μl, 15% w/v) and protein removed by centrifugation for 5 min. in a MSE Microcentaur Eppendorf centrifuge. An aliquot of supernatant (200μl) was added to 0.8ml Tris-HCl buffer (0.8M, pH 8.0) containing DTNB (250nmol) and the increase in absorbance at 412nm measured against a sample from a control reaction lacking acyl-CoA substrate. To quantify the increase in free CoASH a molar extinction coefficient of 13 600 $l.mol.^{-1}cm.^{-1}$ was used.

2.8. ANALYTICAL PROCEDURES

2.8.1. PROTEIN DETERMINATION

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.8.2. METHYLATION OF CARBOXYLIC ACIDS

Fatty acids and dicarboxylic acids were methylated with trimethylsulphonium hydroxide (TMSH)(Butte 1983) which methylates both free and esterified carboxylic acids.

2.8.3. G.L.C. OF DICARBOXYLIC ACIDS

A Pye Unicam PU4500 Chromatograph fitted with a flame ionization detector was used in conjunction with a Pye Unicam CDP4 computing integrator. The instrument was fitted with a glass column (1.75m x 4mm) packed with 10% diethylene glycol succinate (DEGS) on Celite 100-120 mesh. The column was maintained at 200°C with injection and detector ports at 220°C. The carrier gas was N_2 supplied at approx. 35ml/min.. Dicarboxylic acids were methylated with TMSH prior to injection of 1µl aliquots onto the column. Dicarboxylic acids were identified by comparison of retention times with those of authentic dicarboxylic acids. Dicarboxylic acids were quantified by comparison of integrated peak area with that of an internal standard (DC13 unless stated otherwise).

2.8.4. H.P.L.C. OF INTACT ACYL-CoA ESTERS

2.8.4.1. H.P.L.C. PROTOCOL

H.P.L.C. was performed essentially as described by Causey and Bartlett (1986) with minor modifications.

A Kontron 420 HPLC pump and a Kontron 425 HPLC gradient former were used with a Lichrosorb 10μ RP C18 column held at 30°C. Column eluates were detected by measuring absorbance at 260nm. Peak heights and peak areas were measured by a Kontron I-459 integrator. The following gradient of acetonitrile in KH_2PO_4 (50mM, pH 5.3) was used: isocratic 5% (v/v) acetonitrile (for 5 min.), linear to 40% (v/v) acetonitrile (7.5 min.), linear to 50% (v/v) acetonitrile (5 min.). The flow rate was 1ml/min.. The mobile phases were continuously sparged with helium.

2.8.4.2. EXTRACTION OF ACYL-CoA ESTERS FROM REACTION MIXTURES

Intact acyl-CoA esters were extracted from β-oxidation reaction mixtures essentially as described by Causey and Bartlett (1986) with minor modifications. An aliquot (0.5ml) from a β-oxidation assay was quenched by the addition of glacial acetic acid (100μl). An acyl-CoA easter internal standard (50nmol) was added and the reaction mixture extracted 3 times with a total of 15ml of diethyl ether to remove fatty acids. To the aqueous phase was added saturated ammonium sulphate (50 μl). Methanol/chloroform (2:1 v/v, 3ml) was added slowly and the mixture left for 20 mins. at room temperature. The salt-protein precipitate was sedimented by centrifugation (1000g, 5 mins.) and the supernatant removed. The salt-protein pellet was extracted with a further 3ml of chloroform/methanol (2:1 v/v) and the supernatants combined. The solvent was removed by evaporation under a stream of N at 55°C. The residue was

redissolved in 200µl of KH_2PO_4 (50 mM, pH 5.3) and an aliquot (20 µl) analysed by H.P.L.C.

2.8.5. ULTRACENTRIFUGATION

to determine the subcellular location of enzyme activities crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C. The supernatant was then carefully removed with a Pasteur pipette. The pellet was resuspended in the minimum volume of ice-cold Hepes buffer (20mM, pH 7.5). Fractions were assayed immediately for the appropriate enzyme activity.

2.9. SYNTHESIS OF ACYL-CoA ESTERS

2.9.1 CHEMICAL SYNTHESIS OF ω-HYDROXYFATTY ACYL- AND DICARBOXYL-CoA ESTERS

The acyl-CoA esters of DC_{10}, DC_{12}, DC_{14}, DC_{16}, 12-hydroxylaurate and 16-hydroxypalmitate were synthesized via the acyl-imidazole as described by Kawaguchi *et al* (1981). Fatty acid (5µmol) tetrahydrofuran (0.1ml) was added to N,N'-carbonylimidazole (6µmol) dissolved in tetrahydrofuran (0.1ml). After reaction for 30 minutes the solvent was removed under a N_2 stream and the residue redissolved in tetrahydrofuran/water (2:1 v/v, 0.2ml). To this was added CoASH (5µmol) dissolved in tetrahydrofuran/water (2:1 v/v, 0.5ml). The pH was adjusted to 7.0 - 7.5 with 1M NaOH and the reaction with CoASH alowed to proceed for 4 hours at room temperature under a N_2 atmosphere. The tetrahydrofuran was then remobved under a N_2 stream and the residual aqueous solution acidified to pH 3 - 4 by adding Dowex 50 (H^+). The solution was pipetted from the Dowex beads and then extracted with diethyl ether (3 x 0.5ml) to remove

free faty acids. The aqueous layer was then freeze-dried and stored at -15C.

2.9.2. ENZYMIC SYNTHESIS OF *trans*-Δ^2-ENOYL-CoA AND 3-HYDROXYACYL-CoA ESTERS

trans-Δ^2-enoyl-CoA esters were synthesized from the corresponding saturated acyl-CoA esters using acyl-CoA oxidase from *Candida* sp. (Sigma).

The reaction mixture (1 ml) contained: potassium -phosphate buffer (100 µmol), ABTS (0.8 µmol), horseradish peroxidase (0.1 unit), acyl-CoA oxidase (0.1 unit) and acyl-CoA ester (80 nmol). The reaction was initiated by additinon of the acyl-CoA ester. The reaction-course was followed by measuring the increase in absorbance at 405nm (ε_{412} = 36 800 $l.mol.^{-1}cm.^{-1}$). When an absorbance increase of 1.4 (≡ 40nmol acyl-CoA oxidized) was obtained the reaction was quenched by the addition of glacial acetic acid (200µl). The acyl-CoA esters (saturated and *trans* Δ^2 unsaturated) were then extracted as described in 2.8.4.2..

3-Hydroxyacyl-CoA esters were synthesised as described above except that crotonase (0.1 units)(Sigma) was added to the reaction mixture. All other procedures were identical.

2.9.3. ASSAY OF ACYL-CoA CONCENTRATION

2.9.3.1. ALKALINE HYDROLYSIS

The acyl-CoA-containing sample (approx. 20 nmol acyl-CoA) was mixed with an equal volume of 0.1M NaOH and heated at 90°C for 2 minutes. A known volume was then added to Tris-HCl buffer (0.8M, pH 8.0) containing DTNB (250nmol) and the increase in absorbance at 412nm measured against an

unhydrolysed sample.

2.9.3.2. HYDROXYLAMINE CLEAVAGE

The acyl-CoA-containing sample (approx. 20nmol acyl-CoA) was added to 1ml Tris-HCl buffer (0.8M, pH 8.0) containing DTNB (250nmol). Neutral hydroxylamine (50µl, 0.5M) was added and the increase in absorbance at 412nm measured against a control lacking acyl-CoA.
Acyl-CoA was quantified using a molar extinction coefficient of 13 600 $l.mol.^{-1}cm.^{-1}$ for the reaction of CoASH with DTNB.

2.9.4. PURIFICATION OF CHEMICALLY SYNTHESIZED ACYL-CoA ESTERS

Chemically synthesized acyl-CoA esters were purified by H.P.L.C. on a Lichrosorb 10µ RP C18 column (250 x 4.6 mm) as described in 2.8.4.1..
The acyl-CoA sample (up to 2 µmol) was loaded onto the column and the acyl-CoA-containing fractions collected. The acyl-CoA containing eluate fractions were evaporated to dryness under a stream of N_2 at 55°C. The residue contained acyl-CoA and phosphate buffer salts. The buffer salts were removed by extracting the acyl-CoA into methanol/chloroform. The residue was mixed with 6 ml of methanol/chloroform (2:1 v/v) and left to stand for 20 mins.at room temperature. The salt precipitate was sedimented by centrifugation (1000g, 5 mins.). The supernatant was removed and the pellet extracted with another 6 ml of methanol/chloroform (2:1 v/v). The supernatants were combined and evaporated to dryness under a N_2 stream at 55°C. The residue was resuspended in distilled water.

2.10. PROTEIN PURIFICATION

2.10.1. ACYL-CoA SYNTHETASE

Acyl-CoA synthetase activity was purified using an LKB F.P.L.C. system. Crude cell-free extract was centrifuged at 100 000g for 90min. at 4°C and the supernatant used for subsequent FPLC work. Extract (typically 1.5ml, 4mg/ml) was loaded onto a polyethylene imine FPLC column (Bio-Rad)(6 cm * 1.5 cm) which had beeen equilibrated with Hepes buffer (20mM, pH 7.5) and unbound protein eluted with the same buffer. Bound protein was eluted with a linear gradient of 0 to 1M KCl in 15ml of Hepes buffer (20mM, pH 7.5). The flow rate was 1.5ml/min. and 0.75ml fractions were collected.

2.10.2.1. ALCOHOL DEHYDROGENASE

Crude cell-free extract was centrifuged at 100 000g for 90min. at 4°C. The supernatant (typically 5ml, 4mg/ml) was mixed with an equal volume of acetone/water (95:5 v/v) which had been cooled to -15°C. The mixture was centrifuged at 48 000g for 10min. at -10°C. The supernatant was decanted and the protein pellet resuspended in 2.5ml of ice-cold Hepes buffer (20mM, pH 7.5). The extract thus obtained was then subjected to PEI-FPLC as described in 2.10.1..

MATERIALS

Citrate synthase (Pigeon breast muscle), NADase (*Neurospora crassa*), DNAase I, Peroxidase (Type I from Horseradish), Acyl-CoA oxidase (*Candida* sp.), Crotonase (bovine liver), ATP (Type II), GTP (Type III), CTP (Type III), UTP (Type III), ITP (Type III), N,N'-carbonyldiimidazole, Coenzyme A (Li salt from yeast) and all acyl-CoA esters (apart from dicarboxyl- and ω-hydroxymonocarboxyl-CoAs) were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K.. NAD^+ (Grade II) and NADH (Grade II) were from Boehringer Mannheim UK, Lewes, East Sussex. $NADP^+$ was from Park Scientific Ltd., Northampton, U.K.. All alkanes, alcohols, aldehydes, α,ω-diols, fatty acids, fatty acid methyl esters (except methyl palmitate) ω-hydroxyfatty acids and dicarboxylic acids were obtained from Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.. Methyl palmitate was obtained from Lancaster Synthesis Ltd., Morecambe, Lancs., U.K.. Dimethyl sulphoxide was obtained from Fisons Scientific Apparatus, Loughborough, Leics, U.K.. Nutrient broth was obtained from Oxoid Ltd., Basingstoke, Hants, U.K.. Yeast extract (Lab M) was obtained from London Analytical and Bacteriological Media Ltd., Salford, U.K.. All other chemicals were of the highest grade commercially available.

3. DICARBOXYLIC ACID PRODUCTION AND WHOLE-CELL EXPERIMENTS

3.1. SELECTION OF A DICARBOXYLIC ACID-PRODUCING BACTERIUM

Bacteria of the genus *Corynebacterium* are known producers of dicarboxylic acids (Kester and Foster 1963; du Pont 1973, 1974; Bacchin *et al* 1974) so bacteria of this genus seemed likely candidates for the purposes of this study. *Acinetobacter calcoaceticus* strains are well documented alkane-utilizers (eg: Makula and Finnerty 1968; Kleber *et al* 1983; Singer and Finnerty 1985 a,b; Jirausch *et al* 1986) and although there have apparently been no investigations of their ability to produce dicarboxylic acids during growth on alkanes they have been shown to degrade dialkylethers via dicarboxylic acids (Modrzakowski *et al* 1977; Modrzakowski and Finnerty 1980; Modrzakowski and Finnerty 1989). Therefore *Acinetobacter calcoaceticus* also seemed a good organism to test for dicarboxylic acid production from alkanes.

Bacteria were tested for their ability to produce DC_{12} during growth on dodecane. To test for DC production cells were grown on dodecane (20g/l) for 5 days, after which the entire culture was acidified and extracted into diethyl ether. The culture extract was analysed for DC content by GLC (see Methods). The DC accumulation by the various bacteria are shown in Table 3.1. The best DC producers all belong to the genus *Corynebacterium* although small but significant quantities of DC_{12} were produced by *Acinetobacter calcoaceticus* HO1-N. With all the bacteria, except *A.calcoaceticus* RAG-1 the main DC accumulated had the same carbon chain-length as the parent alkane ie: 12 carbons. *Corynebacterium* 21744 to 21747 are mutants derived from *Corynebacterium* 7E1C by U.V. and chemical mutagenesis and reported to produce increased yields of dicarboxylic acid as compared to the wild-type organism, *Corynebacterium* 7E1C (du Pont 1973, 1974). The results reported here are in agreement with this as evidenced by the 2 to 3 fold increase in the levels of

Table 3.1: Dicarboxylic acid production by a number of strains of *Acinetobacter* and *Corynebacterium*

Bacterium	DC produced (mg/l)			
	DC_6	DC_8	DC_{10}	DC_{12}
Acinetobacter calcoaceticus HO1-N	12	13	43	50
Acinetobacter calcoaceticus RAG-1	28	17	9	9
Corynebacterium dioxydans	n.d.	10	21	30
Corynebacterium 7E1C	n.d.	13	19	301
Corynebacterium 21744	n.d.	7	11	587
Corynebacterium 21745	n.d.	47	58	955
Corynebacterium 21746	n.d.	34	39	614
Corynebacterium 21747	n.d.	2	6	840

Bacteria were grown on dodecane (20g/l) for 5 days then the entire culture was extracted with diethyl ether

n.d. = not detected

DC_{12} accumulated. The DC yields reported in this study are much lower than reported in the du Pont patents which described yields of up to 26g/l of DC_{12} which corresponded to a 34% conversion of dodecane to DC_{12} (du Pont 1973, 1974). This high yield is probably due to the very different culture conditions used in the du Pont investigation which were selected to obtain maximum yields of dicarboxylic acid. In the experiments reported in the du Pont patents cells were grown in a medium containing both dodecane (up to 78g/l) and acetate as an additional carbon-source to increase the biomass yield. Also, the cultures were subjected to forced aerationd which is also likely to increase DC production (see Shiio and Uchio 1971). In the experiments carried out in the present study no attempt was made to optimize the culture conditions for maximum dicarboxylic acid production.

3.2. RANGE OF DICARBOXYLIC ACIDS PRODUCED

Dicarboxylic acid production from C_{10} to C_{16} alkanes and fatty acid methyl esters (FAME) was investigated using both *Corynebacterium* 7E1C and *Corynebacterium* sp. 21747. The range of dicarboxylic acids produced by *Corynebacterium* 21747 was investigated to determine if the mutation(s) resulting in increased dicarboxylic acid production altered the the spectrum of dicarboxylic acids produced, as compared to the wild-type organism *Corynebacterium* 7E1C. The culture conditions were the same as in the initial screening experiments. The range of dicarboxylic acids accumulated by these 2 organisms are shown in Tables 3.2 and 3.3. The range of substrates on which *Corynebacterium* sp. 21747 will grow are identical to the wild-type organism. Furthermore, the spectrum of dicarboxylic acids produced by the 2 strains of *Corynebacterium* was the same. With both organisms dodecane is the best substrate for

Table 3.2: Dicarboxylic acid production by *Corynebacterium* 7E1C during growth on a range of carbon-sources

Growth sustrate (20g/1)		DC produced (mg/1)			
		DC_{10}	DC_{12}	DC_{14}	DC_{16}
Decane	NG	n.d.	n.d.	n.d.	n.d.
Methyl decanoate	NG	n.d.	n.d.	n.d.	n.d.
Dodecane		12	301	n.d.	n.d.
Methyl laurate	NG	n.d.	24	n.d.	n.d.
Tetradecane		10	6	17	n.d.
Methyl myristate		2	2	45	n.d.
Hexadecane		n.d.	n.d.	n.d.	n.d.
Methyl palmitate		n.d.	n.d.	n.d.	n.d.

Bacteria were grown on the appropriate carbon-source for 5 days then the entire culture was extracted with diethyl ether

NG = no growth

n.d. = not detected

Table 3.3: Dicarboxylic acid production by *Corynebacterium* 2174 during growth on a range of carbon-sources

Growth substrate (20g/l)		DC produced (mg/l)			
		DC_{10}	DC_{12}	DC_{14}	DC_{16}
Decane	NG	n.d.	n.d.	n.d.	n.d.
Methyl decanoate	NG	n.d.	n.d.	n.d.	n.d.
Dodecane		15	1192	n.d.	n.d.
Methyl laurate	NG	n.d.	36	n.d.	n.d.
Tetradecane		n.d.	n.d.	59	n.d.
Hexadecane		n.d.	n.d.	n.d.	n.d.
Methyl palmitate		n.d.	n.d.	n.d.	n.d.

Bacteria were grown on the appropriate carbon-source for 5 days then the entire culture was extracted with dietyl ether

NG = no growth

n.d. = no growth

dicarboxylic acid (DC_{12}) production. Small amounts of DC_{12} were produced from methyl laurate, despite the lack of growth on this carbon-source, and was probably due to transformation by the cells present in the initial inoculum. Some DC_{14} is produced from C_{14} substrates and in both cases better yields are obtained from methyl myristate than from tetradecane. No DC_{16} was produced by either organism.

Interestingly the results described here are somewhat different to those reported by Kester and Foster (1963) who first reported on dicarboxylic acid production by *Corynebacterium* 7E1C. These authors found that no dicarboxylic acids were produced from fatty acids which is rather curious since during growth on decane (which would not support growth in the present study) they detected decanoic acid early in the fermentation followed later by ω-hydroxydecanoic acid and decanedioic acid, the order of appearance and disappearance indicating a precursor-product relationship between the C_{10} acids. This implies that dicarboxylic acid production from alkane can proceed via the fatty acid in this organism (although the diol pathway may be operating simultaneously) thus one would expect dicarboxylic acids to be produced from fatty acids, as was observed in the present study (see Tables 3.2 and 3.3). The production of dicarboxylic acids from fatty acid methyl esters in this study may indicate a difference in the metabolism of free fatty acids and of the corresponding methyl esters. It is possible that fatty acid methyl esters are better inducers of the enzymes of the diterminal oxidation pathway than the corresponding free fatty acids. In this study it was shown that the ω-hydroxyfatty acid and α,ω-diol dehydrogenase activities are constitutive as is the β-oxidation system (the only system required for growth on fatty acids). However, ω-hydroxylase activity (essential for the ω-oxidation of fatty acids ie: for diterminal oxidation) was found to be induced by alkane in a previous investigation (Cardini and Jurtshuk 1968, 1970). It is conceivable that fatty acid methyl esters

are better inducers of ω-hydroxylase activity than free fatty acids, which would be consistent with the diterminal oxidation (and resultant dicarboxylic acid production) of fatty acid methyl esters and the monoterminal oxidation of free fatty acids. Another possibility is that the strain used in the present study was slightly different (due to a spontaneous mutation) to that used by Kester and Foster (1963). It should be noted that in the present study the production of dicarboxylic acids from free fatty acids was not investigated. Therefore it is possible that dicarboxylic acids would also have been produced from free fatty acids as well as from the corresponding fatty acid methyl esters.
Despite the increased yields of DC_{12} and DC_{14} by *Corynebacterium* sp. 21747 as compared to *Corynebacterium* 7E1C there is still no DC_{16} production. The inability to produce DC_{16} was investigated in some detail. The period of incubation was varied from 1 to 8 days, the degree of aeration was increased by incubating the 100ml culture volume in a 2 litre flask instead of the usual 250ml flask but still no DC_{16} could be detected. One possible explanation for the lack of DC_{16} production from hexadecane or methyl palmitate was that the ω-hydroxylase cannot ω-hydroxylate hexadecanol or palmitate. The ω-hydroxylase reaction was circumvented by growing *Corynebacterium* 7E1C on 16-hydroxypalmitate. However, even during growth on 16-hydroxypalmitate no DC_{16} could be detected. Interestingly, no dicarboxylic acids of any chain-length could be detected in cultures grown on C_{16} substrates, including 16-hydroxypalmitate.

3.3. DEGRADATION OF DICARBOXYLIC ACIDS BY WASHED WHOLE CELLS

In the present investigation entire cultures were acidified and the dicarboxylic acids extracted into diethyl ether so that intracellular and extracellular dicarboxylic acid could not be distinguished. However,

in the du Pont patents it was the culture filtrates that were analysed indicating an extracellular location of the dicarboxylic acids.

A factor influencing the accumulation of dicarboxylic acids may be the permeability of the cell to dicarboxylic acids. There are 2 aspects to the problem of dicarboxylic acid permeability. There is the problem of intracellularly formed dicarboxylic acid getting out of the cell whilst on the other hand there is the question of whether dicarboxylic acids excreted into the culture medium are metabolically inert. Therefore the degradation of exogenous C_6 to C_{16} dicarboxylic acids by washed-whole cells of *Corynebacterium* 7E1C was investigated. Succinate-grown cells were used so that the results would not be affected by endogenous fatty acids that would be present in cells grown on alkanol, fatty acid or alkane. The latter could not be used anyway due to extensive flocculation during growth on alkanes. It was possible to use succinate-grown cells for such an experiment since the β-oxidation system of *Corynebacterium* 7E1C is constitutive (see Chapter 6). The results of a series of experiments with different incubation periods are shown in Table 3.4 (a) to (c). These results demonstrate that *Corynebacterium* 7E1C can degrade exogenous dicarboxylic acids. Degradation is seen to proceed via a homologous series of chain-shortened (by multiples of 2) dicarboxylic acids which is consistent with the degradation occuring via β-oxidation. DC_8 and DC_6 were poorly degraded, over 50% of the parent DC remaining after incubation for 30 hours, and significant amounts of DC_8 accumulated from DC_{14}, DC_{12} and DC_{10} in the 30 hour incubation experiment. Interestingly, no such accumulation was observed from DC_{16} which may reflect the slower rate of DC_{16} degradation; regardless of incubation period the extent of degradation of DC_{16} was less than that of DC_{10} to DC_{14}. The slower degradation of DC_{16} may well be due to the fact that under the

Table 3.4: Degradation of dicarboxylic acids by washed-whole cells of succinate-grown *Corynebacterium* 7E1C

(a)

Parent DC (80mg)	DC detected in incubation medium (mg)					
	DC_{16}	DC_{14}	DC_{12}	DC_{10}	DC_8	DC_6
DC_{16}	1	0	0	0	0	0
DC_{14}		0	0	0	0	0
DC_{12}			0	0	0.6	0.8
DC_{10}				0	0.7	3.3
DC_8					14.9	44.9

Washed-whole cells (5ml, 9mg dry weight/ml) incubated for 120 hrs. at 30°C on a shaker (160 rpm).

(b)

Parent DC (80mg)	DC detected in incubation medium (mg)					
	DC_{16}	DC_{14}	DC_{12}	DC_{10}	DC_8	DC_6
DC_{16}	17.3	0	0	2.1	0.9	0
DC_{14}		0	0	13.6	32.6	2.3
DC_{12}			14	20.9	18.8	6
DC_{10}				3	19.2	7.2
DC_8					42.0	0.6
DC_6						42.8

Washed-whole cells (5ml, 9.2mg dry weight/ml) were incubated for 30 hrs. at 30°C on a shaker (160 rpm).

(c)

Parent DC (80mg)	DC detected in incubation medium (mg)					
	DC_{16}	DC_{14}	DC_{12}	DC_{10}	DC_8	DC_6
DC_{16}	39.6	0	1.4	0.5	2.1	0.2
DC_{14}		12.5	5.1	6.8	4.7	0.2
DC_{12}			12.3	7.4	5.1	1.2
DC_{10}				0.7	3.7	14.7
DC_8					47.8	0
DC_6						43.5

Washed-whole cells (5ml, 8.1mg dry wright/ml) were incubated for 18 hrs. at 30°C on a shaker (160 rpm).

experimental conditions DC_{16} formed a precipitate. This is likely to reduce its availability to the cell. DC_6 to DC_{14} on the other hand were completely soluble under these conditions. Despite this difficulty with DC_{16} these results demonstrate that *Corynebacterium* 7E1C is able to degrade (via β-oxidation) exogenous long-chain dicarboxylic acids. Furthermore, since DC_{12} and DC_{14} are degraded faster than DC_{16}, it seems that the degradation of exogenous dicarboxylic acids is not a determining factor in the specificity of dicarboxylic acid accumulation. It is possible to envisage a situation whereby exogenous DC_{12} could not be degraded whereas exogenous DC_{16} could be. Such a situation would favour accumulation of DC_{12} but not of DC_{16}. However, the results obtained here make such an explanation untenable for *Corynebacterium* 7E1C. It is worth noting that these results are rather diffferent to those reported by Uchio and Shiio (1972a,b) who performed similar whole-cell degradation experiments with *Candida cloacae*. These authors found that the extent of degradation, for a fixed incubation period, increased dramatically with increasing carbon chain-length; DC_{14} and DC_{16} being degraded completely in the time taken for DC_{12} to be degraded 50%.

4. ALCOHOL DEHYDROGENASE

Starting from alkane, dicarboxylic acids can be formed either via the fatty acid and/or the α,ω-diol (Yi and Rehm 1982b)(see also Fig. 1.1). Regardless of which pathway operates the ω-hydroxyfatty acid is an obligatory intermediate in dicarboxylic acid formation. Therefore the alcohol dehydrogenase activity with ω-hydroxyfatty acids of different chain-lengths is of great interest with respect to dicarboxylic acid production by this organism.

Bacchin *et al* (1974) showed that a strain of *Corynebacterium* produced large amounts of 1,10-decanediol when grown on decane. This indicates that in *Corynebacterium* significant dicarboxylic acid may be produced via the α,ω-diol. Therefore the specificity of α,ω-diol dehydrogenase activity is also of interest. Therefore the specificity of alcohol dehydrogenase activity in *Corynebacterium* 7E1C was investigated with respect to n-mono-ols, α,ω-diols and ω-hydroxyfatty acids.

4.1. ASSAY OF ALCOHOL DEHYDROGENASE AND ASSOCIATED PROBLEMS

Alcohol dehydrogenase activity was measured fluorimetrically by following the increase in fluorescence on reduction of $NAD(P)^+$ to $NAD(P)H + H^+$.

A problem associated with assaying alcohol dehydrogenase activity was the high endogenous rate of $NAD(P)^+$ reduction when using crude extracts. The cause of this endogenous $NAD(P)^+$ reduction is not certain but the rate decreased with time, falling to zero after approx. 10 minutes (when using 20 µl of extract of 3mg/ml protein). Thus assays were preincubated for approx. 10 minutes, to allow the endogenous rate to fall to zero, before substrate was added. The endogenous $NAD(P)^+$ reduction was more pronounced at high salt concentrations. The practical consequence of the endogenous $NAD(P)^+$ reduction is that it limited the amount of extract that could be added to assays. If large amounts of extract were added,

as would be necessary when studying the oxidation of poor substrates, the period of preincubation required for the endogenous rate to fall to zero became excessively long. Hence poor substrates would not then be studied very satisfactorily.

4.2. INDUCTION OF ALCOHOL DEHYDROGENASE ACTIVITY

Alcohol dehydrogenase activity was measured in crude cell-free extracts of *Corynebacterium* 7E1C grown on a range of different carbon sources. Activity was assayed with n-octanol, 1,12-dodecanediol and 12-hydroxylaurate since these were the optimal mono-ol, α,ω-diol and ω-hydroxyfatty acid respectively (in terms of maximum rate under standard assay conditions). As shown in Table 4.1., alcohol dehydrogenase activity is remarkably similar after growth on a wide range of carbon-sources with the exception of $NADP^+$-dependent octanol dehydrogenase activity in succinate-grown cells. When compared to succinate-grown cells there appears to be an increase in $NADP^+$-dependent n-octanol dehydrogenase activity by growth on alkyl-containing substrates. In succinate-grown cells 1,12-dodecanediol dehydrogenase is the highest $NADP^+$-dependent activity whereas in cells grown on alkyl-containing substrates n-octanol dehydrogenase is the highest $NADP^+$-dependent activity. This is consistent with the induction of additional $NADP^+$-dependent n-octanol dehydrogenase activity by alkyl-containing substrates.

No $NAD(P)^+$-independent alcohol deydrogenase activity could be detected, when assayed using phenazine methosulphate (PMS) and 2,6-dichloroindophenol (DCPIP) as electron acceptors, in cells grown on either succinate or hexadecanol.

In other alkane-utilizing bacteria there are reports of both constitutive and inducible $NAD(P)^+$-dependent alcohol dehydrogenases.

Table 4.1: Specific activities of alcohol dehydrogenase activities of *Corynebacterium* 7E1C after growth on a range of carbon-sources

Carbon-source	*Assay substrate*	*Activity (nmol/min/mg)*	
		NAD^+	$NADP^+$
Succinate	octanol	9.4 ± 4.2 (3)	3.5 ± 1.5 (3)
	dodecanediol	18.1 ± 7.8 (3)	5.1 ± 2.1 (3)
	12-hydroxylaurate	9.0 ± 3.0 (3)	3.5 ± 1.6 (3)
Glucose	octanol	15.0 (1)	not done
	dodecanediol	20.0 (1)	not done
	12-hydroxylaurate	10.0 (1)	not done
Dodecanediol	octanol	9.4 (1)	6.7 (1)
	dodecanediol	12.7 (1)	3.8 (1)
	12-hydroxylaurate	6.2 (1)	2.9 (1)
Hexadecane	octanol	9.2 ± 3.6 (3)	9.0 ± 3.4 (3)
	dodecanediol	12.4 ± 3.7 (3)	7.2 ± 2.6 (3)
	12-hydroxylaurate	6.3 ± 2.6 (3)	4.8 ± 1.2 (3)
Hexadecanol	octanol	13.7 ± 4.0 (8)	12.9 ± 4.1 (8)
	dodecanediol	16.4 ± 5.6 (8)	7.9 ± 1.9 (8)
	12-hydroxylaurate	8.9 ± 2.6 (8)	4.7 ± 1.0 (8)

Values are mean ± S.D. for (n) independent determinations

[octanol] = 330 μM, [dodecanediol] = 67 μM, [12-hydroxylaurate] = 330 μM

Assayed in potassium phosphate buffer (0.8M, pH 8.0)

Pseudomonas aeruginosa strain 196 possesses 2 NAD^+-dependent and 2 $NADP^+$-dependent alcohol dehydrogenases (Vandecasteele *et al* 1983) all of which are constitutive ie: they are present in both heptane-grown and glucose-grown cells at similar specific activities. The only inducible activity was a dye-linked, particulate alcohol dehydrogenase (Vandecasteele *et al* 1983; Tassin *et al* 1973; Tassin and Vandecasteele 1972). In *Acinetobacter calcoaceticus* there have been reports of an inducible (by n-alkanol) NAD^+-dependent long-chain specific alcohol dehydrogenase (Fewson 1966, *A. calcoaceticus* NCIB 8250). Similarly, Singer and Finnerty (1985a) reported an inducible (by hexadecane or hexadecanol) NAD^+-dependent hexadecanol dehydrogenase in *A. calcoaceticus* HO1-N which is presumably analogous to the enzyme reported by Fewson. These authors also reported an ethanol-induced NAD^+-dependent ethanol dehydrogenase. *A. calcoaceticus* also possesses a constitutive $NADP^+$-dependent alcohol dehydrogenase (Fixter and Nagi 1984; Singer and Finnerty 1985a; Jirausch *et al* 1986).

It is worth noting that all the above mentioned bacteria are Gram-negative. There are, apparently, no reports of long-chain alcohol dehydrogenase in alkane-grown Gram-positive bacteria.

4.3. VARIATION OF ALCOHOL DEHYDROGENASE ACTIVITIES WITH PERIOD OF GROWTH ON HEXADECANOL

In view of the apparent induction of $NAD(P)^+$-dependent octanol dehydrogenase activity by growth on alkyl-containing substrates, which indicated a possible role in the dissimilation of these sustrates, (see section 4.1.) it was decided to investigate the variation in the various alcohol dehydrogenase activities with the period of growth on hexadecanol (Fig. 4.1). Early in growth $NADP^+$-dependent octanol dehydrogenase is the highest activity and then decreases quite rapidly

Figure 4.1.: Variation of alcohol dehydrogenase activities in *Corynebacterium* 7E1C with the period of growth on hexadecanol (0.2%w/v)

Assayed in potassium phosphate buffer (0.8M, pH 8)

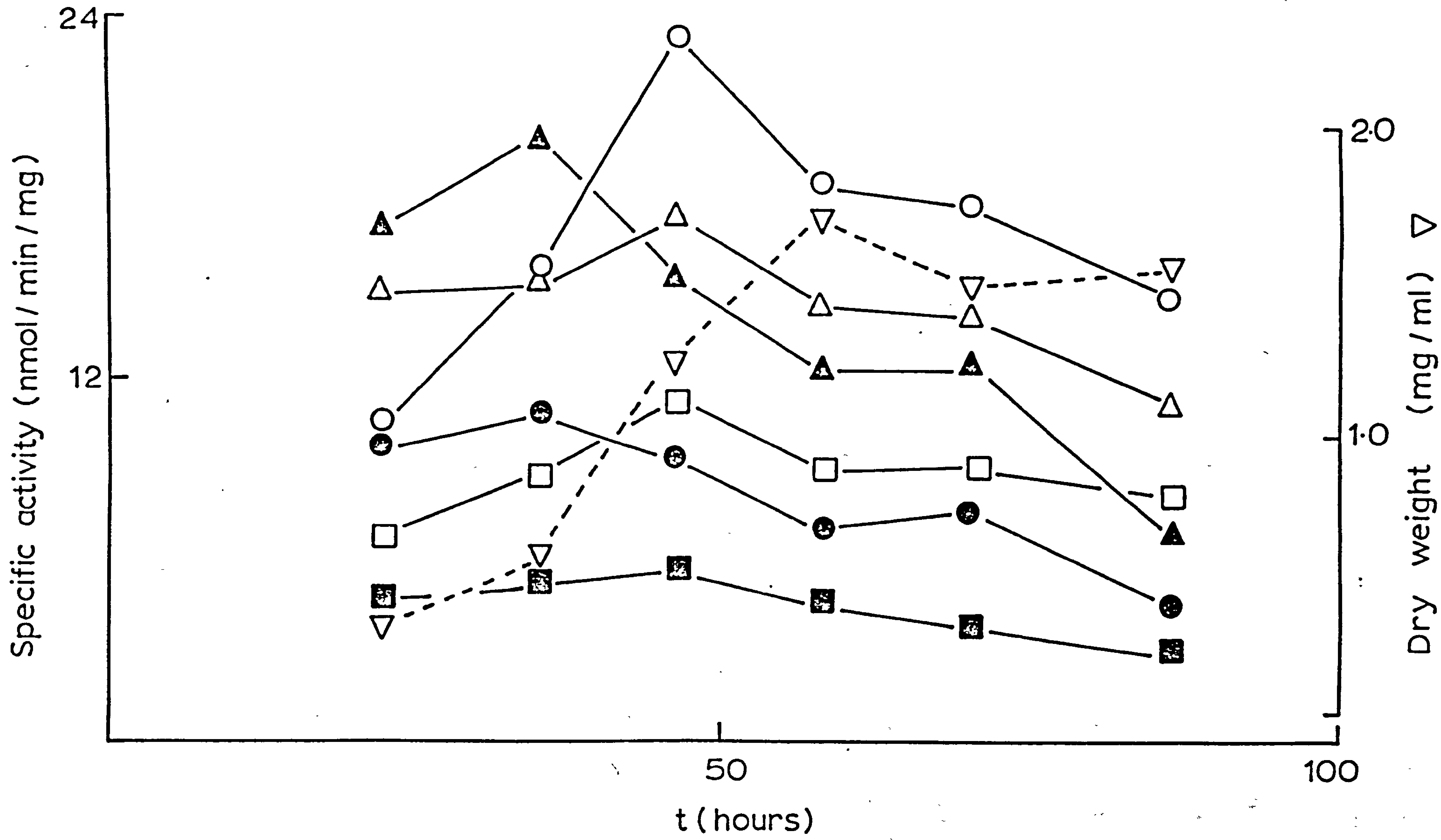
[octanol] = 330 μM, [dodecanediol] = 67 μM, [12-hydroxylaurate] = 330 μM

△ = octanol/NAD^+ ▲ = octanol/$NADP^+$

○ = dodecanediol/NAD^+ ● = dodecanediol/$NADP^+$

□ = 12-hydroxylaurate/NAD^+ ■ = 12-hydroxylaurate/$NADP^+$

▽ = growth



Specific activity (nmol / min / mg)
24
12
Dry weight (mg / ml) ▽
2·0
1·0
50
100
t (hours)

whereas the corresponding NAD^+-dependent acivity is relatively constant over the period investigated. NAD^+-dependent 1,12-dodecanediol dehydrogenase was the main activity during the mid-exponential to stationary phases of growth. The high levels of $NADP^+$-dependent octanol dehydrogenase activity during the early stages of growth on hexadecanol may indicate some specific involvement in long-chain alcohol dissimilation. This contention is supported by the observation that $NADP^+$-dependent alcohol dehydrogenase activity is increased by growth on various long-chain alcohols (see section 4.2.).

4.4. SUBCELLULAR LOCATION OF ALCOHOL DEHYDROGENASE(S)

The subcellular location of NAD^+-dependent and $NADP^+$-dependent octanol, 1,12-dodecanediol and 12-hydroxylaurate dehydrogenase activities was investigated in cells grown on succinate, 1,12-dodecanediol, hexadecanol and hexadecane. Crude cell-free extract was ultracentrifuged at 100 000g for 90 min. at 4°C and the pellet and supernatant fractions assayed for each activity (Tables 4.2, 4.3, 4.4 and 4.5).

With hexadecane-grown and dodedecanediol-grown cells there is significant particulate $NADP^+$-dependent octanol dehydrogenase activity (87% in hexadecane-grown cells, 44% in dodecanediol-grown cells)(see Tables 4.2 and 4.3). However, in both succinate-grown and hexadecanol-grown cells the amount of $NADP^+$-dependent octanol dehydrogenase activity associated with the 100 000g pellet is less than 20% and is no greater than for the $NADP^+$-dependent 1,12-dodecanediol or 12-hydroxylaurate dehydrogenase activities (see Tables 4.4 and 4.5). The apparent lack of particulate $NADP^+$-dependent octanol dehydrogenase activity in cells grown on succinate or hexadecanol can be explained as follows. In cells grown on succinate the highest $NADP^+$-dependent activity is with 1,12-dodecanediol whereas in cells grown on

Table 4.2: Subcellular location of alcohol dehydrogenase activities in hexadecane-grown *Corynebacterium* 7E1C

Fraction	Assay substrate	Units* (%)		Specific activity**	
		NAD^+	$NADP^+$	NAD^+	$NADP^+$
Crude extract	8ol	217 (100)	172 (100)	7.4	5.8
	12diol	294 (100)	195 (100)	10.0	6.6
	12HO	186 (100)	67 (100)	6.3	2.3
Supernatant	8ol	136 (63)	59 (34)	7.9	5.8
	12diol	223 (76)	99 (51)	12.9	5.3
	12HO	136 (73)	43 (64)	7.9	2.3
Pellet	8ol	40 (18)	150 (87)	4.2	15.8
	12diol	38 (13)	33 (17)	4.0	3.4
	12HO	26 (14)	23 (34)	2.7	2.4

Crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C

8ol = octanol, 12diol = 1,12-dodecanediol, 12HO = 12-hydroxylaurate

[octanol] = 330 μM, [dodecanediol] = 67 μM, [12-hydroxylaurate] = 330 μM

* = 1 unit ≡ 1 nmol/min/mg

** = nmol/min/mg

Table 4.3: Subcellular location of alcohol dehydrogenase activities in dodecanediol-grown *Corynebacterium* 7E1C

Fraction	Assay substrate	Units* (%) NAD⁺	Units* (%) NADP⁺	Specific activity** NAD⁺	Specific activity** NADP⁺
Crude extract	8ol	210 (100)	105 (100)	8.1	4.0
	12diol	335 (100)	75 (100)	12.9	2.9
	12HO	135 (100)	50 (100)	5.2	1.9
Supernatant	8ol	158 (75)	72 (69)	6.7	3.1
	12diol	257 (77)	68 (90)	11.0	2.9
	12HO	101 (75)	41 (82)	4.4	1.7
Pellet	8ol	38 (18)	46 (44)	14.6	35.0
	12diol	17 (5)	8 (11)	13.3	5.8
	12HO	9 (7)	2 (4)	6.7	1.2

Crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C

8ol = octanol, 12diol = 1,12-dodecanediol, 12HO = 12-hydroxylaurate

[octanol] = 330 µM, [dodecanediol] = 67 µM, [12-hydroxylaurate] = 330 µM

* = 1 unit ≡ 1nmol/min

** = nmol/min/mg

Table 4.4: Subcellular location of alcohol dehydrogenase activities in succinate-growwn *Corynebacterium* 7E1C

Fraction	Assay substrate	Units (%)		Specific activity	
		NAD^+	$NADP^+$	NAD^+	$NADP^+$
Crude extract	8ol	43 (100)	18 (100)	13.3	5.6
	12diol	86 (100)	25 (100)	26.5	7.8
	12HO	35 (100)	14 (100)	10.8	4.4
Supernatant	8ol	36 (84)	14 (78)	13.5	5.2
	12diol	68 (79)	19 (76)	25.1	7.0
	12HO	29 (83)	11 (78)	11.0	4.2
Pellet	8ol	3 (7)	2 (10)	3.6	2.3
	12diol	4 (5)	1 (4)	4.4	1.6
	12HO	2 (6)	1 (4)	2.3	0.8

Crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C

8ol = octanol, 12diol = 1,12-dodecanediol, 12HO = 12-hydroxylaurate

[octanol] = 330 μM, [dodecanediol] = 67 μM, [12-hydroxylaurate] = 330 μM

* = 1 unit ≡ 1 nmol/min

** = nmol/min/mg

Table 4.5.: Subcellular location of alcohol dehydrogenase activities in hexadecanol-grown *Corynebacterium* 7E1C

Fraction	Assay substrate	Units (%) NAD$^+$	Units (%) NADP$^+$	Specific activity NAD$^+$	Specific activity NADP$^+$
Crude extract	8ol	212 (100)	319 (100)	8.6	13.0
	12diol	216 (100)	176 (100)	8.8	7.1
	12HO	184 (100)	140 (100)	7.5	5.7
Supernatant	8ol	173 (82)	231 (72)	7.0	9.3
	12diol	225 (104)	154 (88)	9.1	6.2
	12HO	161 (88)	135 (96)	6.5	5.5
Pellet	8ol	0 (0)	53 (17)	0	4.7
	12diol	0 (0)	12 (7)	0	1.1
	12HO	0 (0)	7 (6)	0	0.7

Crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C

8ol = octanol, 12diol = 1,12-dodecanediol, 12HO = 12-hydroxylaurate

[octanol] = 330 μM, [dodecanediol] = 67 μM, [12-hydroxylaurate] = 330 μM

* = 1 unit ≡ 1 nmol/min

** = nmol/min/mg

dodecanediol, hexadecanol or hexadecane the highest $NADP^+$-dependent activity is observed with octanol (see section 4.2.) indicating the induction of additional $NADP^+$-dependent octanol dehydrogenase activity by growth on these substrates. The presence of particulate $NADP^+$-dependent octanol dehydrogenase in hexadecane-grown and dodecanediol-grown cells is consistent with the additional $NADP^+$-dependent octanol dehydrogenase activity induced by growth on these substrates being membrane-associated. This would explain the lack of particulate activity in succinate-grown cells; the activity has not been induced. However, this does not explain the lack of particulate $NADP^+$-dependent activity in hexadecanol-grown cells. In hexadecanol-grown cells the highest $NADP^+$-dependent activity is observed with octanol as is the case with cells grown on dodecanediol or hexadecane. The lack of membrane-association of $NADP^+$-dependent octanol dehydrogenase could be explained by assuming that a different $NADP^+$-octanol dehydrogenase enzyne is induced by growth on hexadecanol. An alternative, and perhaps more likely explanation is that the detergent properties of hexadecanol may solubilize the membrane-associated $NADP^+$-dependent octanol dehydrogenase activity. This latter explanation would imply that the particulate $NADP^+$-dependent octanol dehydrogenase is only loosly membrane associated ie: an extrinsic, rather than an intrinsic, membrane protein. This possibility has not been examined any further. However, the observation that there is negligible NAD^+-dependent alcohol dehydrogenase activity associated with the particulate fraction in hexadecanol-grown cells (as opposed to approx. 10-15% in cells grown on other carbon-sources) is consistent with the contention that hexadecanol can act as a solubilizing detergent. Another possibility is that the membrane(s) with which the $NADP^+$-dependent octanol dehydrogenase is associated in hexadecane-grown cells are only present in alkane-grown cells.

From these results it can be concluded that the NAD^+-dependent alcohol dehydrogenases of *Corynebacterium* 7E1C are soluble (ie: cytoplasmic) as are the $NADP^+$-dependent 1,12-dodecanediol and 12-hydroxylaurate dehydrogenase activities. In contrast $NADP^+$-dependent octanol dehydrogenase activity appears to have a dual location; cytoplasmic in succinate-grown cells and both particulate and soluble in cells grown on hexadecanol or dodecanediol, indicating that the dual distribution is due to the existence of different enzymes.

4.5. STIMULATION OF ACTIVITY BY HIGH SALT CONCENTRATIONS

Early on in these investigations it became apparent that alcohol dehydrogenase activity was markedly stimulated by high concentrations of potassium phosphate buffer (pH 8). Therefore, the effect of potassium phosphate concentration on alcohol dehydrogenase activity was investigated in some detail. The effect of increasing concentrations of potassium phosphate buffer (pH 8) on NAD^+-dependent alcohol dehydrogenase activity, in crude cell-free extracts of succinate-grown *Corynebacterium* 7E1C, are shown in Fig. 4.2. With 12-hydroxylaurate as substrate the effect proved to be more pronounced at lower substrate concentrations. Therefore, the effect on dodecanediol and octanol dehydrogenase activities were investigated at substrate concentrations lower than those used in the standard assay. Dodecanediol was used at 13μM rather than 67μM whilst octanol was used at 165μM rather than 330μM. Similar stimulation by high potassium phosphate concentrations was observed with all 3 substrates. The fact that the stimulatory effect was more pronounced at low substrate concentrations indicated that the stimulation may be due, at least in part, to a change in the K_m of the enzyme for the alcohol substrate. In order to determine whether this stimulatory effect of high potassium phoshate concentrations was due to

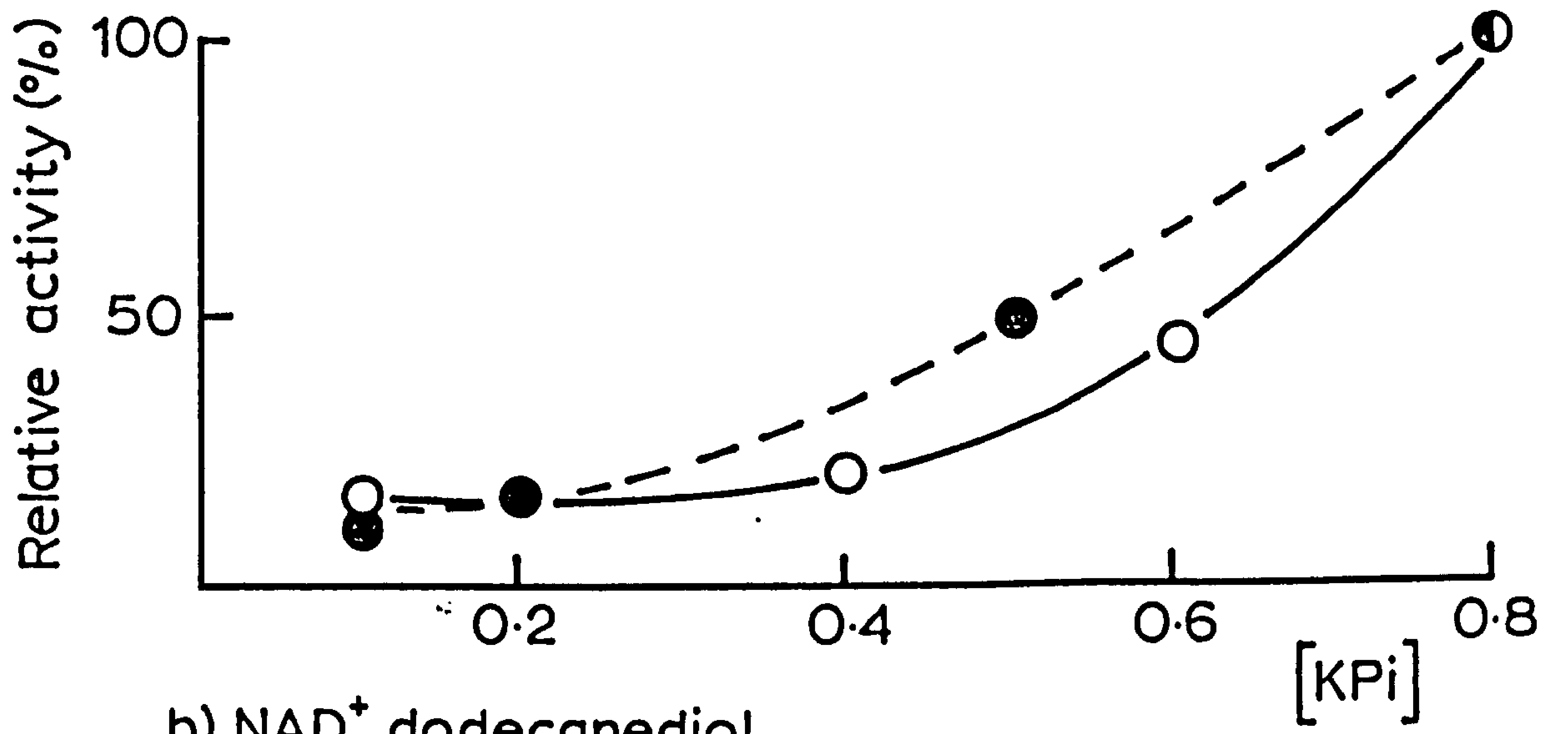
Figure 4.2.: Effect of potassium phosphate concentration on NAD^+-dependent alcohol dehydrogenase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C

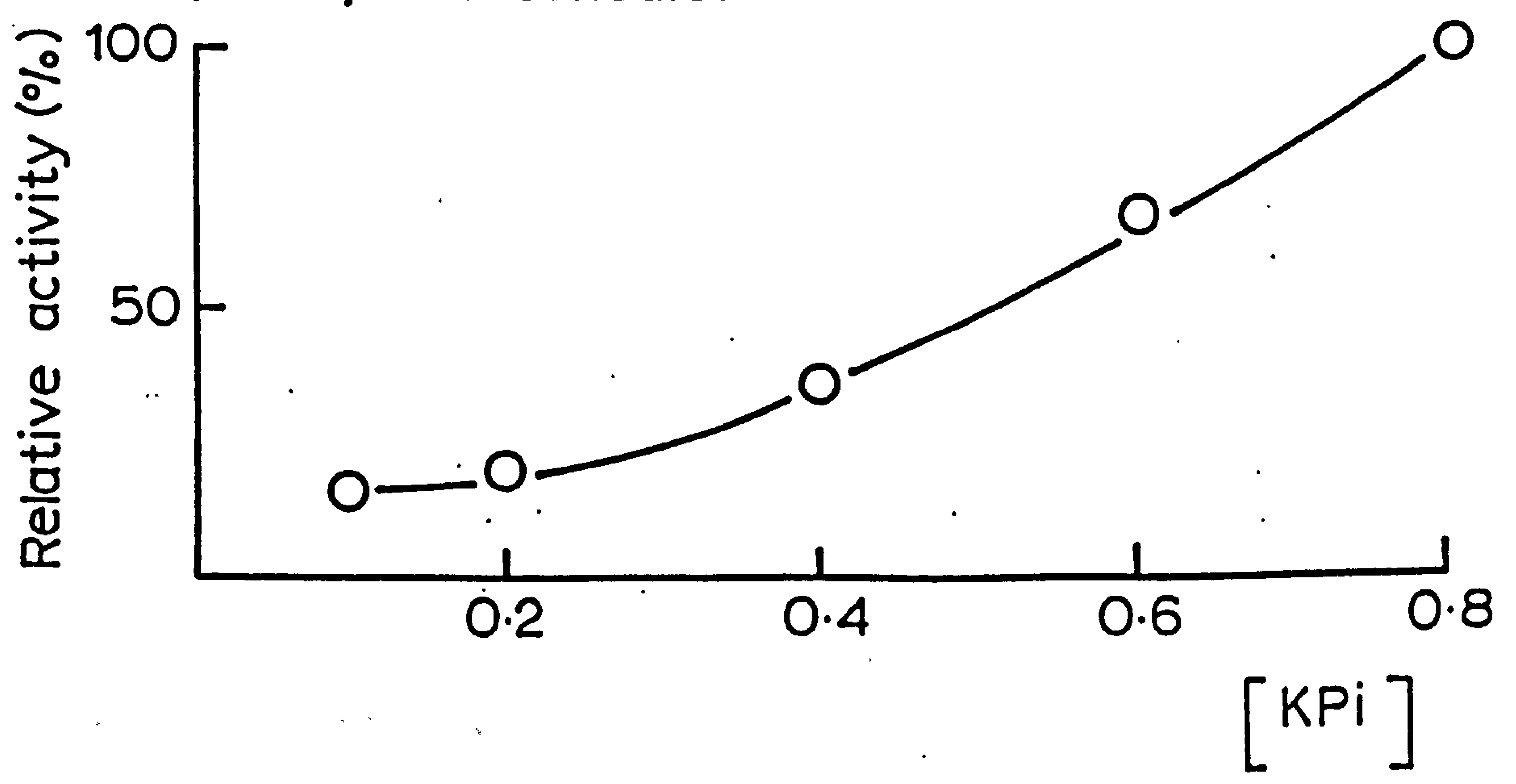
Assayed in potassium phosphate buffer (pH 8)

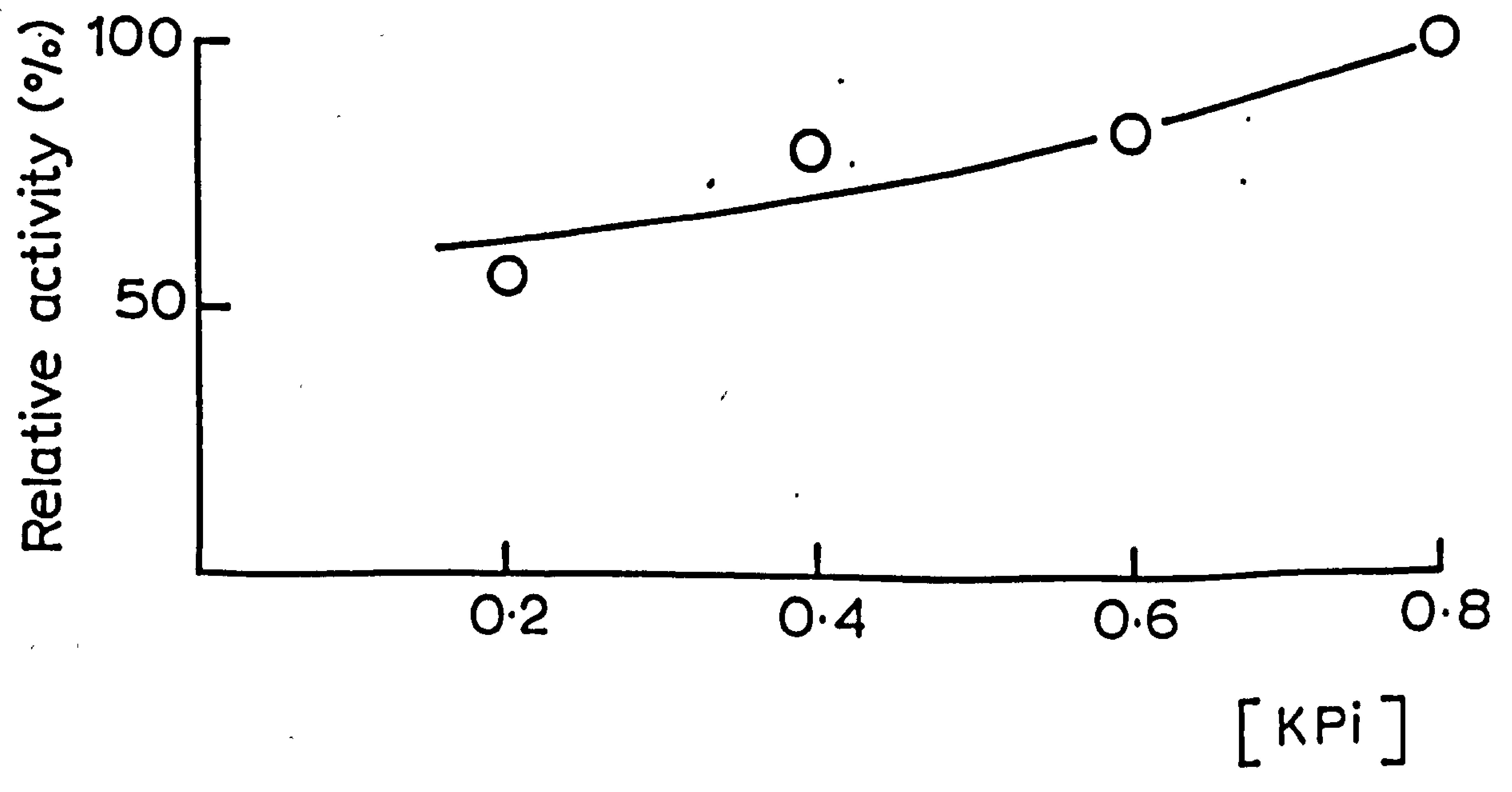
[octanol] = 165 µM, [dodecanediol] = 13µM

[12-hydroxylaurate] = 330 µM ●

[12-hydroxylaurate] = 165 µM ○


a) NAD⁺, 12, hydroxylaurate
Relative activity (%)
100
50
0·2
0·4
0·6
0·8
[KPi]


b) NAD⁺, dodecanediol
Relative activity (%)
100
50
0·2
0·4
0·6
0·8
[KPi]


c) NAD⁺, octanol
Relative activity (%)
100
50
0·2
0·4
0·6
0·8
[KPi]

an increase in V_{max} and/or a decrease in K_m the kinetics o 12-hydroxylaurate dehydrogenase activity were investigated at different potassium phosphate concentrations (Fig. 4.3). V_{max} is essentially independent of potassium phosphate concentration. However, the K_m for 12-hydroxylaurate is highly dependent on potassium phosphate concentration, increasing 6.3-fold (0.35mM to 2.22mM) as the potassium phosphate concentration is decreased 2-fold (from 0.8M to 0.4M). Thus the stimulation of activity is due to a decrease in K_m at high salt concentrations whilst the maximum rate is unaffected. Since these measurements were made in crude cell-free extracts the aldehyde product of the alcohol dehydrohenase would be acted on by the aldehyde dehydrogenase activity present in crude extracts with a concoimitant reduction of NAD^+. Thus if alcohol and aldehyde dehydrogenase activities were tightly linked (which may not be the case with soluble enzymes in a crude extract) 2 mol $NAD(P)^+$ would be reduced for every mol of alcohol oxidized. Thus the possibility remained that the stimulatory effect of high potassium phosphate concentrations was due to an effect on the linked aldehyde dehydrogenase rather than an effect on the alcohol dehydrogenase itself. Although, this will only be a possibility if the activity of the aldehyde dehydrogenase is lower than that of the alcohol dehydrogenase reaction. If the activity of the aldehyde dehydrogenase with 12-oxolaurate was much higher than the alcohol dehydrogenase activity with 12-hydroxylaurate then such a possibility could be discounted. However, no 12-oxolaurate was available to test this. The effect of high potassium phosphate concentrations on aldehyde dehydrogenase activity was investigated using dodecanal as substrate. The kinetics of NAD^+-dependent dodecanal dehydrogenase were determined at 0.2M and 0.8M potassium phosphate concentrations (Fig. 4.4). As with 12-hydroxylaurate dehydrogenase activity V_{max} is essentially unafffected. However, the K_m for dodecanal increased 3-fold (9.6μM to

Figure 4.3.: Effect of potassium phosphate concentration on the kinetics of NAD^+-dependent 12-hydroxylaurate dehydrogenase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C

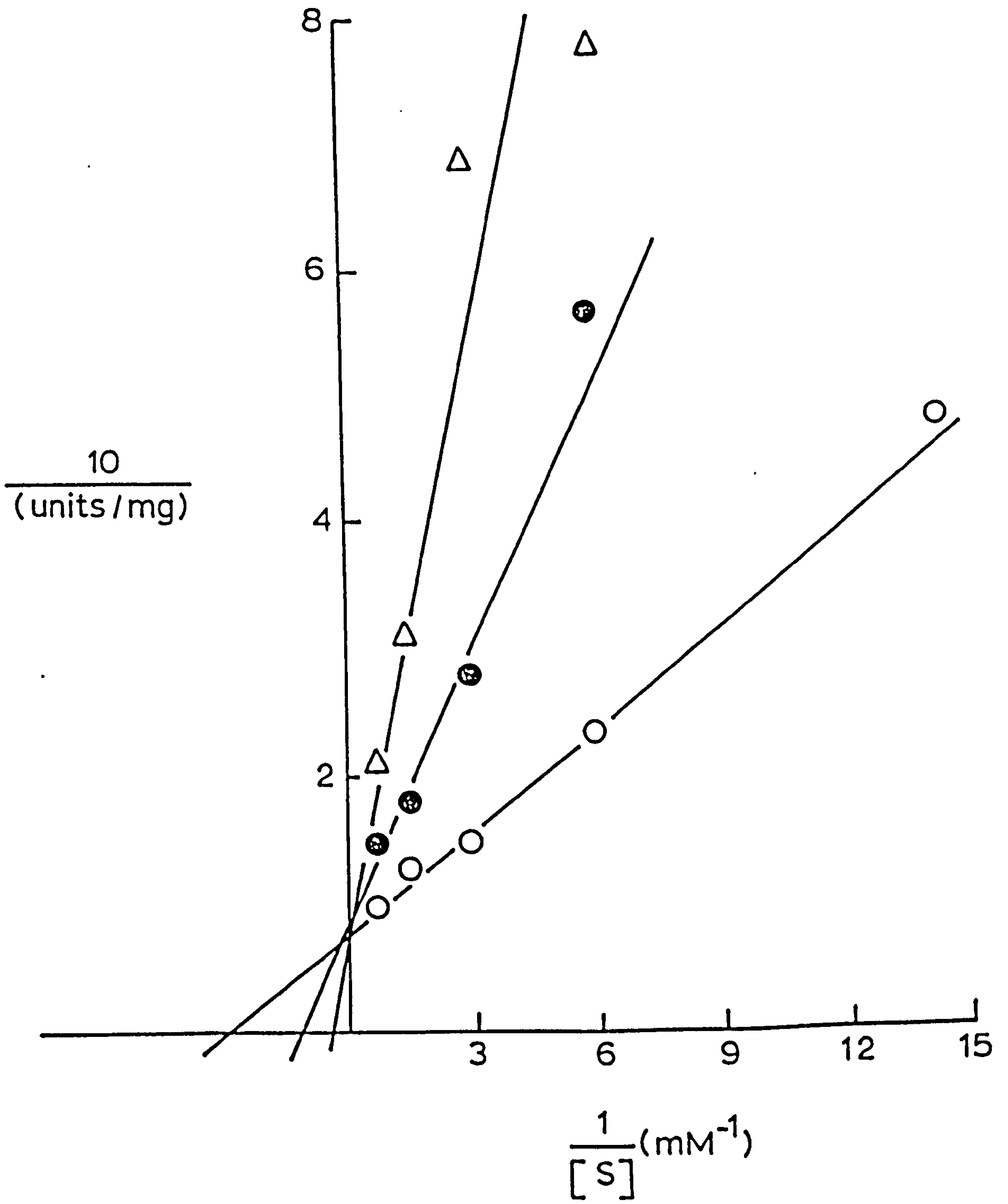
Assayed in potassium phosphate buffer (pH 8)

○ = 0.8M potassium phosphate, K_m = 350 μM

● = 0.6M potassium phosphate, K_m = 830 μM

△ = 0.4M potassium phosphate, K_m = 2220 μM

In all cases V_{max} = 12.5 nmol/min/mg



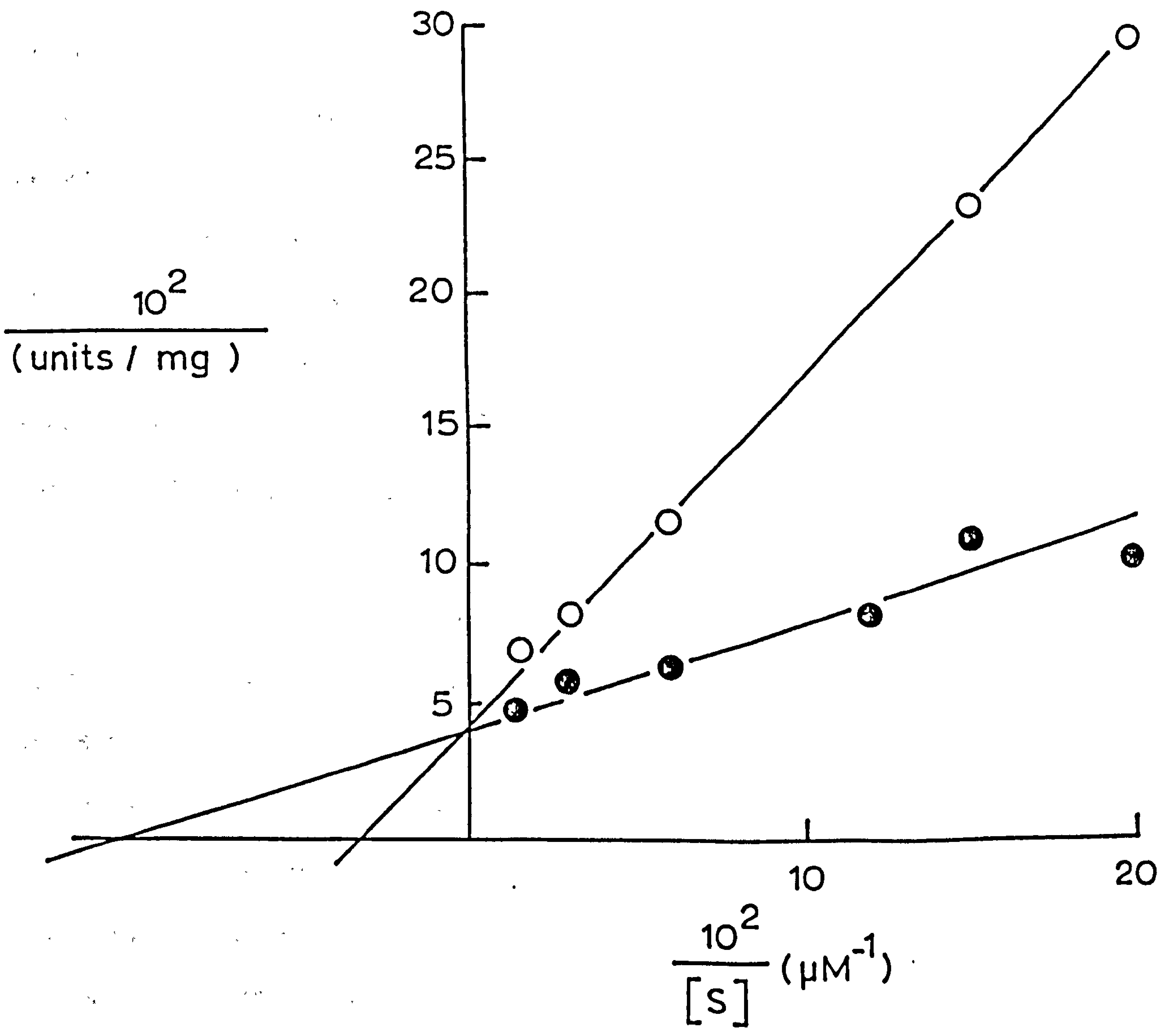
8
6
4
2
10
(units/mg)
3
6
9
12
15
1/[S] (mM⁻¹)

Figure 4.4.: Effect of potassium phosphate concentration on the kinetics of NAD^+-dependent dodecanal dehydrogenase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C

○ = 0.8M potassium phosphate, K_m = 32.0 µM

● = 0.2M potassium phosphate, K_m = 9.6 µM

In both cases V_{max} = 25 nmol/min/mg


30
25
20
15
10
5
$\frac{10^2}{(\text{units / mg})}$
10
20
$\frac{10^2}{[S]}$ (μM^{-1})

32μM) as the potassium phosphate concentration was increased 4-fold (from 0.2M to 0.8M). This increase in K_m with increasing potassium phosphate concentration is the reverse of the situation with NAD^+-dependent 12-hydroxylaurate dehydrogenase activity. This indicated that the stimulation of alcohol dehydrogenase activity was due to an effect on the alcohol dehydrogenase itself rather than to some effect on the linked aldehyde dehydrogenase activity. Although it is possible that dodecanal and 12-oxolaurate are acted on by different enzymes the effect of potassium phosphate concentration on dodecanal dehydrogenase activity is certainly consistent with the stimulation of NAD^+-dependent octanol dehydrogenase being due to stimulation of the alcohol dehydrogenase itself. That the alcohol dehydrogenase(s) are indeed the enzyme stimulated by high potassium phosphate concentrations was conclusively demonstrated by the fact that a partially purified alcohol dehydrogenase preparation was stimulated by high potassium phosphate concentrations. The partially purified preparation was prepared as described in section 4.6.. The NAD^+-dependent activity peak was purified 21-fold (as assayed with 12-hydroxylaurate) and was active with 12-hydroxylaurate, 1,12-dodecanediol and octanol. No activity was observed with $NADP^+$ and no $NAD(P)^+$-dependent aldehyde dehydrogenase activity (assayed with dodecanal) could be detected. With 330μM 12-hydroxylaurate as substrate the specific activities (nmol/min/mg protein) at 0.8M, 0.6M and 0.2M potassium phosphate were 208 (100%), 112 (56%) and 25 (12.5%) respectively. This result confirms that it is indeed the alcohol dehydrogenase, rather than the linked aldehyde dehydrogenase, that is stimulated by high potassium phosphate concentrations.

$NADP^+$-dependent alcohol dehydrogenase activity was also stimulated by high concentrations of potassium phosphate buffer. The $NADP^+$-dependent activities were not investigated in as much detail as the NAD^+-dependent activities since the lower activities made them more difficult to work

with. However, the effects of potassium phosphate concentration were investigated using a partially purified $NADP^+$-dependent alcohol dehydrogenase preparation. The $NADP^+$-dependent alcohol dehydrogenase was prepared as described in section 4.6. and was active with 12-hydroxylaurate, 1,12-dodecanediol and octanol. The effect of potassium phosphate concentration on 1,12-dodecanediol dehydrogenase activity is shown in Fig. 4.5. This stimulation is essentially the same as that observed with the NAD^+-dependent activities. The effect of potassium phosphate concentration on the particulate $NADP^+$-dependent octanol dehydrogenase has not been investigated.

As both NAD^+-dependent and $NADP^+$-dependent activities were stimulated by high concentrations of potassium phosphate buffer it was decided to investigate the effect of a number of different salts on alcohol dehydrogenase activity. It was hoped to determine whether this activation is due to a non-specific effect of high ionic strength or due to activation by a specific ion (or group of ions). The effects of a number of salts on $NAD(P)^+$-dependent 12-hydroxylaurate, 1,12-dodecanediol and octanol dehydrogenase activities are shown in Tables 4.6 and 4.7. NAD^+-dependent 12-hydroxylaurate, 1,12-dodecanediol and octanol dehydrogenase activities showed similar activations. Similarly $NADP^+$-dependent 12-hydroxylaurate, 1,12-dodecanediol and octanol dehydrogenase activities showed similar activations. However, the NAD^+-dependent and $NADP^+$-dependent activities display markedly different activations to one another. The similar properties of all 3 NAD^+-dependent activities is not surprizing since purification studies indicated that a single enzyme may be responsible for most of the NAD^+-dependent alcohol dehydrogenase activity (see section 4.6.). Similarly, a single $NADP^+$-dependent alcohol dehydrogenase is active with all 3 alcohol substrate although it should be noted that that there appeares to be a separate $NADP^+$-dependent octanol dehydrogenase that is

Figure 4.5: Effect of potassium phosphate concentration on $NADP^+$-dependent 1,12-dodecanediol dehydrogenase activity using a partially purified preparation

100% ≡ 37 nmol/min/mg

The fraction was purified 11-fold

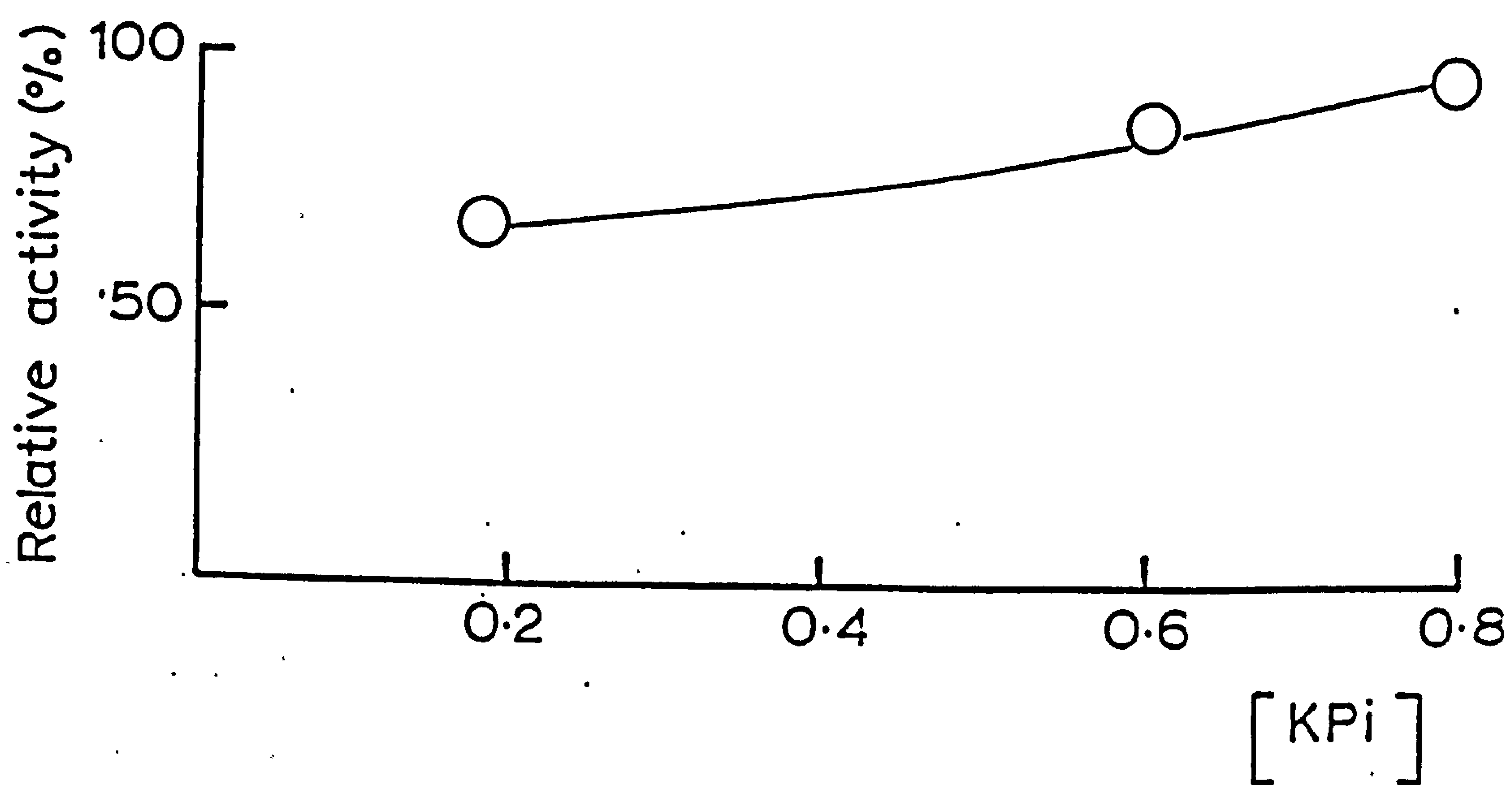

Relative activity (%)
100
50
0·2
0·4
0·6
0·8
[KPi]

Table 4.7.: Effect of high salt concentrations on $NADP^+$-dependent alcohol dehydrogenase activities in crude cell-free extracts of hexadecanol-grown *Corynebacterium* 7E1C

Salt[1]		Relative activity (%)		
		octanol	dodecanediol	12-hydroxylaurate
potassium phosphate[2]		100	100	100
None[3]		43	34	40
KCl	(1.6M)	31	26	54
NaCl	(1.6M)	32	26	not done
Na_2SO_4	(0.8M)	99	97	106
$(NH_4)_2SO_4$	(0.8M)	93	not done	not done
$MgCl_2$	(1.6M)	0	0	0
KI	(1.6M)	0	0	0

1 = unless stated otherwise assays were performed in Hepes buffer (50 mM, pH 8) containing the appropriate salt

2 = assayed in potassium phosphate buffer (0.8M, pH 8)

3 = assayed in Hepes buffer (50 mM, pH 8) without added salt

[octanol] = 330 µM, [dodecanediol] = 67 µM, [12-hydroxylaurate] = 330 µM

Specific activities: octanol, 100% = 16 nmol/min/mg

dodecanediol, 100% = 11.1 nmol/min/mg

12-hydroxylarate, 100% = 5.7 nmol/min/mg

responsible for a significant proportion of the $NADP^+$-dependent octanol dehydrogenase activity.

With NAD^+-dependent activities all the salts tested, except $MgCl_2$ and KI, resulted in a stimulation of activity as compared to Hepes (50mM, pH 8.0). The greatest stimulation was observed with Na_2SO_4. No activity was observed in the presence of $MgCl_2$ or KI, the reason for which is unknown. With $NADP^+$-dependent activities no activity was observed in the presence of $MgCl_2$ or KI, as was the case with the NAD^+-dependent activities. Na_2SO_4 and $(NH_4)_2SO_4$ stimulate all 3 $NADP^+$-dependent activities (relative to Hepes) as they do the NAD^+-dependent activities. However, KCl and NaCl are slightly inhibitory to $NADP^+$-dependent activities in contrast to their stimulatory effect on NAD^+-dependent activities. The reason for these differences is not known.

It has been demonstrated that high concentrations of potassium phosphate stimulate activity by decreasing the K_m for the alcohol substrate whilst V_{max} is unaffected. However, the mechanism by which this change in substrate affinity is mediated is unknown. A possible explanation is that high salt concentrations will tend to promote hydrophobic interactions. Thus it is possible that high salt concentrations increase substrate affinity by promoting interaction between the hydrophobic alkyl-chain of the alcohol and a putative hydrophobic binding site on the alcohol dehydrogenase.

Halophphilic bacteria possess enzymes that are maximally active at high salt concentrations (typically molar NaCl)(see Lanyi 1974), specific examples being menadione reductase (Lanyi 1970) and the NADH dehydrogenase (Hochstein 1970) of *Halobacterium salinarium*. With the latter enzyme the K_m for 2,6-dichlorophenolindophenol (DCPIP) decreased with increasing NaCl concentration whereas the K_m for NADH and V_{max} were unaffected (Hochstein 1970). The enzymes from halophilic organisms are not only stimulated by high salt concentrations, they are also

stabilized by high salt concentrations. Many halophiles possess enzymes that denature at low salt concentration (Lanyi 1974). The effect of high salt concentration on the stability of the alcohol dehydrogenases from *Corynebacterium* 7E1C has not been investigated.

4.6. PURIFICATION OF SOLUBLE ALCOHOL DEHYDROGENASES

The alcohol dehydrogenases were investigated with a view to determining the number and specificity of the enzymes. Of particular interest was the question of whether the same or different enzymes acted on mono-ols, α,ω-diols and ω-hydroxyfatty acids. The alcohol dehydrogenases in a 100 000g (for 90min.) supernatant fraction from hexadecane-grown cells was subjected to analytical FPLC on a polyethyleneimine (PEI) ion-exchange column (Bio-Rad). The supernatant fraction was very coarse and would not pass through a 0.45μ pore-size filter. Therefore the supernatant was 'cleaned-up' prior to loading on the column. The supernatant was mixed with 2 volumes of acetone/water (95:5 v/v) which had been precooled to -15°C. The mixture was stirred for 5 minutes then centrifuged at 48 000g for 10 min. at -10°C. The supernatant from this step was discarded and the protein pellet resuspended to approx. half the original volume in ice-cold Hepes buffer (20mM, pH 7.5) and was designated the "acetone pellet". The 'acetone pellet' was then chromatographed on PEI ion-exchange column (6 cm * 1.5 cm). Unbound protein was eluted with Hepes buffer (20mM, pH 7.5). Once the A_{280} of the column eluate had fallen to approx. zero, the gradient was started. Bound protein was eluted with a linear gradient of 0 to 1M KCl (in Hepes buffer 20mM, pH 7.5) over 10 minutes at a flow rate of 1.5 ml/min. and 0.75ml fractions were collected. The recovery of activity and the purification profile are shown in Table 4.8 and Fig. 4.6 respectively. The elution profile shows 2 large activity peaks; one NAD^+-dependent the

Figure 4.6.: Purification of the soluble alcohol dehydrogenases of hexadecane-grown *Corynebacterium* 7E1C

● = octanol dehydrogenase (NAD^+)

■ = 1,12-dodecanediol dehydrogenase (NAD^+)

▲ = 12-hydroxylaurate dehydrogenase (NAD^+)

○ = octanol dehydrogenase ($NADP^+$)

□ = 1,12-dodecanediol dehydrogenase ($NADP^+$)

△ = 12-hydroxylaurate dehydrogenase ($NADP^+$)

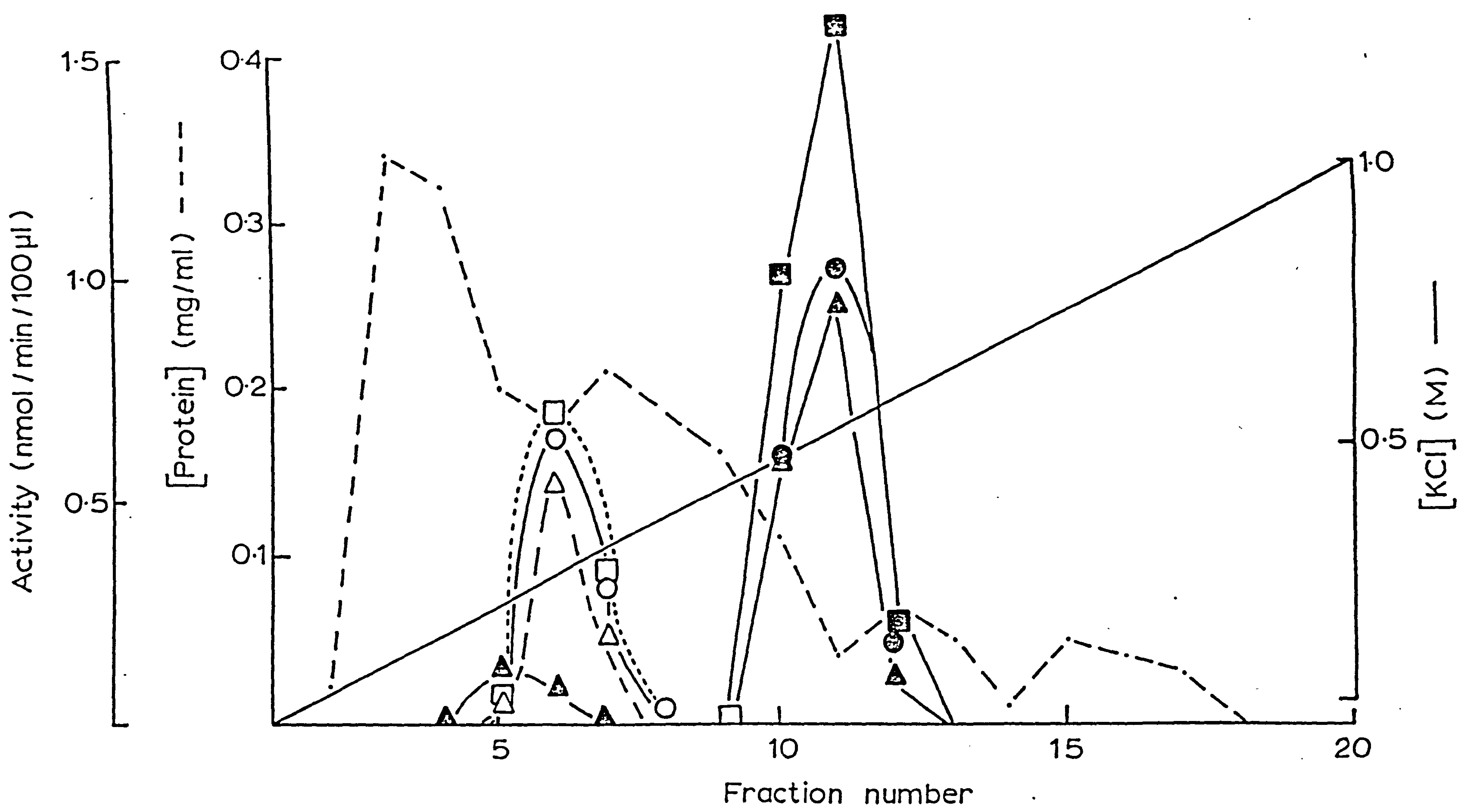

Activity (nmol/min/100µl)
1·5
1·0
0·5
[Protein] (mg/ml) ----
0·4
0·3
0·2
0·1
5
10
15
20
Fraction number
1·0
0·5
[KCl] (M) —

Table 4.8.: Purification of the soluble alcohol dehydrogenases of hexadecane-grown *Corynebacterium* 7E1C by ion-exchange chromatography on polyethylene imine

Recovery of activity

Step		Units' (% recovery)					
			NAD^+			$NADP^+$	
		8ol	12diol	12HO	8ol	12diol	12HO
100 000 g supernatant		435 (100)	665 (100)	372 (100)	166 (100)	213 (100)	80 (100)
Resuspended acetone pellet		318 (72)	504 (76)	295 (79)	105 (63)	143 (67)	57 (71)
PEI* column	loaded	23	27	22	8	11	5
	recovered	14	21	12	7	8	6
		(52)	(50)	(43)	(55)	(49)	(120)

Values in parenthesis are the overall recovery of activity expressed as a percentage

1 = 1 unit ≡ 1 nmol /min

* = only a smal fraction of the acetone pellet was loaded onto the PEI column

Assayed in potassium phosphate buffer (0.8M, pH 8)

8ol = octanol

12diol = 1,12-dodecanediol

12HO = 12-hydroxylaurate

Table 4.8. contined on next page

Table 4.8.: Purification of the soluble alcohol dehydrogenases of hexadecane-grown *Corynebacterium* 7E1C by ion-exchange chromatography on polyethylene imine

Purification of activity

Step		Specific activity² (Fold purification)					
		NAD⁺			NADP⁺		
		8ol	12diol	12HO	8ol	12diol	12HO
100 000 g supernatant		6.8 (1)	10.3 (1)	5.8 (1)	2.6 (1)	3.3 (1)	1.2 (1)
Resuspended acetone pellet		6.2 (0.9)	9.8 (0.95)	5.7 (0.98)	2.0 (0.77)	2.8 (0.85)	1.1 (0.92)
PEI* column	loaded	6.2	9.8	5.7	2.0	2.8	1.1
	recovered	258 (38)	393 (38)	233 (40)	35 (14)	37 (11)	30 (25)

Values in parenthesis are the overall fold-purification

2 = nmol/min/mg

* = only a small fraction of the acetone pellet was loaded onto the PEI column

Assayed in potassium phosphate buffer (0.8M, pH 8)

8ol = octanol

12diol = 1,12-dodecanediol

12HO = 12-hydroxylaurate

other $NADP^+$-dependent. Both peaks are active with 12-hydroxylaurate, 1,12-dodecanediol and octanol. Within each peak the activity with all 3 alcohols are superimposable which indicates that each fraction contains a single enzyme active with all 3 alcohols. There is also a minor NAD^+-dependent peak that displays activity only with 12-hydroxylaurate. The yield for all activities ranges from 43% to 59% with the exception of $NADP^+$-dependent 12-hydroxylaurate dehydrogenase activity for which the overall recovery was 120%. The very high recovery of this latter activity is due to a very high recovery from the column step, rather than from the preparation of the "acetone pellet", and is probably due to the difficulty in accurately measuring such a low activity (of the 6 activities measured this was the lowest). The NAD^+-dependent activities were purified approx. 40-fold whilst the $NADP^+$-dependent activities were purified approx. 10-fold. The lower purification factor for the $NADP^+$-dependent activities reflects the fact that they eluted with the main protein peak.

4.7. pH OPTIMUM OF 12-HYDROXYLAURATE DEHYDROGENASE ACTIVITY

The pH optimum for both NAD^+-dependent and $NADP^+$-dependent activities was determined. For both activities the buffers used were Hepes (pH 6.5 to 8.5), glycine-NaOH (pH 8.5 to 10.5) and sodium carbonate-sdium hydrogencarbonate (pH 10.5 to 11.5). All buffers were 50mM with respect to the buffer component and additionally contained 0.8M Na_2SO_4. These buffers were used so that the activating salt was the same at each pH. Potassium phosphate buffer could have been used over the pH range 6.5 to 8.0 rather than Hepes (50mM + 0.8M Na_2SO_4) but then the degree of ionization of the activating salt would have changed with pH which may have affected the activation effect independently of any pH effect on the enzyme itself.

Figure 4.7.: pH optimum of NAD^+-dependent 12-hydroxylaurate dehydrogenase activity in a crude cell-free extract of hexadecane-grown *Corynebacterium* 7E1C

All buffers contained Na_2SO_4 (0.8M)

○ = Hepes (50mM)

● = glycine (50mM)

□ = sodium carbonate - sodium hydrogen carbonate (50mM)

100% = 38.4 nmol/min/mg

pH optimum = 10

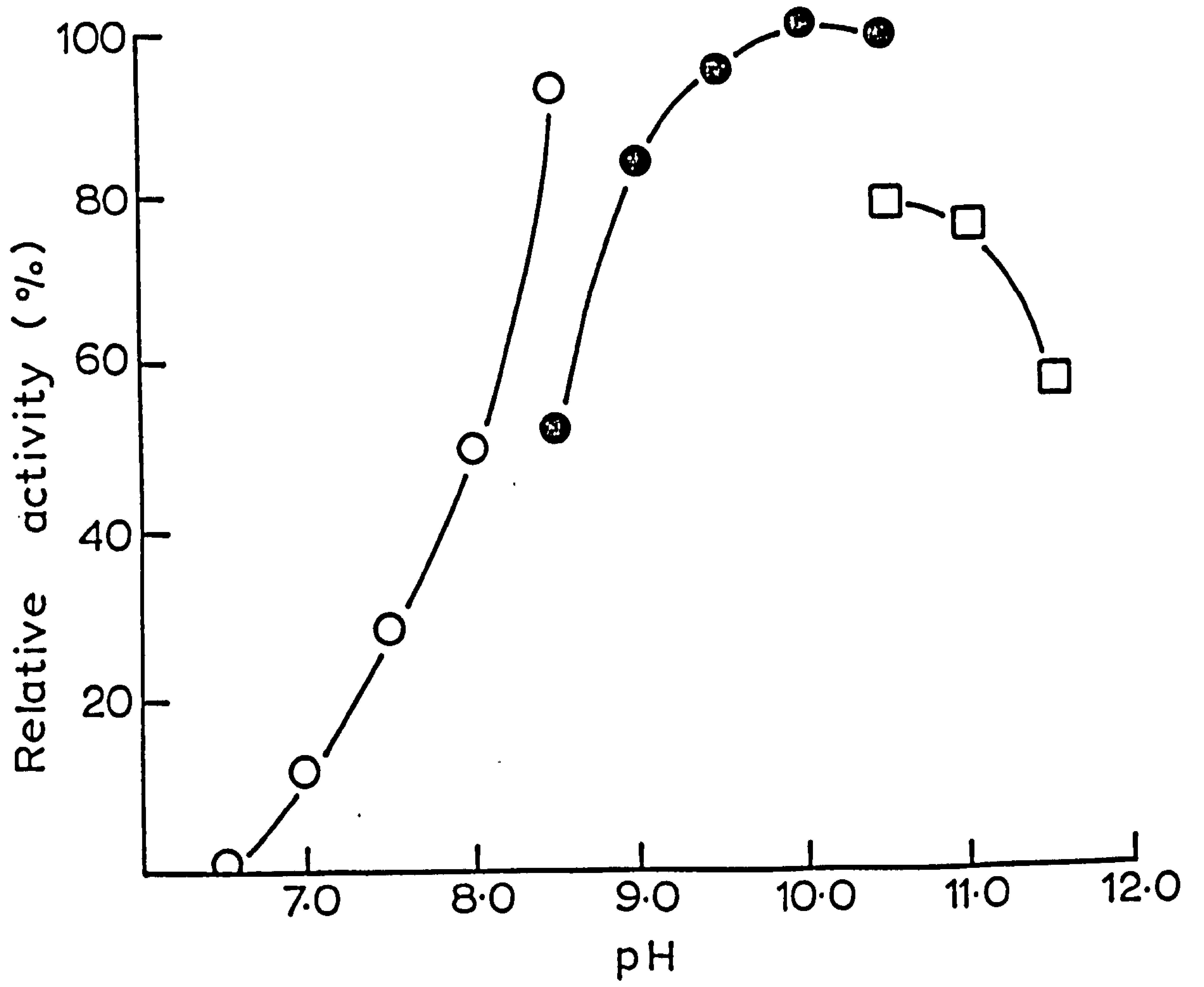

100
80
60
40
20
Relative activity (%)
7.0
8·0
9·0
10·0
11·0
12·0
pH

Figure 4.8.: pH optimum of $NADP^+$-dependent 12-hydroxylaurate dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

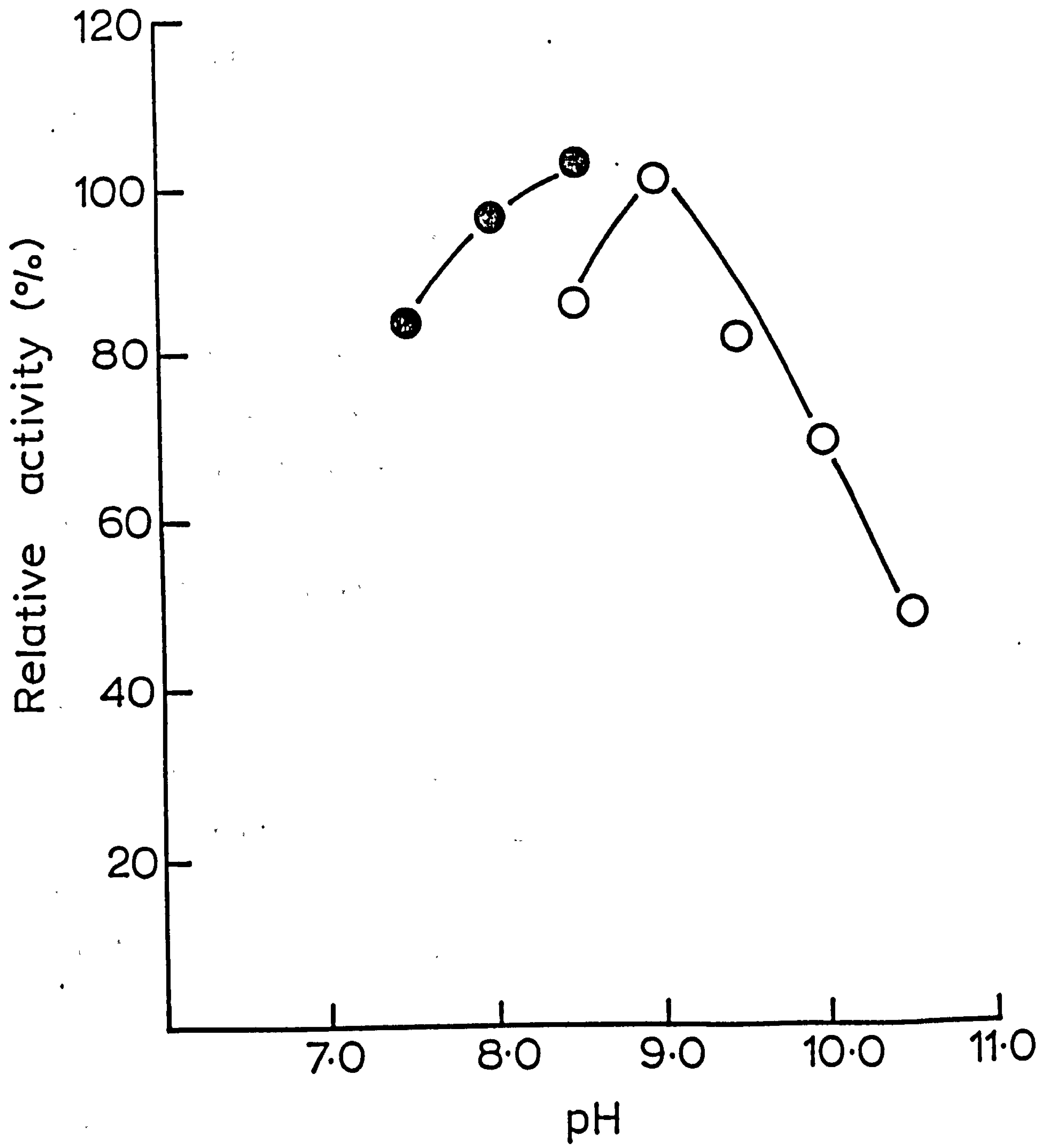
All buffers contained Na_2SO_4 (0.8M)

● = Hepes (50mM)

○ = glycine (50mM)

100% = 4.7 nmol/min/mg

pH optimum = 9



Relative activity (%)
120
100
80
60
40
20
7·0
8·0
9·0
10·0
11·0
pH

The pH optima were 10.0 for the NAD^+-dependent activity and 9.0 for the $NADP^+$-dependent activity (Figs. 5.7 and 5.8). As a comparison the pH optimum of the $NADP^+$-dependent ω-hydroxyfatty acid dehydrogenase of wound-healing potato tuber is pH 9.5. The pH optimum of the NAD^+-dependent 10-hydroxydecanoate dehydrogenase of rabbit liver is 10.5 (Kamei *et al* 1964) and that of the corresponding hog liver enzyme is 10.0 (Mitz and Heinrikson 1961).

4.8. SUBSTRATE SPECIFICITY OF ALCOHOL DEHYDROGENASE

The substrate specificity of alcohol dehydrogenase activity was determined with two different assay conditions. The substrate specificities of both NAD^+-dependent and $NADP^+$-dependent activities were determined in potassium phosphate buffer (0.8M, pH 8.0). The specificity of the NAD^+-dependent activities was also determined in glycine buffer (50mM, pH 10.0 containing 0.8M Na_2SO_4), since of the buffers tested, this one gave the highest rates of NAD^+-dependent activity. The substrate specificities with respect to mono-ols and α,ω-diols in potassium phosphate buffer (0.8M, pH 8.0) with NAD^+ and $NADP^+$ as cofactor are shown in Figs. 4.9 and 4.10. The NAD^+-dependent and $NADP^+$-dependent activities display essentially the same substrate specificity. With both NAD^+- and $NADP^+$-dependent activities the optimum mono-ol is octanol whilst 1,12-dodecanediol is the optimum α,ω-diol. With both mono-ols and diols the activity decreased rapidly on either side of the optimum, no activity being detected with ethanol or hexadecanol. The lack of activity with hexadecanol is somewhat surprising since the cell-free extract used for these expriments was prepared from hexadecanol-grown cells. This lack of activity with hexadecanol may, in part, be due to the poor solubility of hexadecanol compared to the shorter chain-length alcohols. It was difficult to

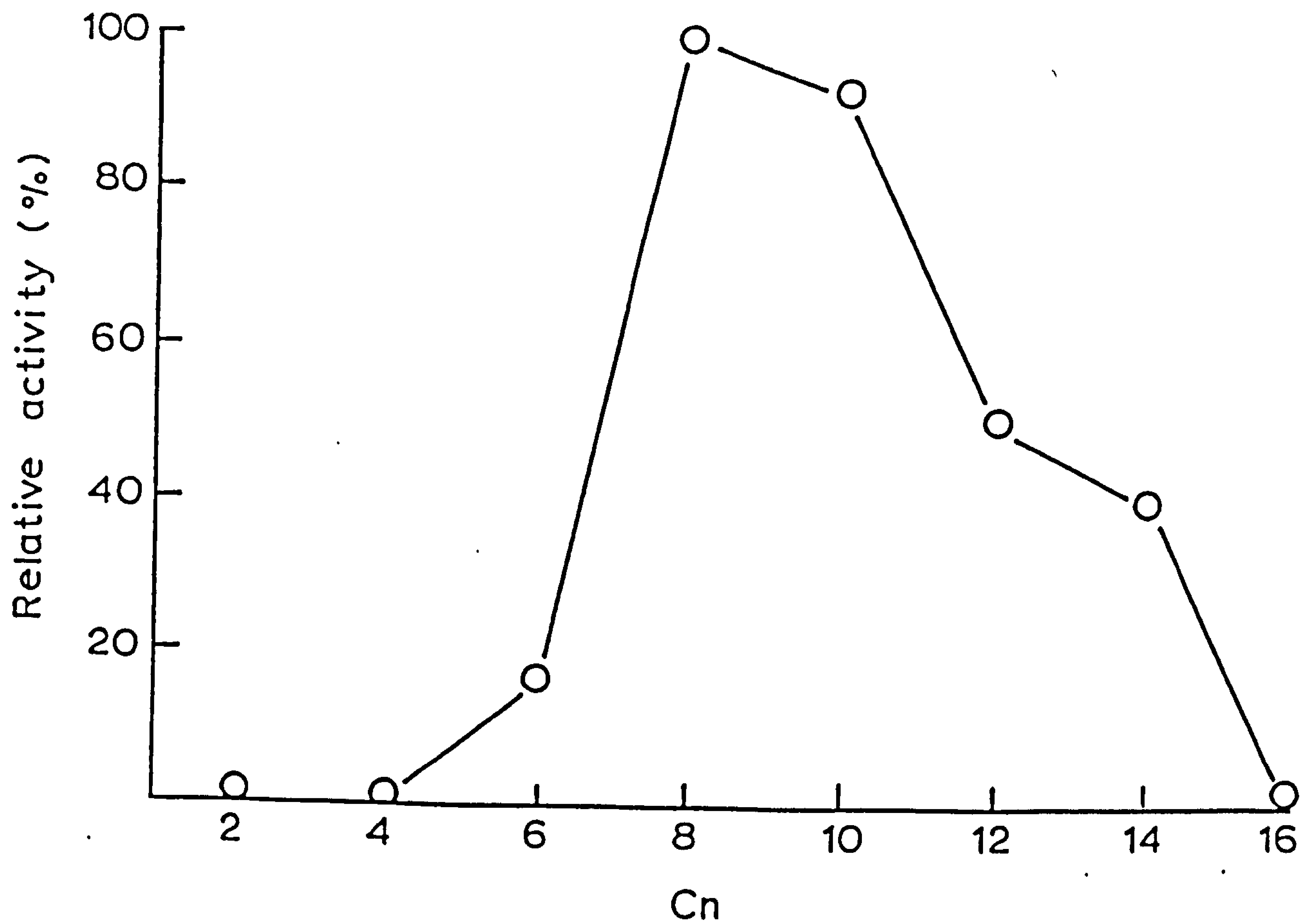
Figure 4.9.: Substrate specificity of NAD^+-dependent alcohol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

[alcohol] = 330 μM

100% = 6.0 nmol/min/mg (mono-ol)

100% = 9.8 nmol/min/mg (diol)


a) MONO-OL
Relative activity (%)
100
80
60
40
20
2
4
6
8
10
12
14
16
Cn

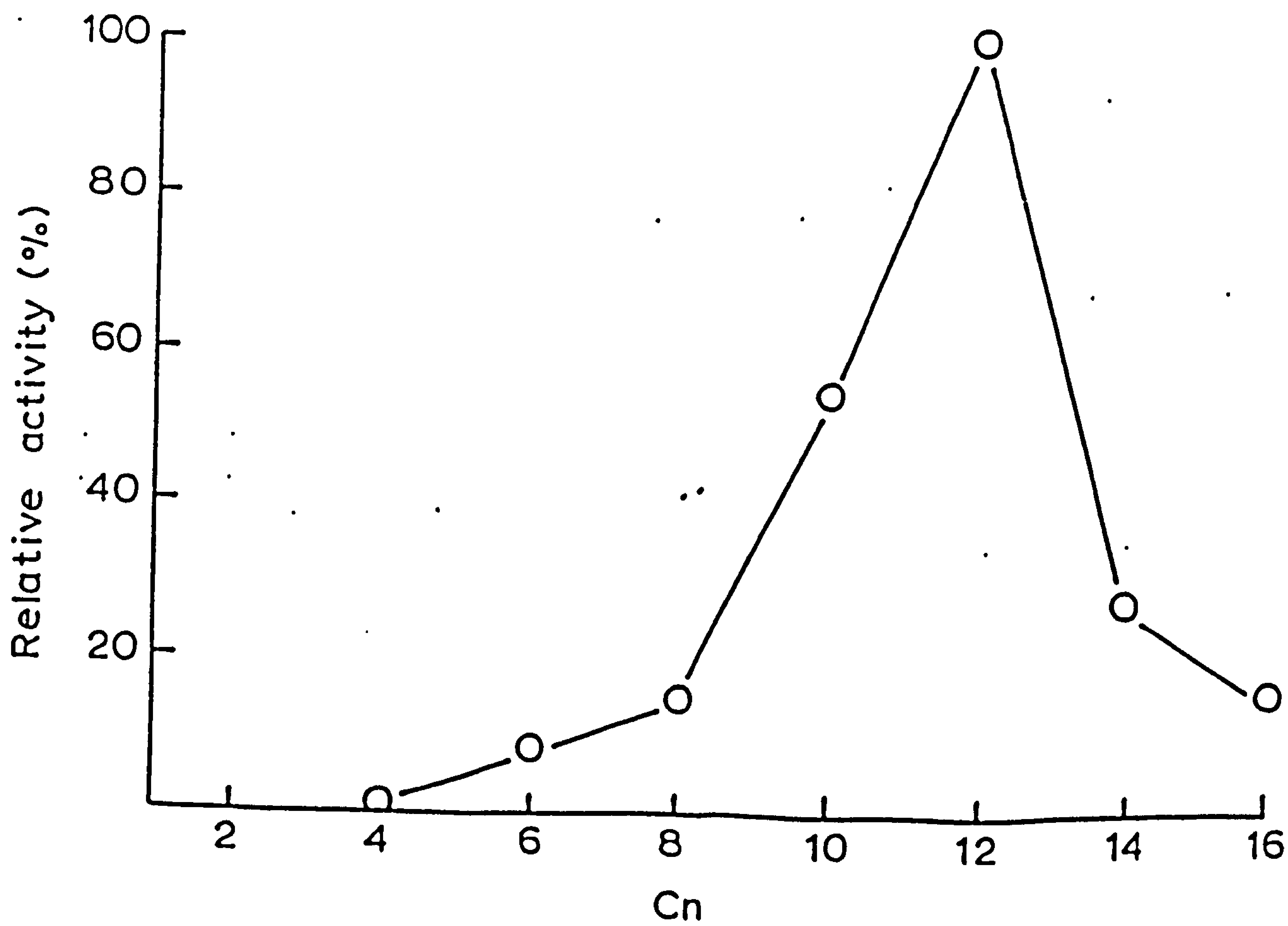
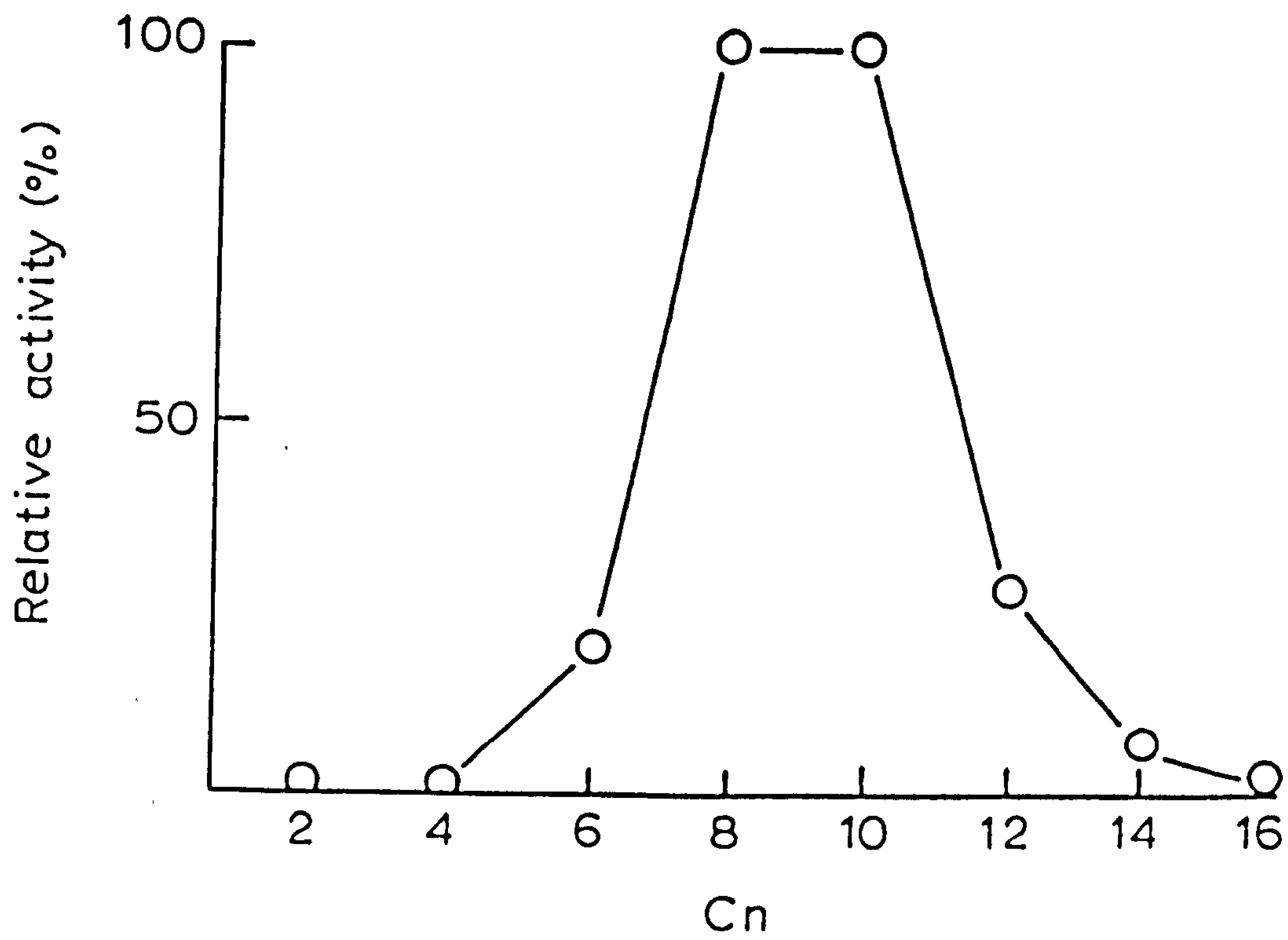

b) DIOL
Relative activity (%)
100
80
60
40
20
2
4
6
8
10
12
14
16
Cn

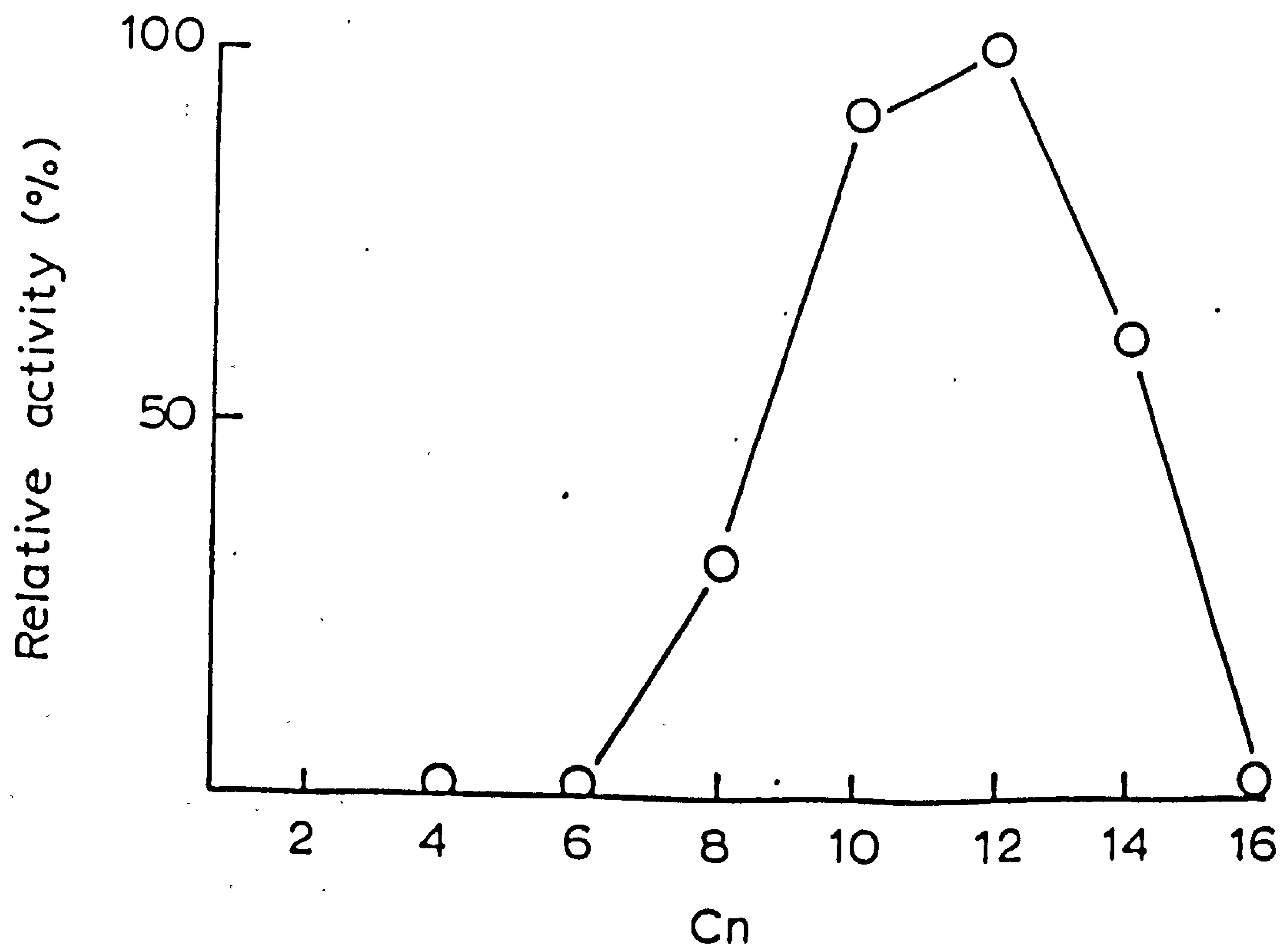
Figure 4.10.: Substrate specificity of $NADP^{+}$-dependent alcohol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

[alcohol] = 330 µM

100% = 5.9 nmol/min/mg (mono-ol)

100% = 3.8 nmol/min/mg (diol)


a) MONO-OLS
100
50
Relative activity (%)
2
4
6
8
10
12
14
16
Cn


b) DIOLS
100
50
Relative activity (%)
2
4
6
8
10
12
14
16
Cn

determine the specificity with respect to ω-hydroxyfatty acids under these assay conditions due to the low rates observed with these substrates and the problem of not being able to add large amounts of cell-free extract to assays due to the problem of high endogenous rates of $NAD(P)^+$ reduction (see section 4.1.). This problem was compounded with the NAD^+-dependent ω-hydroxyfatty acid dehydrogenase activity by the high K_m for these substrates (0.35mM for 12-hydroxylaurate, see below). Rates obtained with 10-hydroxydecanoate were about 65% of those obtained with 12-hydroxylaurate whilst those obtained with 16-hydroxypalmitate were approx. 20% that of 12-hydroxylaurate. The kinetic parameters of alcohol dehydrogenase activity towards 12-hydroxylaurate, 1,12-dodecanediol and octanol with either NAD^+ or $NADP^+$ as cofactor were determined in crude cell-free extracts of hexadecanol-grown cells in potassium phosphate (0.8M, pH 8.0) as buffer. The Lineweavwe-Burk plots are shown in Figs. 4.11 to 4.16 and the kinetic parameters are sumarized in Table 4.9. It was not possible to determine the K_m for NAD^+-dependent octanol dehydrogenase activity as the Lineweaver-Burk plot was highly curved (Fig. 4.11). This could indicate the presence of more than one NAD^+-dependent octanol dehydrogenase, it could be due an allosteric interaction (positive homotropic) or it may be due to micelle formation. Purification studies have revealed the presence of only one NAD^+-dependent octanol dehydrogenase so multiple enzymes does not seem a likely explanation. Since it appears that there is only one NAD^+-dependent octanol dehydrogenase active with all 3 alcohol substrates then one might expect that if allosteric behaviour was exhibited with octanol then similar behaviour would also be observed with 12-hydroxylaurate and 1,12-dodecanediol. Micelle formation may explain the curved Lineweaver-Burk plots. If the curvature is indeed due to micelle formation, then, since maximum rates and the steepest curve are observed

Figure 4.11.: Lineweaver-Burk plot for NAD^+-dependent octanol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

K_m = ?

V_{max} = 6.3 nmol/min/mg

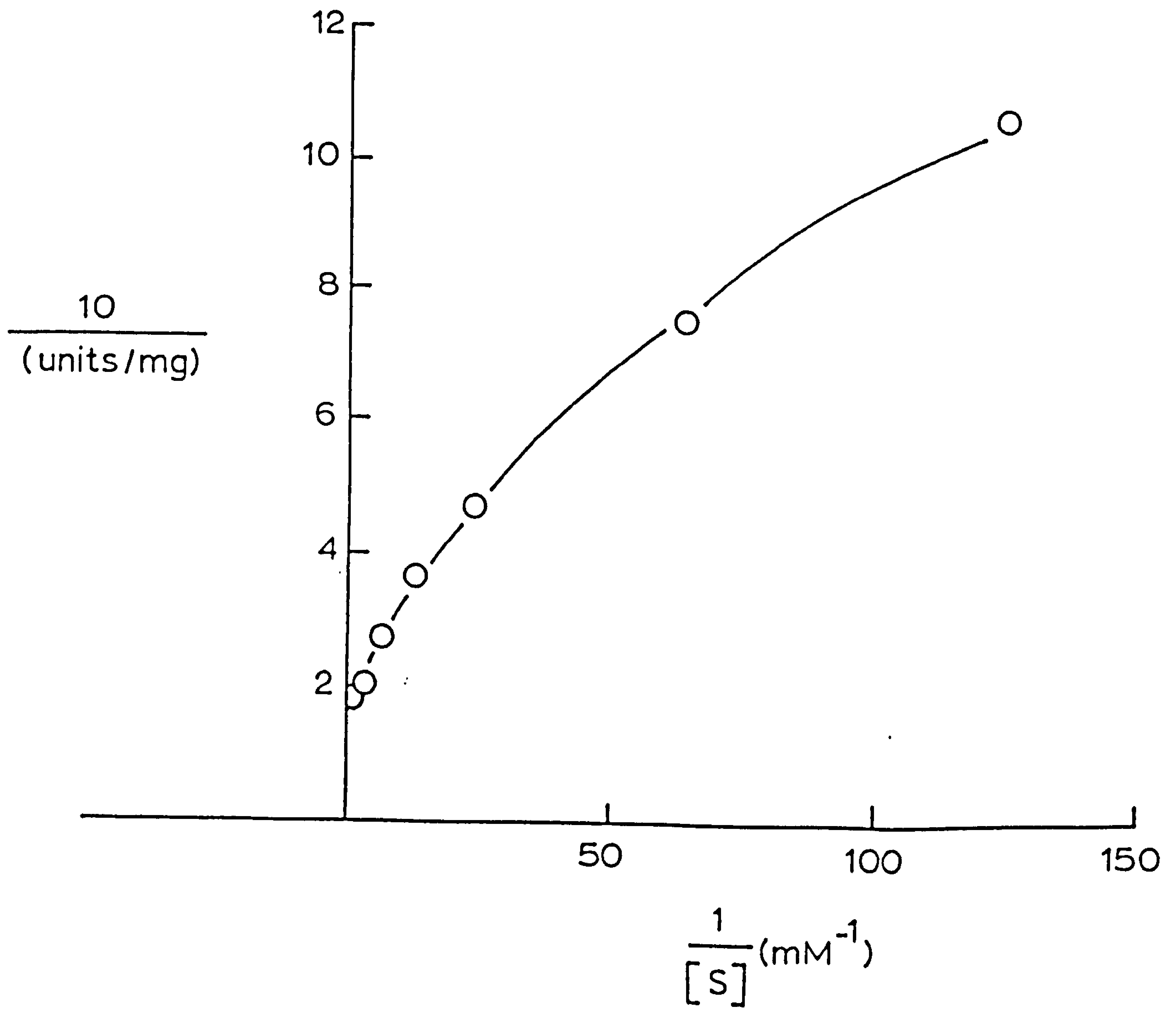

12
10
8
6
4
2
10
(units/mg)
50
100
150
1
[S]
(mM-1)

Figure 4.12.: Lineweaver-Burk plot for NAD^+-dependent 1,12-dodecanediol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

K_m = 67 µM

V_{max} = 10 nmol/min/mg

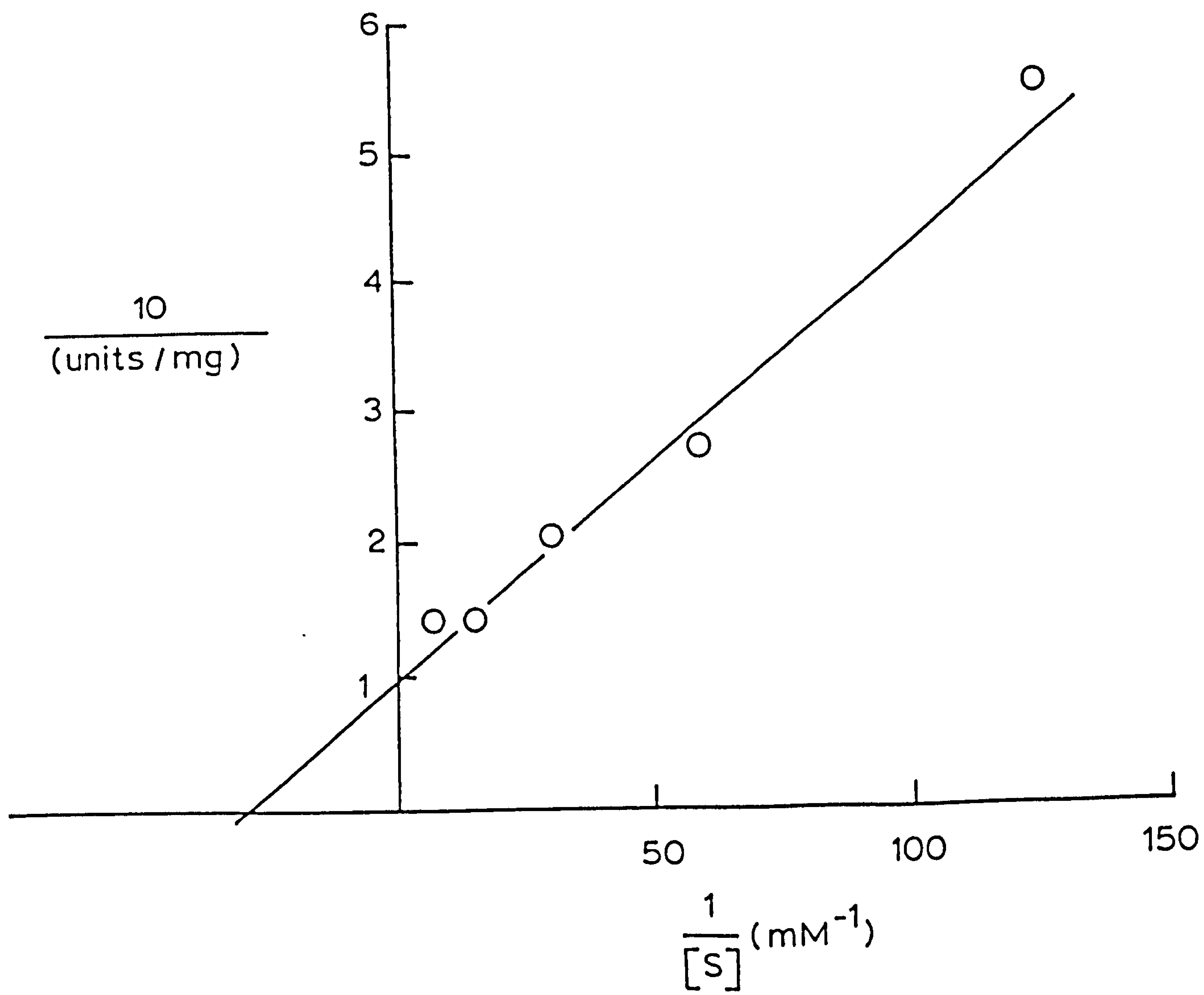

6
5
4
3
2
1
10
(units / mg)
50
100
150
1
[S]
(mM-1)

Figure 4.13.: Lineweaver-Burk ploy for NAD^+-dependent 12-hydroxylaurate dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

K_m = 330 μM

V_{max} = 6.8 nmol/min/mg

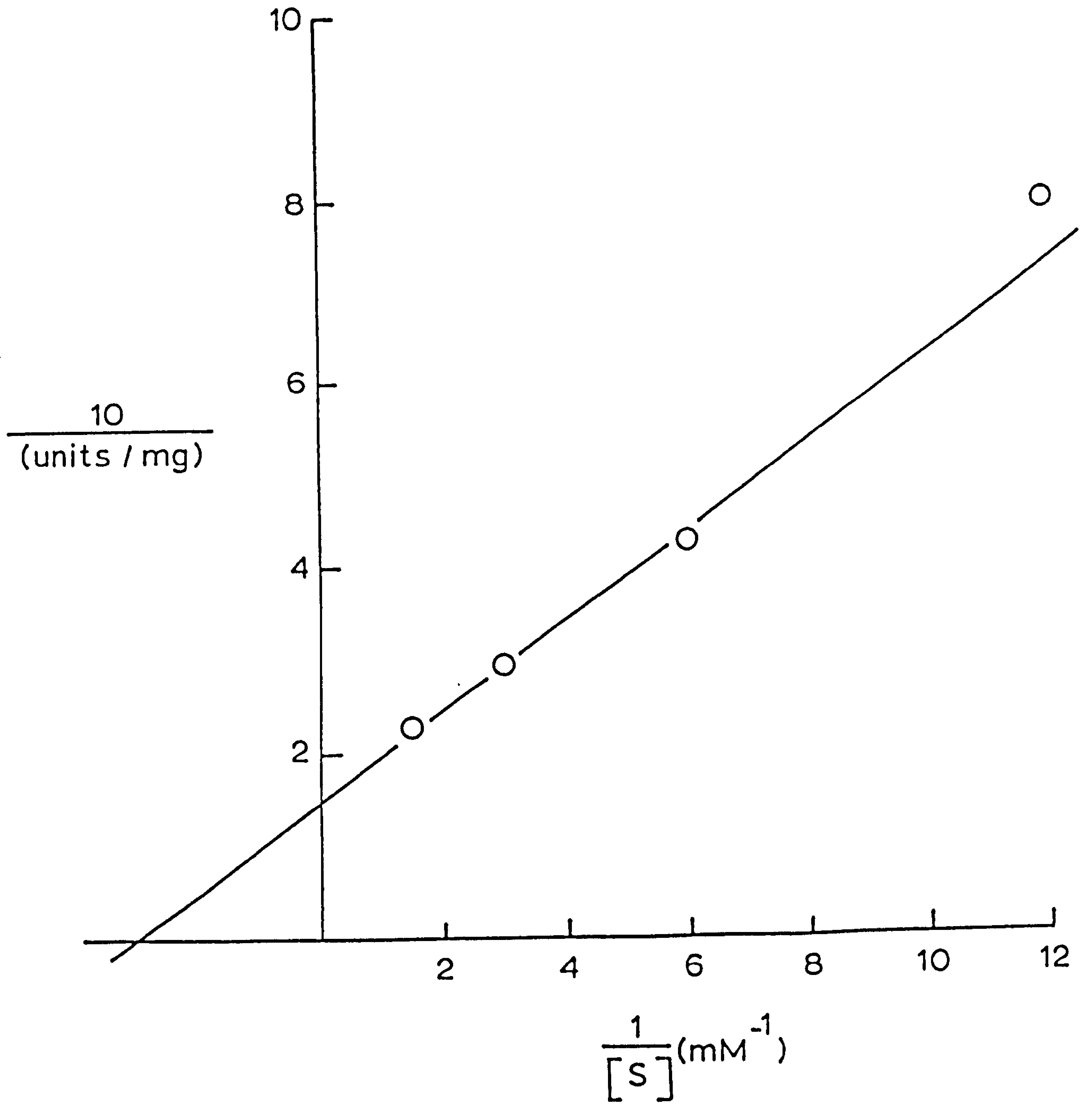
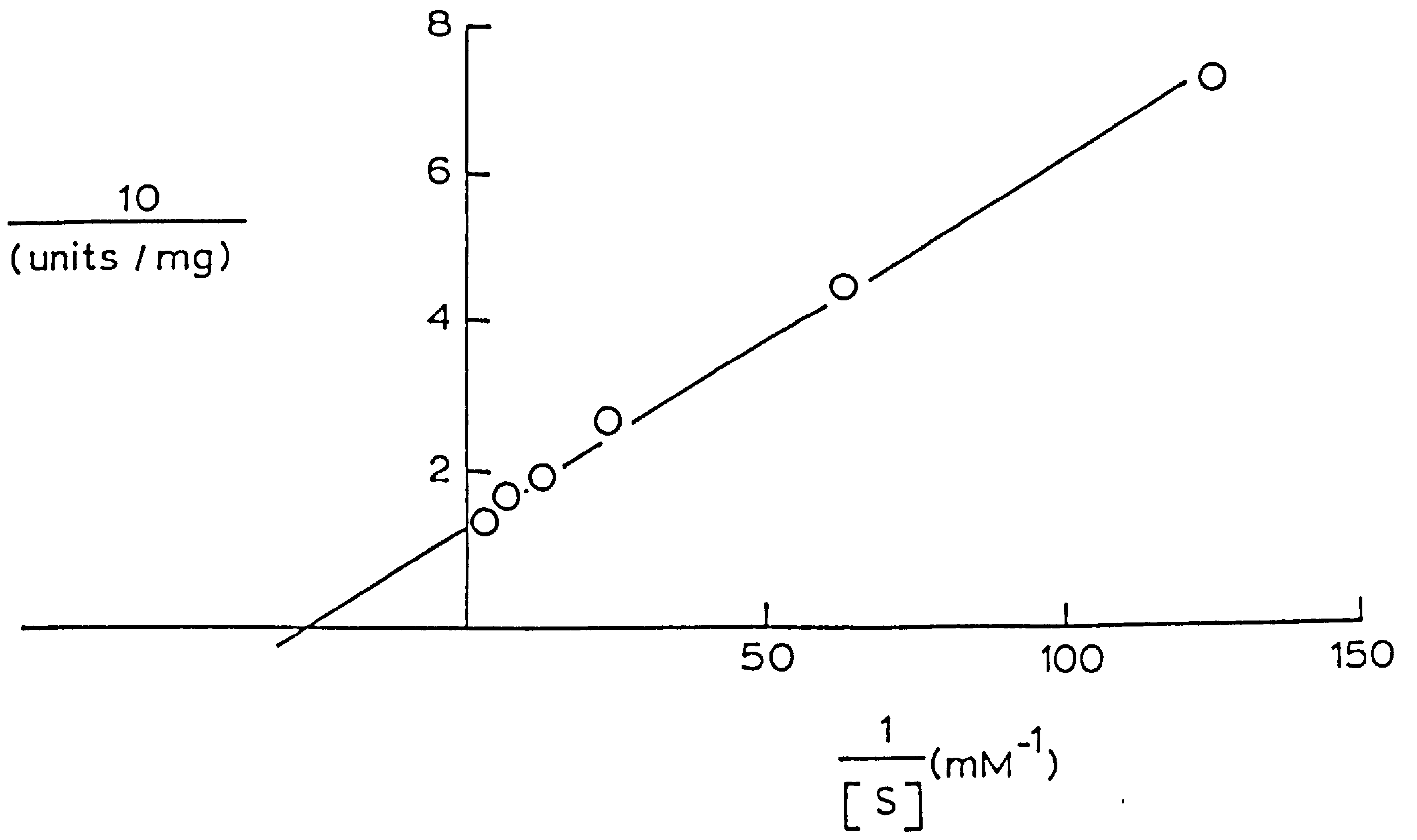

10
8
6
4
2
10
(units / mg)
2
4
6
8
10
12
1
[S]
(mM⁻¹)

Figure 4.14.: Lineweaver-Burk plot for $NADP^+$-dependent octanol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

K_m = 73 μM

V_{max} = 7.7 nmol/min/mg



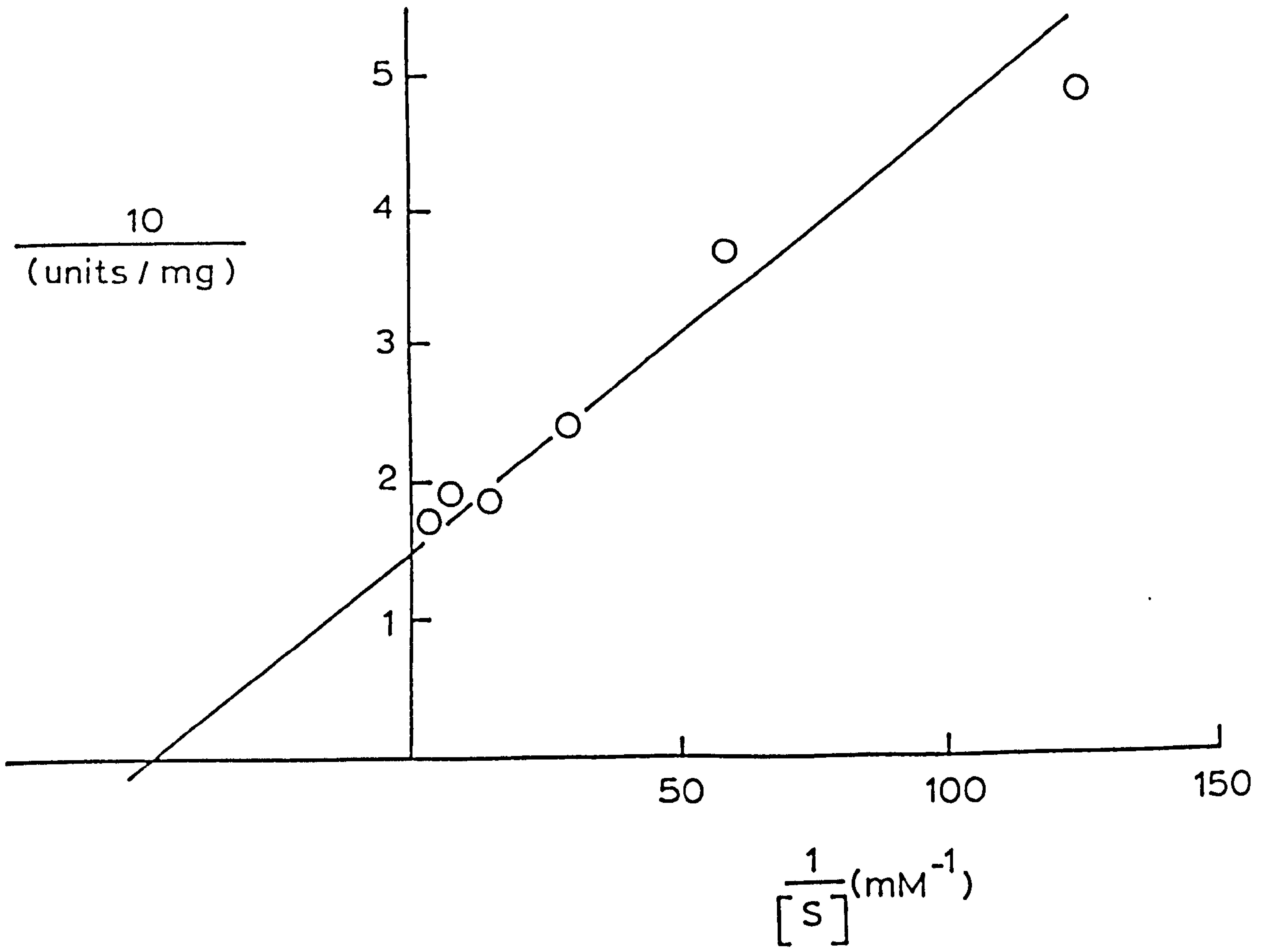
8
6
4
2
10
(units / mg)
50
100
150
1
[S]
(mM-1)

Figure 4.15.: Lineweaver-Burk plot for $NADP^+$-dependent 1,12-dodecanediol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

K_m = 21 µM

V_{max} = 6.7 nmol/min/mg



10
(units / mg)
5
4
3
2
1
50
100
150
1/[S] (mM⁻¹)

Figure 4.16.: Lineweaver-Burk ploy for NADP$^+$–dependent 12-hydroxylaurate dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (0.8M, pH 8)

K_m = 71 µM

V_{max} = 3.4 nmol/min/mg

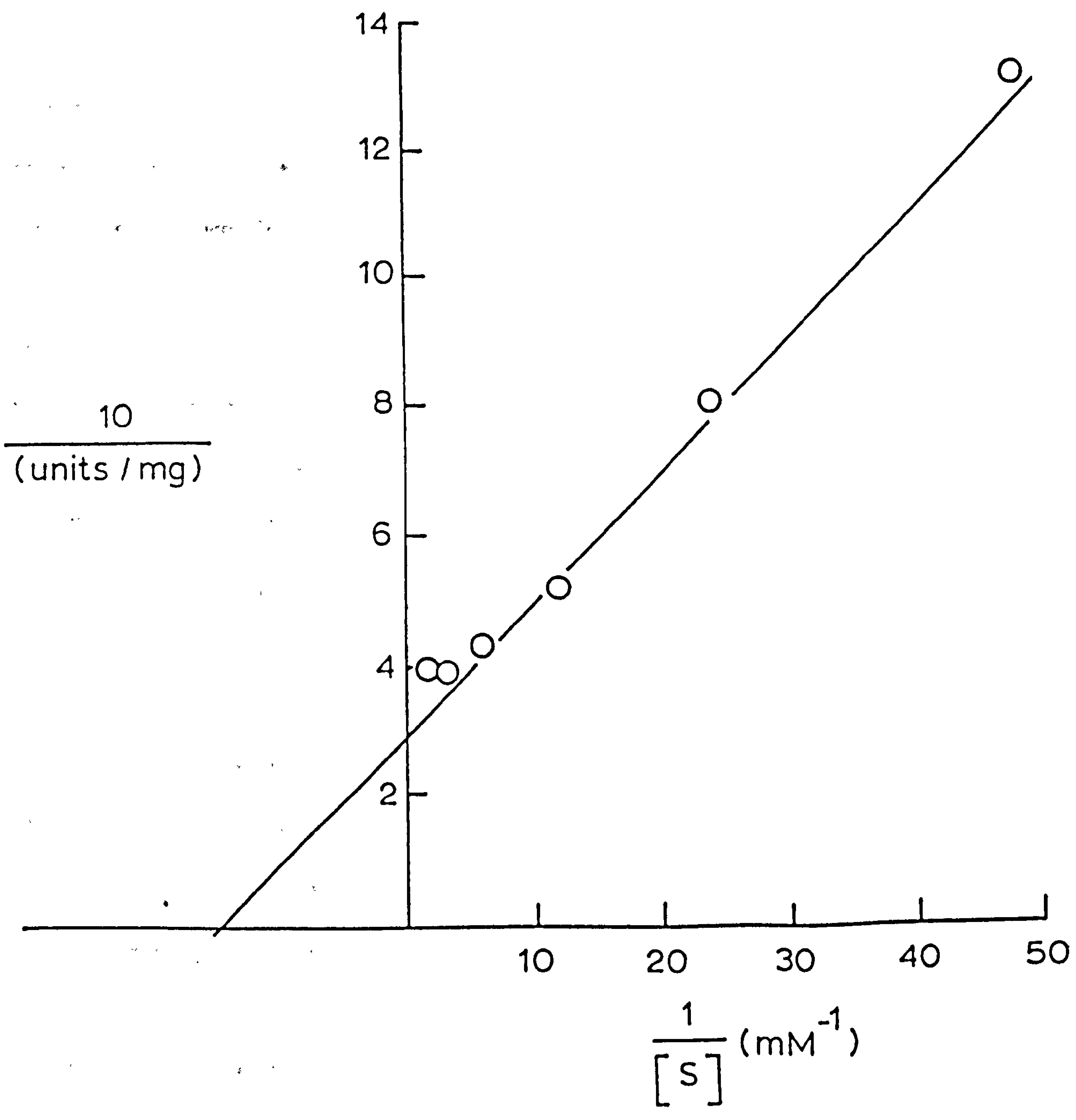

14
12
10
8
6
4
2
10
(units / mg)
10
20
30
40
50
1
[S]
(mM⁻¹)

Table 4.9. Summary of the kinetic parameters of the alcohol dehydrogenase activities in crude cell-free extracts of hexadecanol-grown *Corynebacterium* 7E1C

Cofactor	Substrate	K_m (μM)	V_{max} (nmol/min/mg)
NAD^+	octanol	?	6.3
NAD^+	dodecanediol	67	10.0
NAD^+	12-hydroxylaurate	330	6.8
$NADP^+$	octanol	73	7.7
$NADP^+$	dodecanediol	21	6.7
$NADP^+$	12-hydroxylaurate	71	3.4

Assayed in potassium phosphate buffer (0.8M, pH 8)

? = could not be determined due to highly curved Lineweaver-Burk plot

at high concentrations this would imply that the enzyme is active with micellar octanol but that the K_m is higher for the micellar form than for monomer form of the substrate.

Inspection of the kinetic parameters shows that the $NADP^+$-dependent activities have lower K_m values than the corresponding NAD^+-dependent activities. In particular the K_m for NAD^+-dependent 12-hydroxylaurate dehydrogenase activity (350μM) is approx. 4.9-fold higher than the corresponding $NADP^+$-dependent activity (71μM).

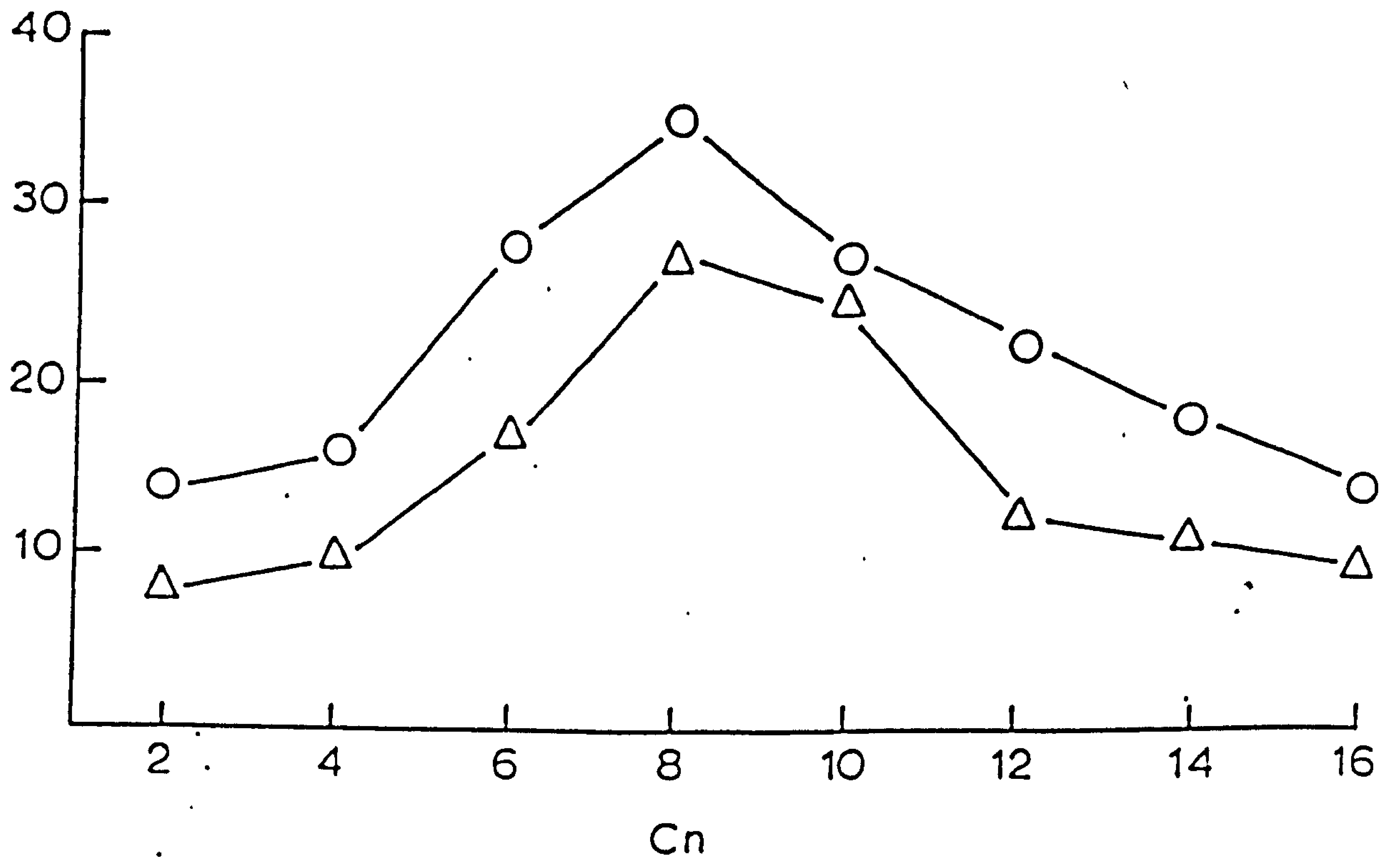
The substrate specificity of NAD^+-dependent activities, in glycine buffer (50mM, pH 10.0 containing 0.8M Na_2SO_4), with respect to mono-ols and α,ω-diols are shown in Fig. 4.17 and Table 4.10. As with the assays performed in potassium phosphate buffer (0.8M, pH 8.0) the optimum mono-ol and α,ω-diol are octanol and 1,12dodecanediol respectively. However, the substrate specificities are significantly broader under these assay conditions. Under these conditions activity is observed with C_2 to C_{16} mono-ols; ethanol and hexadecanol displaying approx. 50% of the activity observed with octanol. α,ω-diol dehydrogenase activity was only investigated with C_{10} to C_{16} substrates but, as was the case when asayed in potassium phosphate buffer, 1,12-dodecanediol was clearly the optimal α,ω-diol substrate. Since ω-hydroxyfatty acids are obligatory intermediates in the formation of dicarboxylic acids the kinetics of ω-hydroxyfatty acid dehydrogenase activity were investigated. It was possible to investigate ω-hydroxyfatty acid dehydrogenase in detail under these assay conditions due to the higher rates and lower K_m as compared to assays performed inpotassium phosphate buffer (0.8M, pH 8.0). Lineweaver-Burk plots obtained for 10-hydroxydecanoate, 12-hydroxylaurate and 16-hydroxypalmitate dehydrogenase activities are shown in Figs. 4.18, 4.19 and 4.20. The kinetic parameters are sumarized in Table 4.10. All 3 substrates displayed very similart values of V_{max} whereas K_m decreased significantly with increasing chain-length. With

Figure 4.17.: Substrate specificity of NAD^+-dependent alcohol dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in glycine buffer (50mM, pH 10) containing 0.8M Na_2SO_4

○ = 330 µM alcohol

△ = 67 µM alcohol


a) MONO-OL
40
30
20
10
2
4
6
8
10
12
14
16
Cn
nmol/min/mg

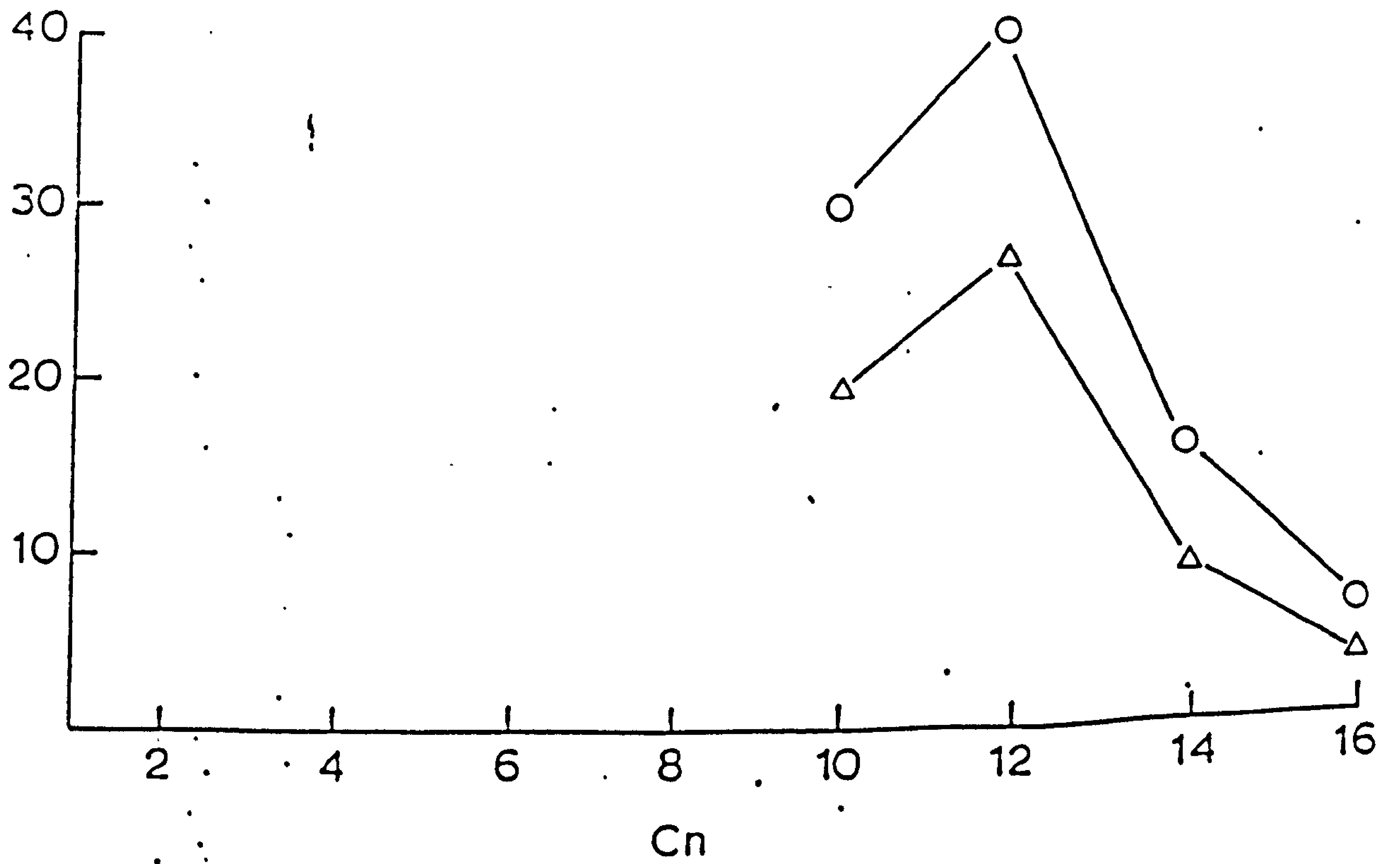
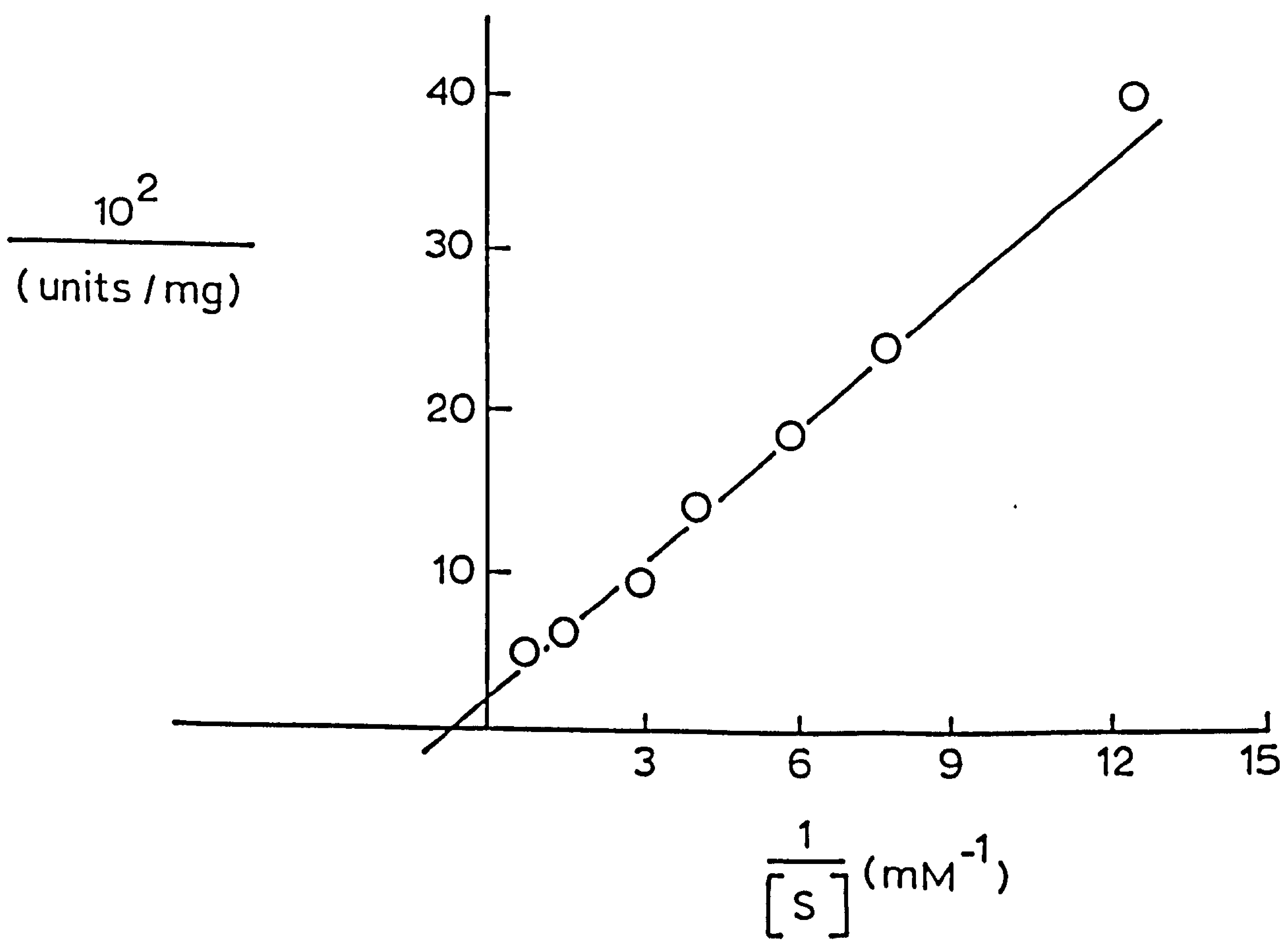

b) DIOL
40
30
20
10
2
4
6
8
10
12
14
16
Cn

Figure 4.18.: Lineweaver-Burk plot for NAD^+-dependent 10-hydroxydecanoate dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in glycine buffer (50mM, pH 10)

K_m = 1520 µM

V_{max} = 50 nmol/min/mg



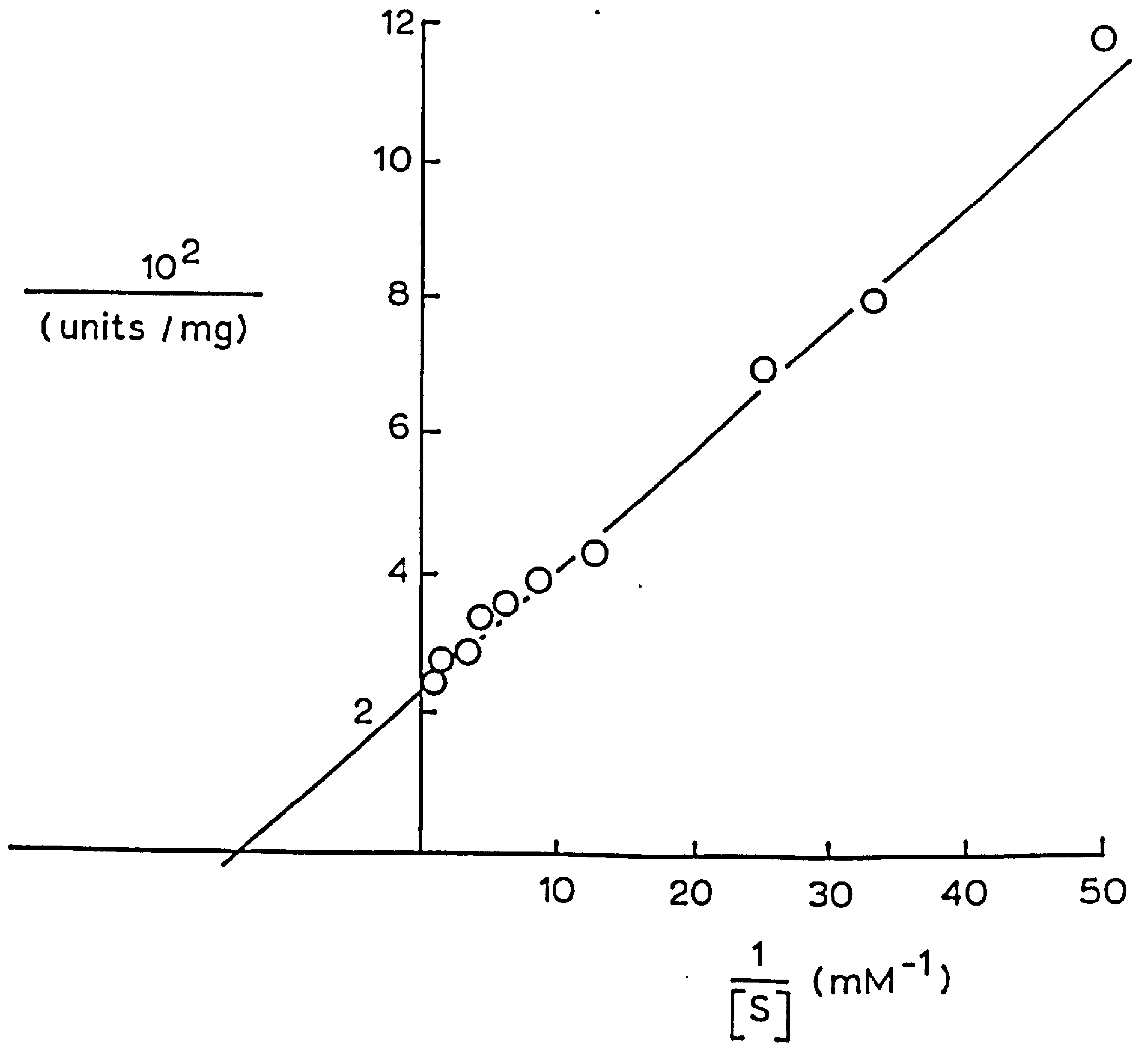
10^2
(units / mg)
40
30
20
10
3
6
9
12
15
$\frac{1}{[S]}$ (mM^{-1})

Figure 4.19.: Lineweaver-Burk plot for NAD^+-dependent 12-hydroxylaurate dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in glycine buffer (50mM, pH 10)

K_m = 77 μM

V_{max} = 44 nmol/min/mg



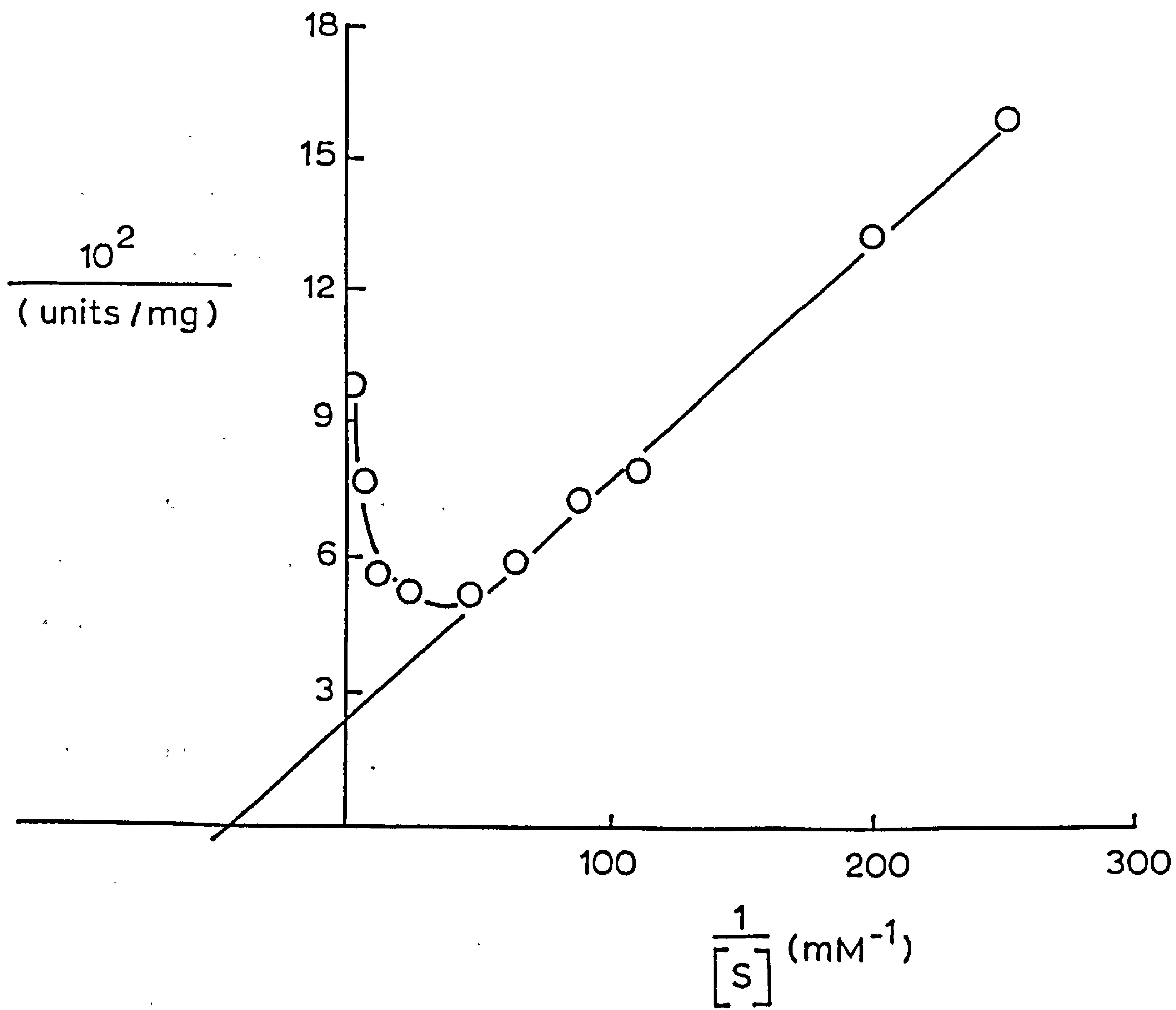
12
10
8
6
4
2
10^2
(units /mg)
10
20
30
40
50
1/[S] (mM^-1)

Figure 4.20.: Lineweaver-Burk plot for NAD^+-dependent 16-hydroxypalmitate dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in glycine buffer (50mM, pH 10)

K_m = 22 μM

V_{max} = 42 nmol/min/mg


18
15
12
9
6
3
$\frac{10^2}{(\text{units/mg})}$
100
200
300
$\frac{1}{[S]}$ (mM^{-1})

16-hydroxypalmitate there is pronounced substrate inhibition at above 50 μM 16-hydroxypalmitate.

The significance of alcohol dehydrogenase specificity with respect to dicarboxylic acid production in *Corynebacterium* 7E1C depends in part on the relative contribitions of the diol and fatty acid pathways to dicarboxylic acid production by this organism and this is currently unknown. With both NAD^+- and $NADP^+$-dependent activities 1,12-dodecanediol is a much better substrate than 1,16-hexadecanediol thus if the diol pathway makes a major contribution to dicarboxylic acid production in this organism then dicarboxylic acid production from C_{12} substrates might be favoured over that from C_{16} substrates. However, at the level of the ω-hydroxyfatty acid the similar values of V_{max} but lower K_m for 16-hydroxypalmitate (than for 12-hydroxylaurate) would mean that the relative rates of conversion of 12-hydroxylaurate and 16-hydroxypalmitate would be highly concentration dependent. However, since it is not known which are the rate limiting steps in the formation of dicarboxylic acids (the ω-hydroxylases are a likely candidate) no information is available as to the relative rates at which the various alcohol intermediates are likely to be formed. Thus the contribution of alcohol dehydrogenase specificity to the specificity of dicarboxylic acid production is unclear. However, these results demonstrate that *Corynebacterium* 7E1C posseses the necessary alcohol dehydrogenase activities to form both DC_{12} and DC_{16} via both the fatty acid and via the α,ω-diol.

Table 4.10.: Summary of the kinetic parameters of NAD^+-dependent ω-hydroxyfatty acid dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Substrate	K_m (μM)	V_{max} (nmol/min/mg)
10-hydroxydecanoate	1520	50
12-hydroxylaurate	77	44
16-hydroxypalmitate	22	42

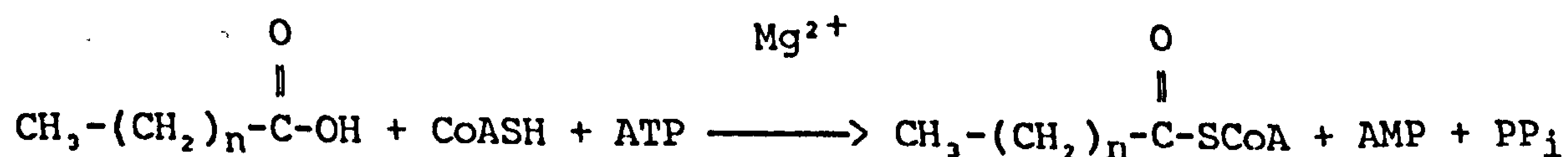
Assayed in glycine buffer (50mM, pH 10) containing 0.8M Na_2SO_4

5. ACYL-CoA SYNTHETASE AND THIOESTERASE

Fatty acids must be activated to their corresponding acyl-CoA esters before they can be degraded via β-oxidation. Thus, the specificity of acyl-CoA synthetase activity was investigated to determine its role in dicarboxylic acid metabolism; in particular whether the specificity of this reaction plays a significant role in determining the specificity of dicarboxylic acid accumulation by *Corynebacterium* 7E1C. Thioesterase (acyl-CoA hydrolase) activity was also investigated. This is an enzyme of uncertain physiological function. The reasons for its study are 2-fold. Firstly, it was hoped to gain some insight as to its role in fatty acid metabolism in *Corynebacterium* 7E1C. Secondly, although the physiological function of thioesterase is obscure its presence in crude extracts is of important practical consequence when assaying acyl-CoA utilizing (eg: β-oxidation) and especially acyl-CoA producing (eg: acyl-CoA synthetase) enzymes or enzyme systems. In particular the presence of such an activity may affect the apparent activity and specificity of acyl-CoA synthetase. For this reason acyl-CoA synthetase and thioesterase are considered together.

5.1. ACYL-CoA SYNTHETASE ASSAY

Acyl-CoA synthetase catalyses the following reaction:



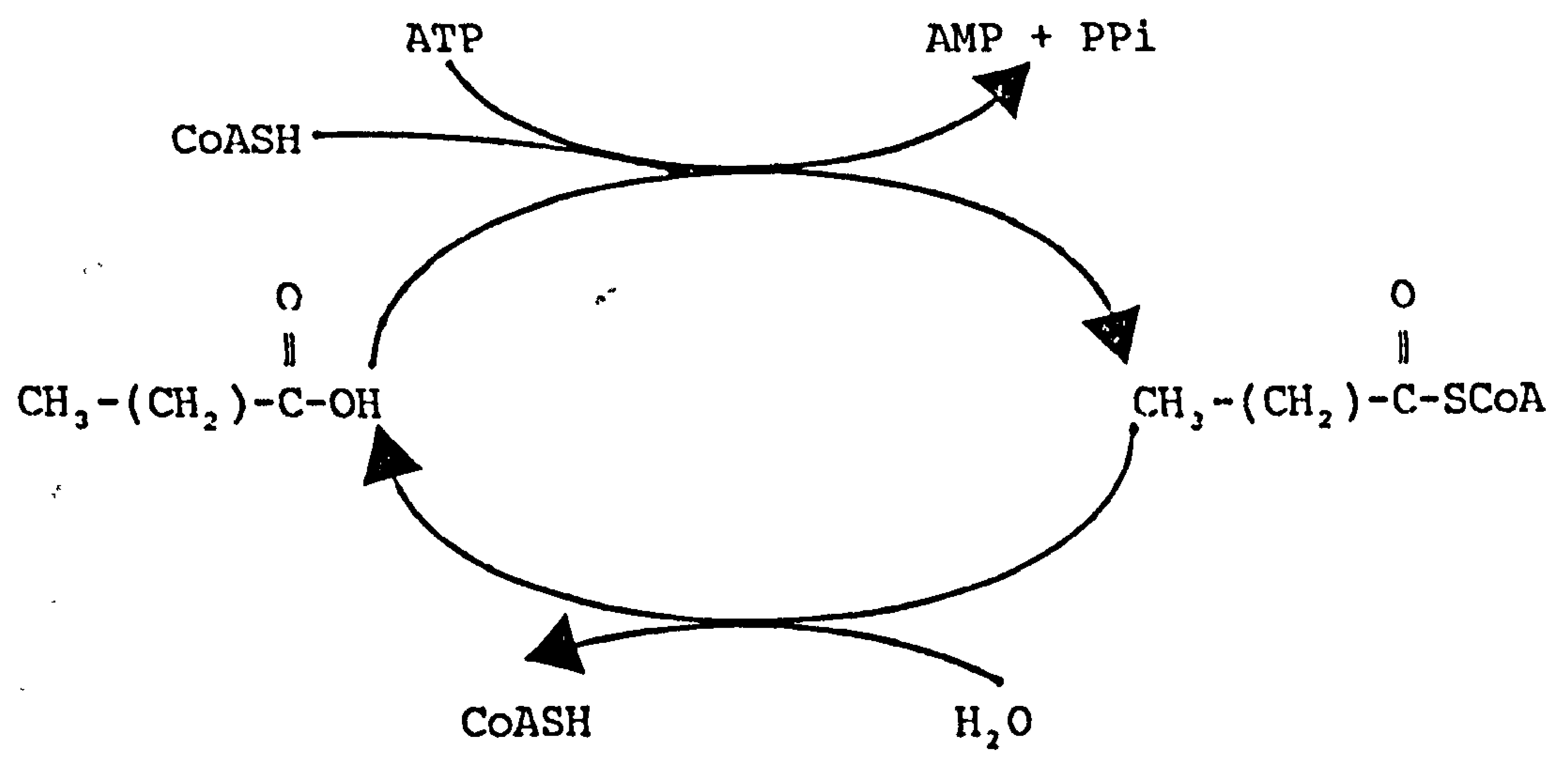
$$CH_3-(CH_2)_n-\overset{O}{\overset{\|}{C}}-OH + CoASH + ATP \xrightarrow{Mg^{2+}} CH_3-(CH_2)_n-\overset{O}{\overset{\|}{C}}-SCoA + AMP + PP_i$$

Acyl-CoA synthetase can be asssayed by a number of methods.
The acyl-CoA ester can be trapped as the corresponding acyl-hydroxamate by reaction with hydroxylamine (Lipmann and Tuttle 1950; Kornberg and Pricer 1953). Radiochemical assays can be carried out using ^{14}C-labelled fatty acid in which the radioactive free acid is removed by solvent

extraction and the radioactive, water soluble acyl-CoA determined by liquid scintillation counting (Samuel and Ailhaud 1969; Mishina *et al* 1978). AMP formation can be quantitatively coupled to NADH oxidation using adenylate kinase, pyruvate kinase and lactate dehydrogenase (Mishina *et al* 1978). Alternatively, activity can be measured by following the disapearance of free CoASH, on incorporation into acyl-CoA esters, by reaction with DTNB (Webster 1969; Vamecq *et al* 1985; Kim and Bang 1987).

The hydroxamate trapping assay suffers from the problem that the high concentrations of hydroxylamine required can be inhibitory (Massaro and Lennarz 1965) and even if the hydroxylamine solution is added once the reaction has been terminated there are still the problems of the longer chain acyl-hydroxamates being insoluble and the poor sensitivity of the method (ε_{520} = 1000 $l.mol^{-1}.cm.^{-1}$).
The radiochemical assay is very sensitive but expensive, especially if one wishes to carry-out chain length specificity studies. Furthermore, radiolabelled ω-hydroxymonocarboxylic and dicarboxylic acids are not commercially available which limits applicability of this assay to the present study.
The coupled NADH oxidation asssay is only suitable for assaying purified preparations.
Therefore, it was decided to measure activity by following CoASH disappearance, with DTNB, due to its simplicity, applicability to almost any carboxylic acid and its sensitivity (ε_{412} = 13 600 $l.mol.^{-1}cm.^{-1}$, Ellman 1959). It sould be noted that with this assay method it is the net CoASH disappearance that is measured. The thioesterase activity present in crude extracts can potentially form a futile-cycle with acyl-CoA synthetase (see Fig. 5.1). Thus it is the NET rate of acyl-CoA formation that is measured ie: acyl-CoA synthetase - thioesterase.

Figure 5.1: Possible futile-cycle catalysed by the combined actions of acyl-CoA synthetase and acyl-CoA thioesterase activities.


Acyl-CoA synthetase
ATP
AMP + PPi
CoASH
O
CH3-(CH2)-C-OH
O
CH3-(CH2)-C-SCoA
CoASH
H2O
Thioesterase

Thioesterase would similarly interfere with the radiochemical assay and with the hydroxamate assay, unless the acyl-CoA ester is "trapped" as its acyl-hydroxamate *in situ*.

5.2. COFACTOR REQURIEMENTS OF ACYL-CoA SYNTHETASE

The effects of various omissions from the standard acyl-CoA synthetase assay are shown in Table 5.1. Activity is dependent on ATP, fatty acid and extract. Since the assay is based on measuring CoASH disappearance it was not possible to determine the effect of omitting CoASH. However, it can be seen that this activity displays the cofactor requirements of an acyl-CoA synthetase.

5.3. INDUCTION OF ACYL-CoA SYNTHETASE

Acyl-CoA synthetase activity was measured in cell-free extracts from cells grown on a range of carbon-sources. The range of activities obtained for each carbon-source are shown in Table 5.2. The level of acyl-CoA synthetase activity in crude cell-free extracts is not drastically different after growth on a number of different carbon-sources (see also Figs. 5.5, 5.6 and 5.7). It should be noted that cell-free extract from succinate-grown cells displayed an approx. zero endogenous rate of CoASH consumption, as was the case with glucose-grown cells, whereas cell-free extract from cells grown on dodecanediol or hexadecanol diplayed a significant endogenous rate of CoASH consumption (typically approx. 10-15 nmol/min/mg protein). This endogenous CoASH consumption was presumably due to endogenous fatty acid derived from the growth substrate. The partial saturation of acyl-CoA synthetase by endogenous substrate in these extracts explains the slightly lower specific activities observed with cells grown on

Table 5.1: Cofactor requirements of lauroyl-CoA synthetase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C

System	Relative activity (%)
Complete	100
– ATP	13
– laurate	0
boiled extract	0

100% ≡ 20nmol/min/mg protein

Asssayed in potassium phosphate buffer (130mM, pH 8)

[laurate] = 2.4mM, [CoASH] = 0.7mM, [ATP] = 4mM

Table 5.2: Induction of acyl-CoA synthetase activity in *Corynebacterium* 7E1C and *E.coli*

Bacterium	Carbon-Source	Activity (nmol/min/mg)	
		C_{12}	DC_{12}
Corynebacterium 7E1C	glucose	22 (1)	n.d
.	succinate	30.7±5.6 (8)	24.6±7.4 (8)
	dodecanediol	19.0 (1)	10.8 (1)
	hexadecanol	23.3±3.3 (2)	14.8±1.4 (2)
E.coli[a]	amino acid	0.33	n.d
.	amino acid+glucose	0.03	n.d
.	amino acid+palmitate	0.69	n.d
	palmitate	1.83	n.d
E.coli[b]	glucose	0.03	n.d.
	succcinate	0.18	n.d
.	oleate	0.97	n.d

a = data of Weeks *et al* (1969) with palmitate as substrate

b = date of O'Connell *et al* (1986) with oleate as substrate

Corynebacterium 7E1C extracts assayed in potassium phosphate buffer (130mM, pH 8) with 2.4mM fatty acid substrate

Values are mean ± S.D. for (n) independent determinations

dodecanediol and hexadecanol as compared to succinate-grown cells. Thus, allowing for the endogenous rate, apparently similar specific activities were found after growth on glucose, succinate, 1,12-dodecanediol or hexadecanol. However, this is drastically different to the situation in *E.coli* where acyl-CoA synthetase is induced by growth on long-chain fatty acids (Overath *et al* 1969; Samuel and Ailhaud 1969; Weeks *et al* 1969). Some results on the induction of *E.coli* acyl-CoA synthetase, obtained by other workers, are included in Table 4.2 to illustrate the difference between *E.coli* and *Corynebacterium* 7E1C. In *E.coli* low levels of acyl-CoA synthetase were found in cells grown on acetate or amino acids; these low activities were repressed still further by growth on glucose. This catabolite repression by glucose was not overcome by adding oleate to glucose-containing media. In contrast *Nocardia asteroides* posseses constitutive acyl-CoA synthetase activity (Calmes and Deal 1973). Constitutive acyl-CoA synthetase has also been reported in *Bacillus megaterium* (Lennarz 1963; Massaro and Lennarz 1965). Similarly, O'Connell *et al* (1986) reported a constitutive long chain acyl-CoA synthetase in *Caulobacter crescentus*. Similar specific activities were observed in cells grown on succinate or oleate whilst activity was repressed no more than 2-3-fold in cells grown on glucose. This is remarkably similar to the behaviour observed in *Corynebacterium* 7E1C.

The yeast *Candida lipolytica* posseses two acyl-CoA synthetases; one is constitutive and has a narrow substrate specificity the other is inducible and has a broad substrate specificity (active with monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids)(Mishina *et al* 1978a, 1978b; Hosaka *et al* 1979, Hosaka *et al* 1981)). In contrast a constitutive dicarboxyl-CoA synthetase has been reported in *Candida tropicalis* (Zhihua and Xiuzhen 1986). Thus *Corynebacterium* 7E1C could conceivably possess both constitutive and inducible acyl-CoA

synthetases. However, the substrate specificity of acyl-CoA synthetase in *Corynebacterium* 7E1C is essentially the same regardless of carbon-source (see 5.6.1. and Figs. 5.5, 5.6 and 5.7) thus it is likely that the same complement of enzymes are present in *Corynebacterium* 7E1C regardless of growth substrate. Shimizu *et al* (1980) found that microbes could be divided into 2 groups on the basis of the induction of acyl-CoA synthetase. In one group acyl-CoA synthetase activity was present after growth on palmitate or on glucose whilst in the other group acyl-CoA synthetase was only present after growth on palmitate. The only *Corynebacterium* tested fitted the former group which is in agreement with the results reported here for *Corynebacterium* 7E1C. The specific activities obtained in this study with *Corynebacterium* 7E1C compare favourably with those reported for long-chain monocarboxyl-CoA synthetase in crude extracts of other bacteria eg: *E.coli*, 1.3 to 9.6 nmol/min/mg protein (Overath *et al* 1969; Samuel *et al* 1970; Kameda and Nunn 1981); *Pseudomonas* sp., 10 nmol/hr/mg (Trust and Millis 1971); *Bacillus megaterium*, 1nmol/min/mg (Massaro and Lennarz 1965); *Caulobacter crescentus* 2.3 to 5.7 nmol/min/mg (O'Connell *et al* 1986); *Nocardia asteroides* 80 nmol/min/mg (Calmes and Deal 1973). Modrzakowski and Finnerty (1989) reported a specific activity of 36.9 nmol/min/mg (with DC_8) for the dicarboxyl-CoA synthetase of didecyl ether-grown *Acinetobacter calcoaceticus* HO1-N.

5.4. INDUCTION OF ACYL-CoA THIOESTERASE

Thioesterase activity is constitutive, being expressed at similar specific activities in both succinate-grown and hexadecanol-grown cells (Table 4.3). This is in contrast to the situation in *E.coli* where thioesterase activity is induced by growth on oleate (Samuel and Ailaud 1969), although high constitutive levels of activity were observed in

Table 5.3: Induction of thioesterase activity in *Corynebacterium* 7E1C

Carbon-Source	Activity (nmol/min/mg protein)
succinate	46 (1)
hexadecanol	44.5±1.8 (3)

Hexadecanol was provided at 0.2% (w/v)

Succinate was provided at 1% (w/v)

Values are mean ± S.D. for (n)independent determinations

Assayed in potassium phosphate buffer (130mM, pH 8) with 80μM lauroyl-CoA as substrate

cells grown on glucose or acetate. The specific activity of *Corynebacterium* 7E1C thioesterase is comparable to that of oleate-grown *E.coli* (upto 43 nmol/min/mg protein; Samuel and Ailaud 1969).

5.5. THIOESTERASE SUBSTRATE SPECIFICITY

5.5.1. CHAIN-LENGTH SPECIFICITY

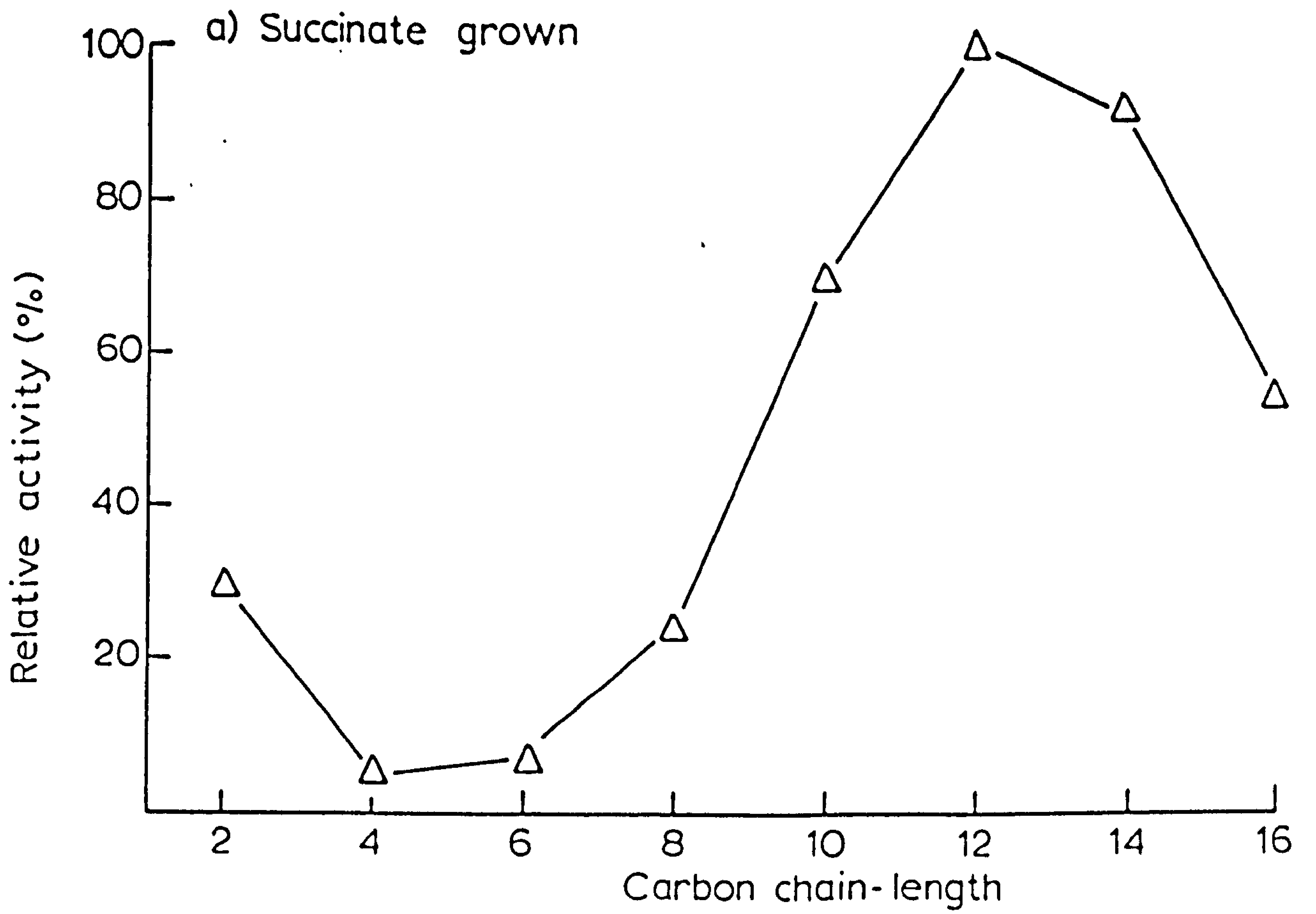
Thioesterase activity with C_2 to C_{16} saturated monocarboxyl-CoA esters is shown in Fig. 5.2. Thioesterase specificity is essentially independent of growth substrate which is consistent with its constitutive nature (see 5.4.). Activity is maximal with long chain substrates, lauroyl-CoA being optimal. The increase in activity with acetyl-CoA may be due to a separate acetyl-CoA thioesterase. The chain-length specifity of thioesterase activity is remarkably similar to that of the β-oxidation system (Chapter 6). The kinetic parameters of lauroyl-CoA and palmitoyl-CoA thioesterase activity were determined in a crude cell-free extract of hexadecanol-grown cell (Figs. 4.3 and 4.4). The values obtained (lauroyl-CoA V_{max} = 118 nmol/min/mg, K_m = 125μM : palmitoyl-CoA V_{max} = 45 nmol/min/mg, K_m = 59μM) are interesting when compared to those of the β-oxidation system. In particular, the K_m values for thioesterase activity are much higher than those obtained for β-oxidation (lauroyl-CoA 43μM; palmitoyl-CoA 8.4μM: see Chapter 6). The thioesterase K_m values reported here are much higher than those reported for the thioesterases purified from *E.coli* (Barnes and Wakil 1968; Barnes *et al* 1970; Barnes 1975) and *R.sphaeroides* (Boyce and Lueking 1984; Seay and Lueking 1986). Thioesterases I and II of both these bacteria had K_m values < 2μM for palmitoyl-CoA.

Figure 5.2.: Substrate specificity of thioesterase activity in crude cell-free extracts of succinate-grown and hexadecanol-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0) with 80μM acyl-CoA.

succinate-grown cells, 100% ≡ 46.0 nmol/min/mg protein

hexadecanolgrown cells, 100% ≡ 44 nmol/min/mg protein


a) Succinate grown
Relative activity (%)
100
80
60
40
20
2
4
6
8
10
12
14
16
Carbon chain-length

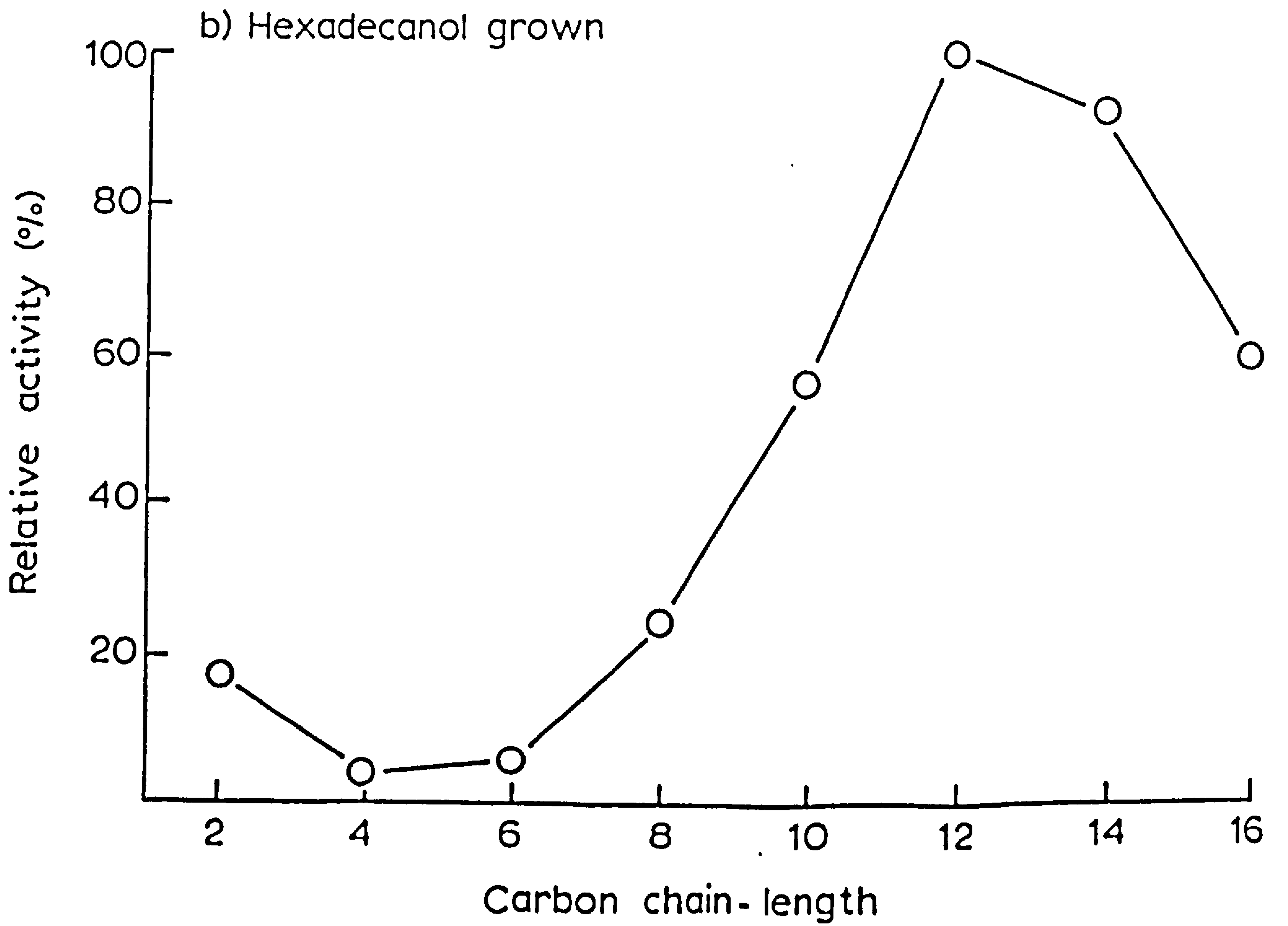

b) Hexadecanol grown
Relative activity (%)
100
80
60
40
20
2
4
6
8
10
12
14
16
Carbon chain-length

Figure 5.3.: Lineweaver-Burk plot of lauroyl-CoA thioesterase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0).

V_{max} = 118 nmol/min/mg protein

K_m = 125μM

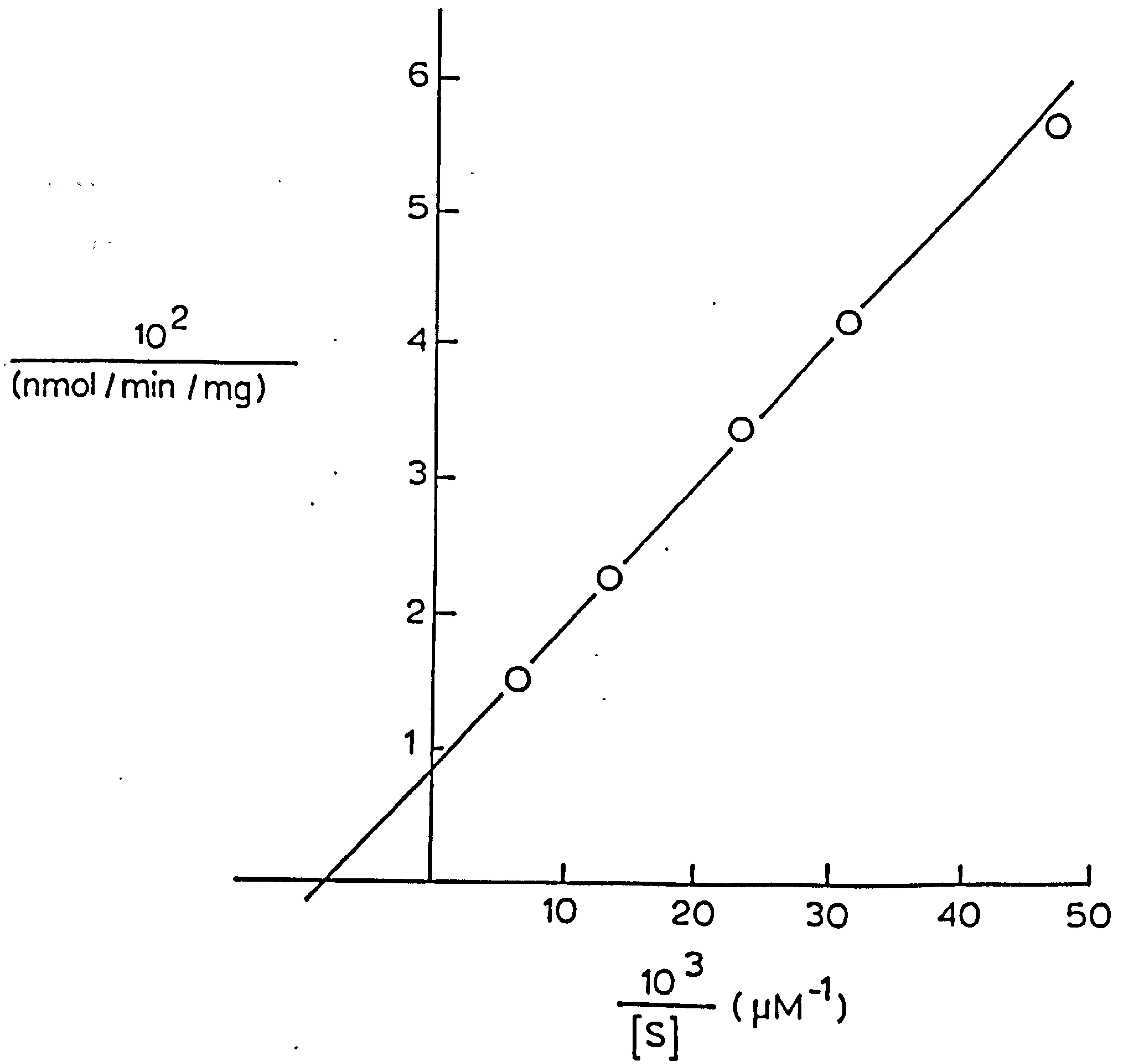
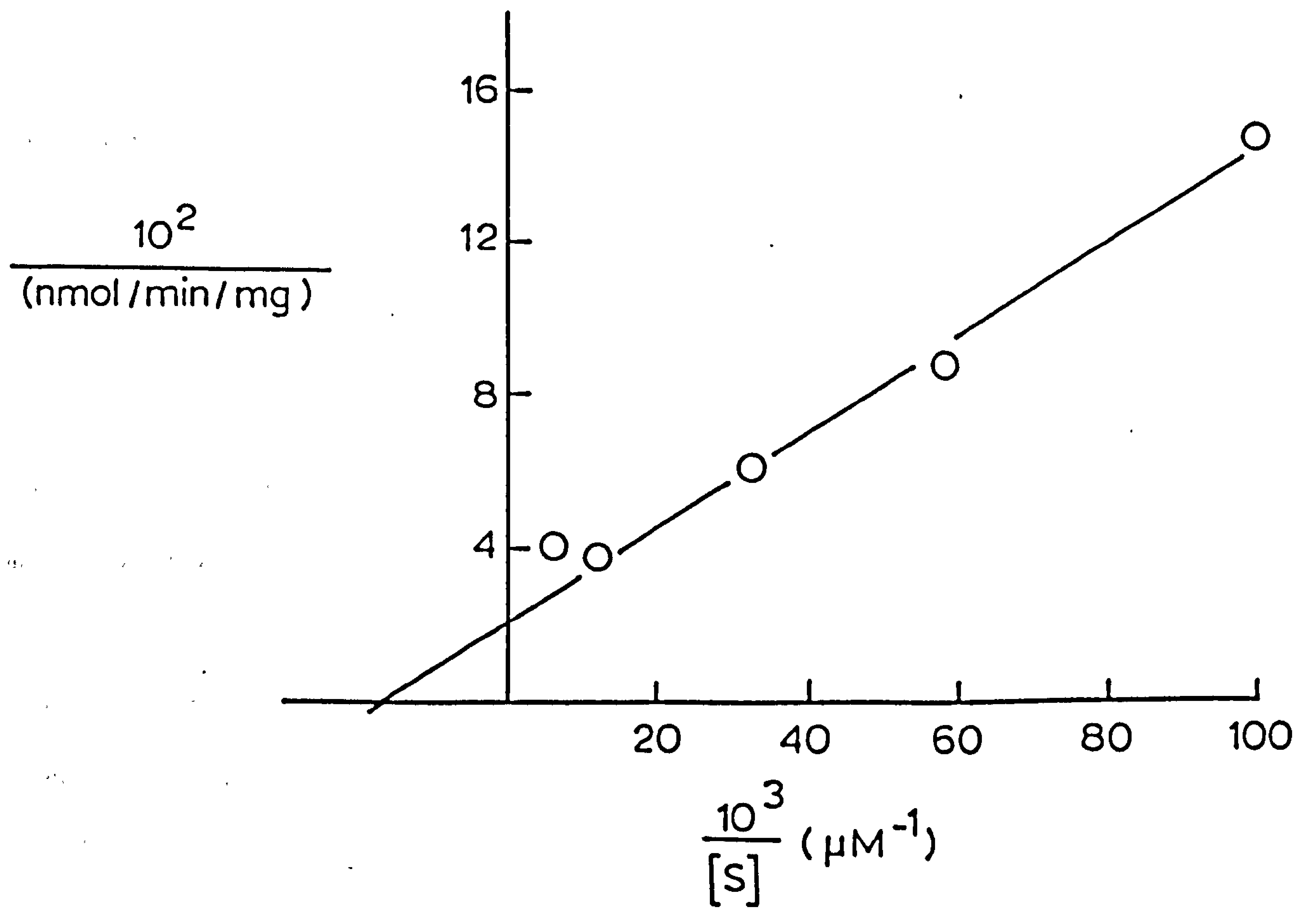

6
5
4
3
2
1
10^2 / (nmol / min / mg)
10
20
30
40
50
10^3 / [S] (μM^-1)

Figure 5.4.: Lineweaver-Burk plot of palmitoyl-CoA thioesterase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0).

V_{max} = 45.0 nmol/min/mg protein

K_m = 59µM



16
12
8
4
$\frac{10^2}{(nmol/min/mg)}$
20
40
60
80
100
$\frac{10^3}{[S]}$ (μM^{-1})

5.5.2. UNSATURATED ACYL-CoA ESTERS

Thioesterase activity with unsaturated acyl-CoA esters of chain-length C_{16} is shown in Table 5.4. Both palmitoleoyl-CoA ($C_{16:1}$ *cis* Δ^9) and palmitelaidoyl-CoA ($C_{16:1}$ *trans* Δ^9) are hydrolysed at a comparable rate to palmitoyl-CoA ($C_{16:0}$); the *cis* isomer being hydrolysed slightly faster than the *trans* isomer. This is different to the specificity of β-oxidation where palmitelaidoyl-CoA and palmitoyl-CoA are oxidided at comparable rates in contrast to palmitoleoyl-CoA which is oxidized much more slowly. The thioesterase of *E.coli* and *R.sphaeroides* hydrolyse palmitoyl-CoA and palmitoleoyl-CoA at comparable rates (Barnes and Wakil 1968; Barnes *et al* 1970; Boyce and Lueking 1984; Seay and Lueking 1986).

5.5.3. ω-SUBSTITUTED ACYL-CoA ESTERS

Thioesterase activity with the acyl-CoA esters of a range of ω-hydroxymonocarboxylic and dicarboxylic acids is shown in Table 4.5. C_{12} and C_{16} substrates behave rather differently. With C_{16} substrates the ω-hydroxy- and dicarboxyl- derivatives are hydrolysed more rapidly than the "parent" compound ie: palmitoyl-CoA. However, with C_{12} substrates the ω-hydroxy and dicarboxyl derivatives are hydrolysed much more slowly than laurate - the "parent" C_{12} compound. Thioesterase activity towards ω-substituted acyl-CoAs has not, apparently, been investigated in other systems although thioesterase II of *E.coli* is active with β-hydroxy acyl-CoA esters (Barnes *et al* 1970; Barnes 1975).

Table 5.4: Thioesterase activity with unsaturated acyl-CoA esters in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Acyl-CoA (80μM)	Activity (nmol/min/mg)	Relative activity (%)
C_{16}-SCoA	27.7	100
trans Δ^9 C_{16}-SCoA	33.7	121.7
cis Δ^9 C_{16}-SCoA	38.5	139.0

Assayed continuously by reaction with DTNB in potassium phosphate buffer (130mM, pH 8.0).

Table 5.5: Thioesterase activity with monocarboxyl-, ω-hydroxymonocarboxyl- and dicarboxyl-CoA esters in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Acyl-CoA (80μM)	Activity (nmol/min/mg)	Relative activity (%)
C_{12}-SCoA	43.5	100
ω-HO C_{12}-SCoA	21.0	48
DC_{12}-$SCoA_1$	10.0	23
DC_{12}-$SCoA_2$	13.5	31
C_{16}-SCoA	26.3	100
ω-HO C_{16}-SCoA	47.1	179
DC_{16}-$SCoA_1$	33.4	127
DC_{16}-$SCoA_2$	31.6	120

Assayed continuously by reaction with DTNB in potassium phosphate buffer (130mM, pH 8.0)

5.6. ACYL-CoA SYNTHETASE SUBSTRATE SPECIFICITY

5.6.1. FATTY ACID SUBSTRATE

Fatty acid substrate specificity was examined in crude cell-free extracts of cells grown on succinate, 1,12-dodecanediol and hexadecanol (Figs. 5.5, 5.6 and 5.7). Both succinate-grown and hexadecanol-grown cells display very broad substate specificity acyl-CoA synthetase activity (dodecanediol-grown cells were only tested with long-chain substrates). Monocarboxylic acids (C_4 to C_{16}; C_2 not tested), ω-hydroxymonocarboxylic acids (C_{10}, C_{12} and C_{16}) and dicarboxylic acids (C_4 plus C_{10} to C_{16}) are good acyl-CoA synthetase substrates. Thus the substrate specificity of *Corynebacterium* 7E1C acyl-CoA synthetase activity is very broad. As a comparison crude extracts of *E.coli* display acyl-CoA synthetase activity with C_8 to C_{18} saturated fatty acids (Overath *et al* 1969; Klein *et al* 1971; Kameda and Nunn 1981). Similarly, crude extracts of *Nocardia asteroides* also activated C_8 to C_{18} fatty acids whilst strains of *Pseudomonas* can activate C_2 to C_{19} fatty acids (Trust and Millis 1971). Bacterial dicarboxyl-CoA synthetase activity has been reported in *Mycobacterium tuberculum* (Kimura and Sasakawa 1956) and *Acinetobacter calcoaceticus* HO1-N (Modrzakowski and Finnerty 1989). Dicarboxyl-CoA synthetase active with ω-hydroxymonocarboxylic and dicarboxylic acids has been reported in rat liver microsomes (Vamecq *et al* 1985; Vamecq and Draye 1987) and the yeast *Candida lipolytica* (Mishina *et al* 1978b) whilst *Candida tropicalis* can activate dicarboxylic acids (Lebault *et al* 1970a, b; Zhihua and Xiuzhen 1986).

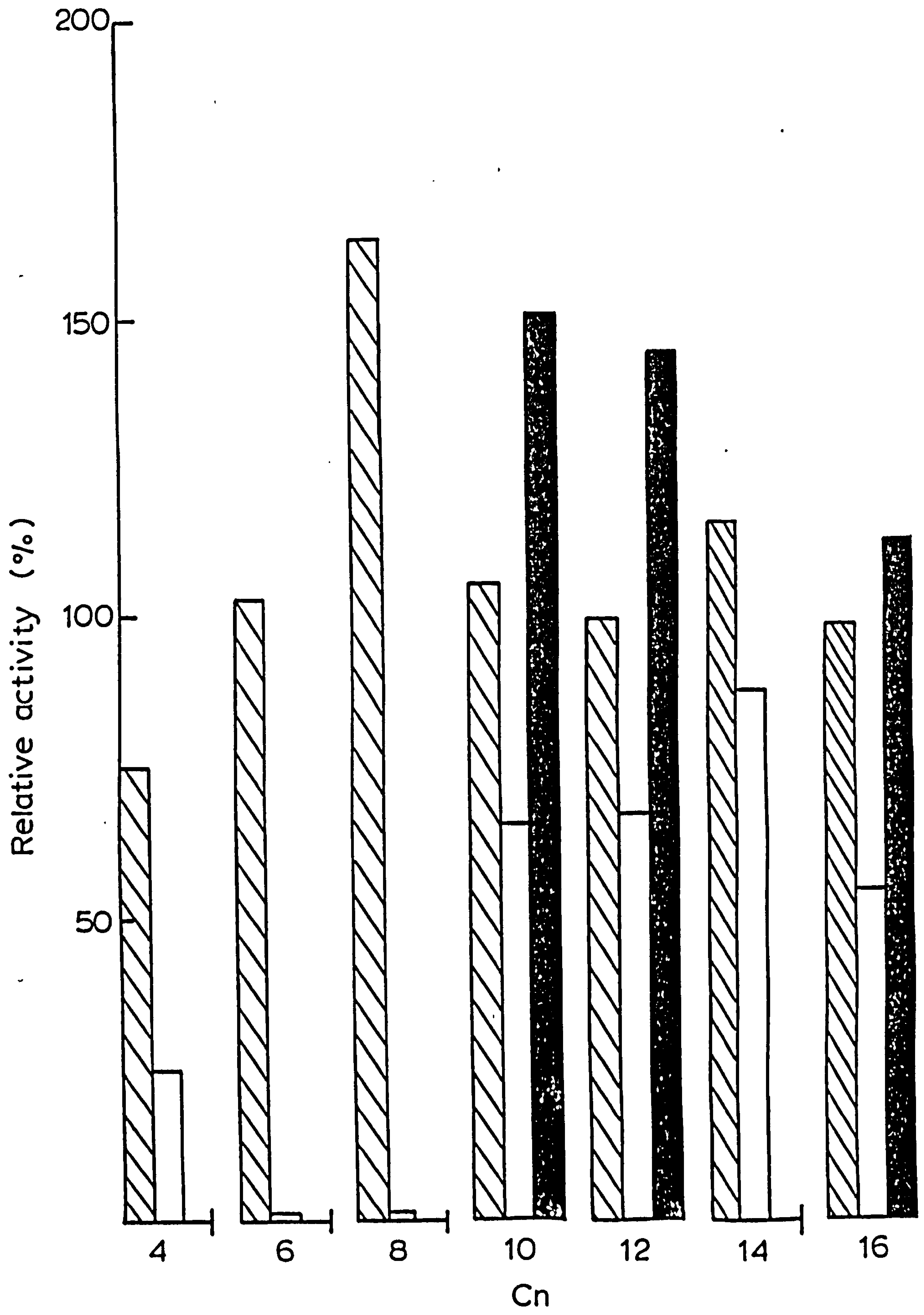
The substrate specificity profile is essentially the same regardless of growth substrate. Of the long-chain (ie: C ≥ 10 carbons) substrates ω-hydroxymonocarboxylic acids are activated at least as rapidly as the corresponding monocarboxylic acids. Long-chain dicarboxylic acids are

Figure 5.5: Substrate specificity of acyl-CoA synthetase activity, with respect to C_4 to C_{16} mono, ω-hydroxymono- and dicarboxylic acids, in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C.

All activities were assayed in potassium phosphate buffer (130mM, pH 8.0)

[fatty acid] = 2.4mM, [CoASH] = 0.7mM, [ATP] = 4mM

100% ≡ 20.0 nmol/min/mg protein



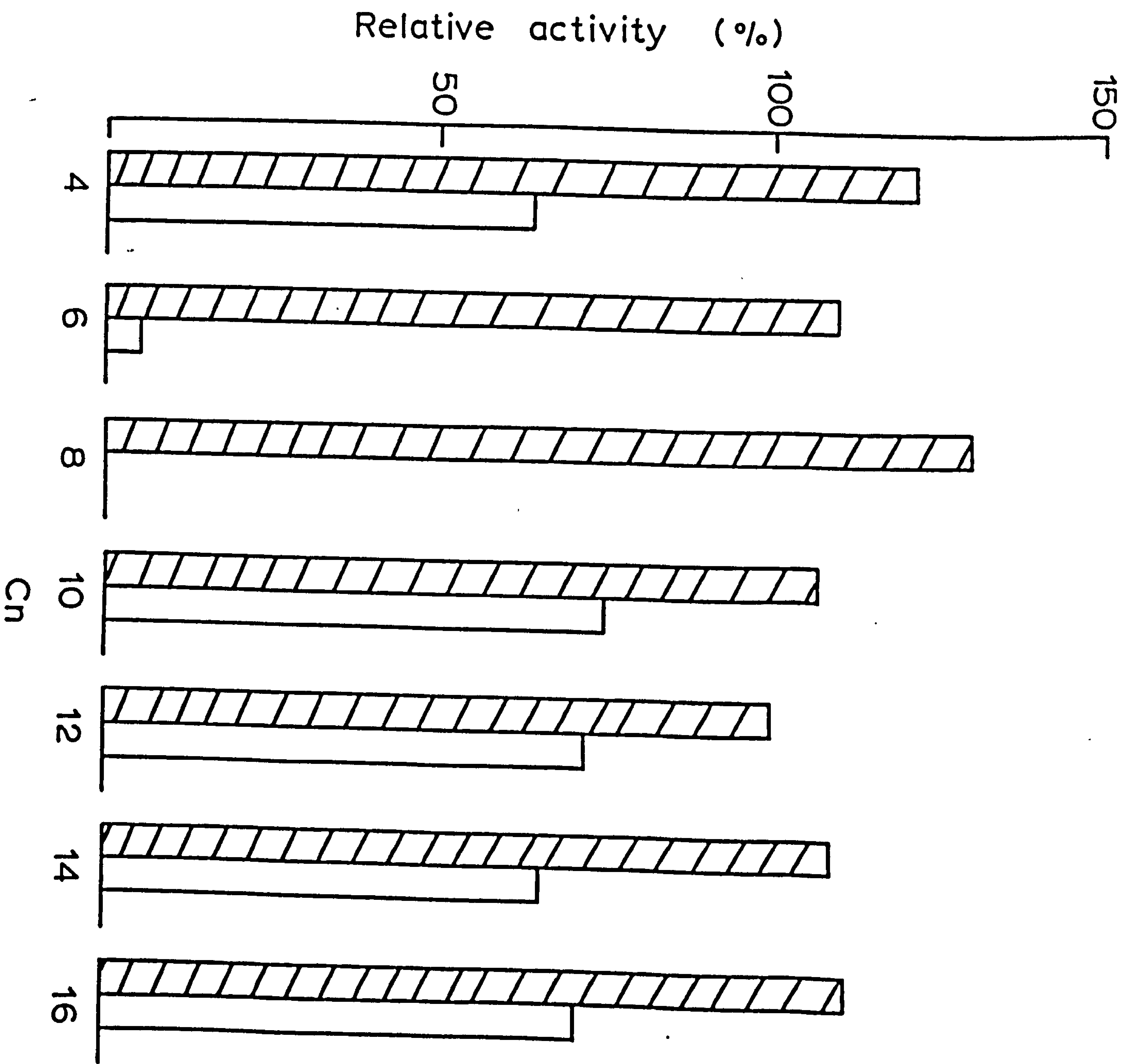
200
150
100
50
Relative activity (%)
4
6
8
10
12
14
16
Cn

Figure 5.6.: Substrate specificity of acyl-CoA synthetase activity, with respect to C_4 to C_{16} mono- and dicarboxylic acids, of a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0)

[fatty acid] = 2.4mM, [CoASH] = 0.7mM, [ATP] = 4mM

100% ≡ 39.5 nmol/min/mg protein



Relative activity (%)
50
100
150
4
6
8
10
12
14
16
Cn

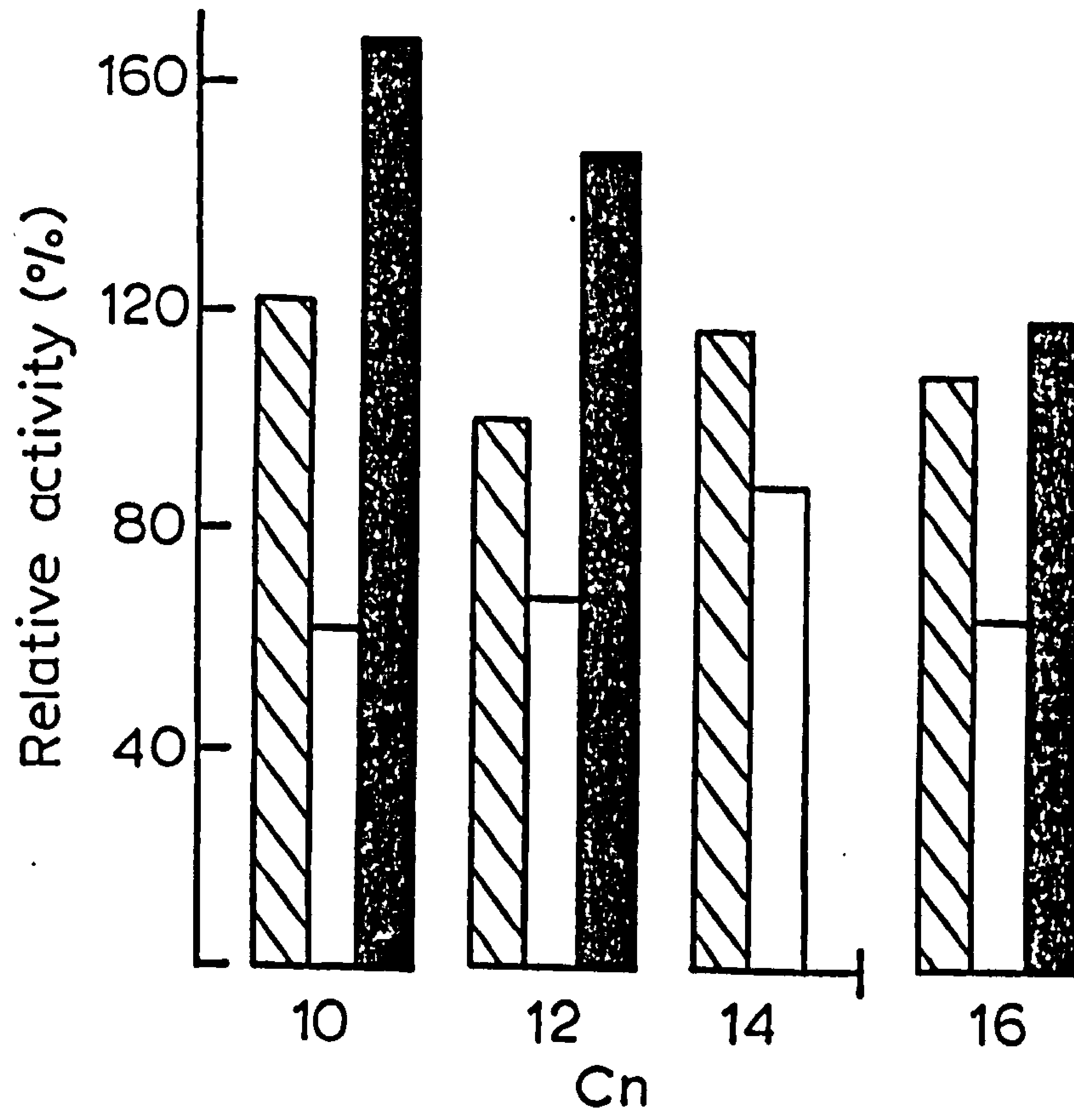
Figure 5.7.: Substrate specificity of acyl-CoA synthetase acivity with respect to C_{10} to C_{16} mono-, ω-hydroxymono- and dicarboxylic acids, in crude cell-free extracts of succinate-grown and dodecanediol-grown *Corynebacterium* 7E1C

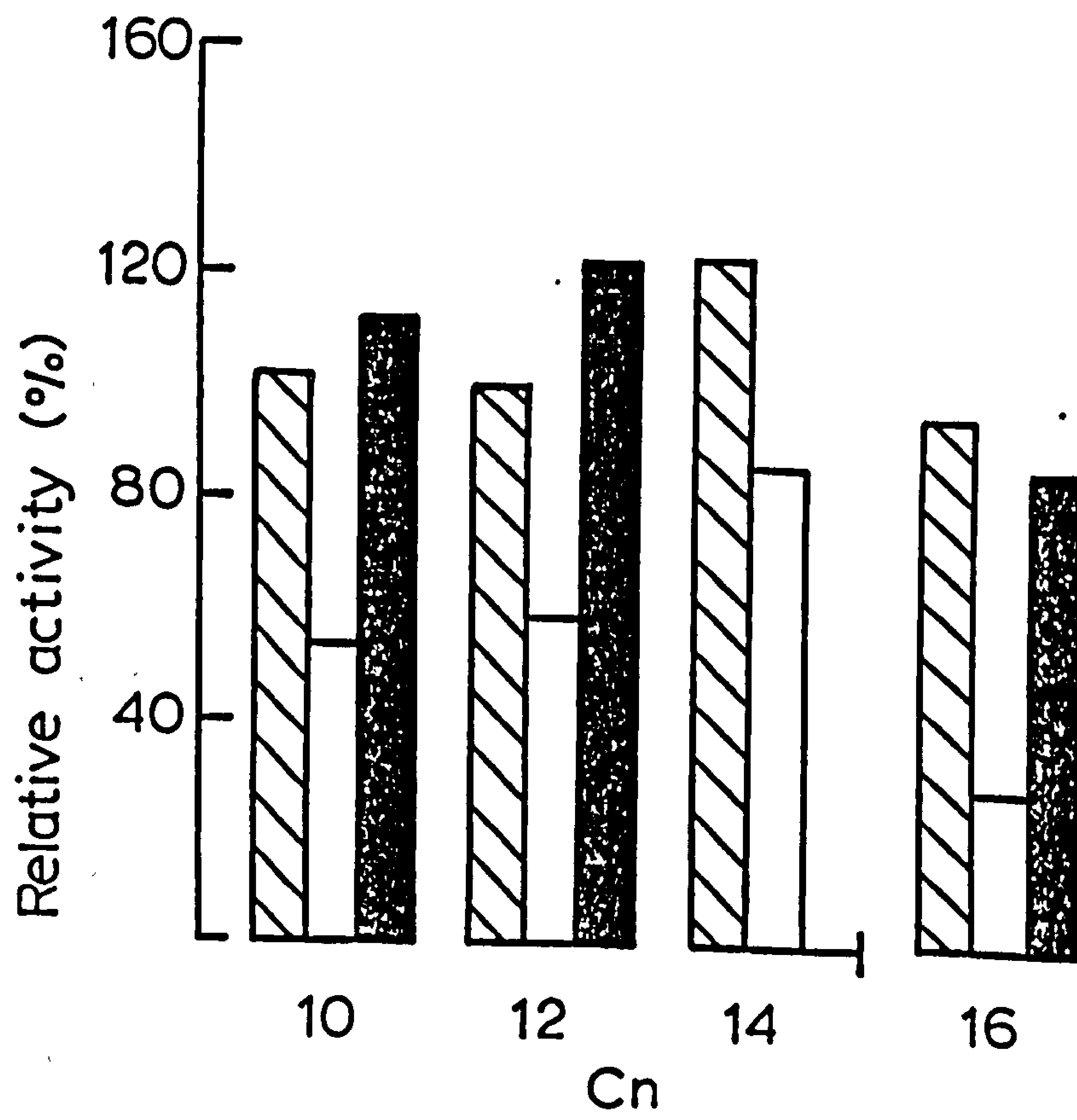
Assayed in potassium phosphate buffer (130mM, pH 8.0)

[fatty acid] = 2.4mM, [CoASH] = 0.7mM, [ATP] = 4mM

succinate-grown cells, 100% ≡ 25.5 nmol/min/mg protein

dodecanediol-grown cells, 100% ≡ 19.0 nmol/min/mg protein


a) Succinate-grown cells
Relative activity (%)
160
120
80
40
10
12
14
16
Cn


b) 1,12-Dodecanediol-grown cells
Relative activity (%)
160
120
80
40
10
12
14
16
Cn

activated at 60-70% the rate of the corresponding monocarboxylate in the data of Figs. 5.5, 5.6 and 5.7; although it should be noted that for any given carbon-source the rate of activation of dicarboxylates varied from 50-100% the the rate of the corresponding monocarboxylates (Table 5.6). This could be due to a change in the relative activities of mono- and dicarboxyl-CoA synthetase *per se* or to a change in the relative levels of the corresponding thioesterase activities. Crude cell-free extracts of *Corynebacterium* 7E1C contain broad substrate specificity acyl-CoA thioesterase (acyl-CoA hydrolase) activity which is active with monocarboxyl-, ω-hydroxymonocarboxyl- and dicarboxyl-CoA esters (see 5.5.). Since the acyl-CoA synthetase assay measures the NET consumption of free CoASH, an increase in thioesterase activity will result in a reduction in the measured acyl-CoA synthetase activity. Therefore, the variation in the relative rates of activation may be a result of changes in the relative rate of hydrolysis of lauroyl-CoA and DC_{12}-CoA rather than a change in their relative rates of synthesis. This possibility has not been investigated. In this connexion it should be noted that the thioesterase activity present in crude extracts will affect the substrate specificity determined for the acyl-CoA synthetase reaction. Thioesterase is optimally active with long-chain substrates so its presence will tend to shift the observed acyl-CoA synthetase specifity towards shorter-chain substrates. With ω-substituted acyl-CoA esters thioesterase is less active with C_{12} substrates than with the corresponding C_{16} substrates (Table 5.5). This may contribute to the observation that C_6 and C_8 are the optimal saturated fatty acid substrates and C_{10} is the optimal ω-hydroxyfatty acid substrate. However, thioesterase activity (assayed with 80μM lauroyl-CoA) is inhibited by CoASH (see 5.10.) so thioesterase activity will be lower in acyl-CoA synthetase assays than in thioesterase assays.

In order to confirm that ω-hydroxymonocarboxylic and dicarboxylic acids

Table 5.6: Variation of the relative rates of lauroyl-CoA and DC_{12}-CoA synthetase activity in crude cell-free extracts of *Corynebacterium* 7E1C

Batch	Activity (nmol/min/mg)		DC_{12} (%)
	C_{12}	DC_{12}	
1	30.4	22.6	74.3
2	39.5	28.4	71.9
3	37.8	36.6	96.8
4	28.1	28.1	100.0
5	28.8	21.7	75.3
6	25.5	17.3	67.8
7	33.6	30.2	89.9
8	22.0	11.7	53.2

Assayed in potassium phosphate buffer (130mM, pH 8) with 2.4mM fatty acid as substrate.

[ATP] = 4mM, [CoASH] = 0.7mM

are indeed good β-oxidation substrates the kinetics of activation of C_{12} and C_{16} monocarboxylates , ω-hydroxymonocarboxylates and dicarboxylates was investigated (Figs. 5.8 and 5.9). The data were too scattered to give accurate Lineweaver-Burk plots. However, from the substrate saturation curves it can be seen that all substrates achieved $V_{max}/2$ at substrate concentrations less than 100μM except for palmitate for which the corresponding value was approx. 150μM. The K_m values reported for other acyl-CoA synthetases vary considerably. For bacterial enzymes the values reported are, *E.coli* 70μM (palmitate)(Samuel *et al* 1970), *Nocardia asteroides* 0.99mM (palmitate)(Calmes and Deal 1973), *P.aeruginosa* 0.44mM (palmitate)(Shimizu *et al* 1980). Vamecq *et al* (1985) reported a K_m of 2.5mM (DC_{12}) for the rat liver dicarboxyl-CoA synthetase. Therefore the kinetic parameters reported here fall within the range of values reported for other systems.

The important point with respect to this study is that long-chain monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids display similar kinetics of activation which confirms that all 3 classes of long-chain carboxylic acid are good acyl-CoA synthetase substrates.

This specificity of acyl-CoA synthetase activity is very interesting with respect to dicarboxylic acid production. The range of dicarboxylic acids produced by *Corynebacterium* 7E1C is very narrow ie: $DC_{12} \gg DC_{14}$; no DC_{16}. However, on the basis of acyl-CoA synthetase specificity one would expect the different classes (ie: mono- , ω-hydroxymono- and dicarboxylic acids) and various chain-lengths to be degraded at similar rates.

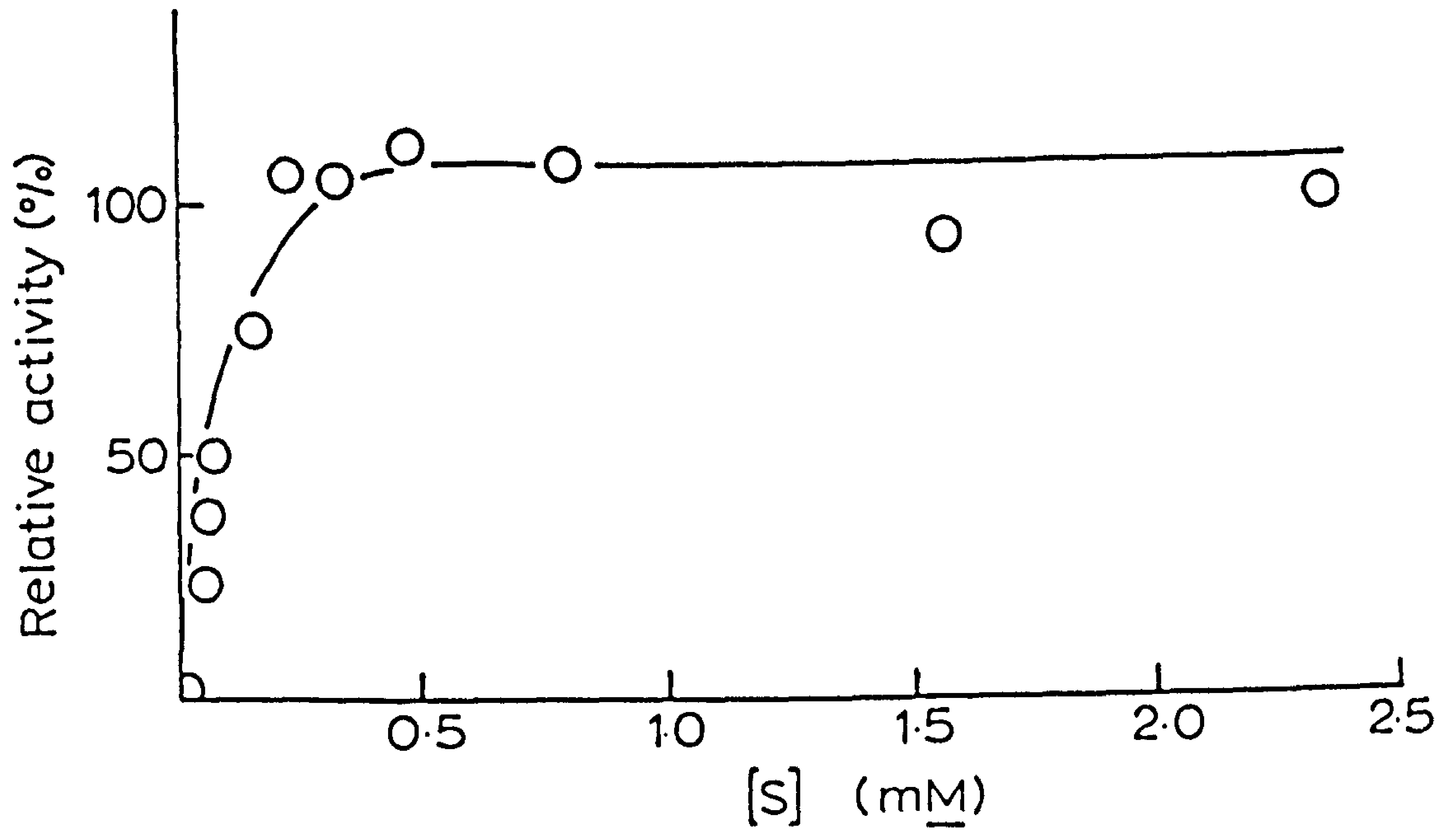
Therefore, on the basis of acyl-CoA synthetase activity one would predict similar accumulation of DC_{12}, DC_{14} and DC_{16}. Thus the specificity of acyl-CoA synthetase does not explain the specificity of dicarboxylic acid accumulation obseved with this organism.

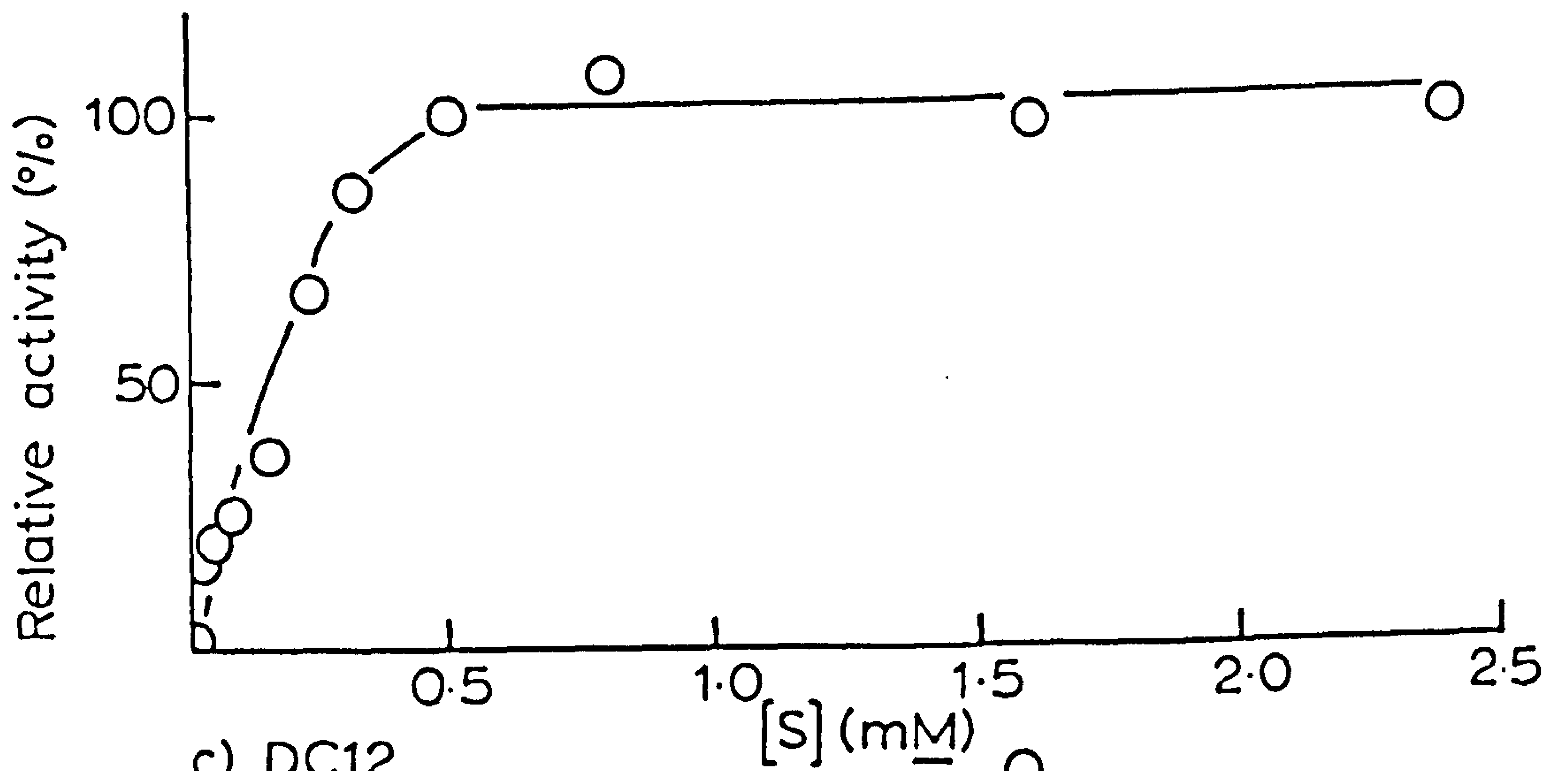
Figure 5.8.: Effect of substrate concentration on lauroyl-, ω-hydroxylauroyl- and dodecanedioyl-CoA synthetase activity in crude cell-free extracts of succinate-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0).

[CoASH] = 0.7mM, [ATP] = 4mM

100% ≡ 28.1 nmol/min/mg protein


a) laurate
Relative activity (%)
100
50
0·5
1·0
1·5
2·0
2·5
[S] (mM)


b) 12 - hydroxylaurate
Relative activity (%)
100
50
0·5
1·0
1·5
2·0
2·5
[S] (mM)

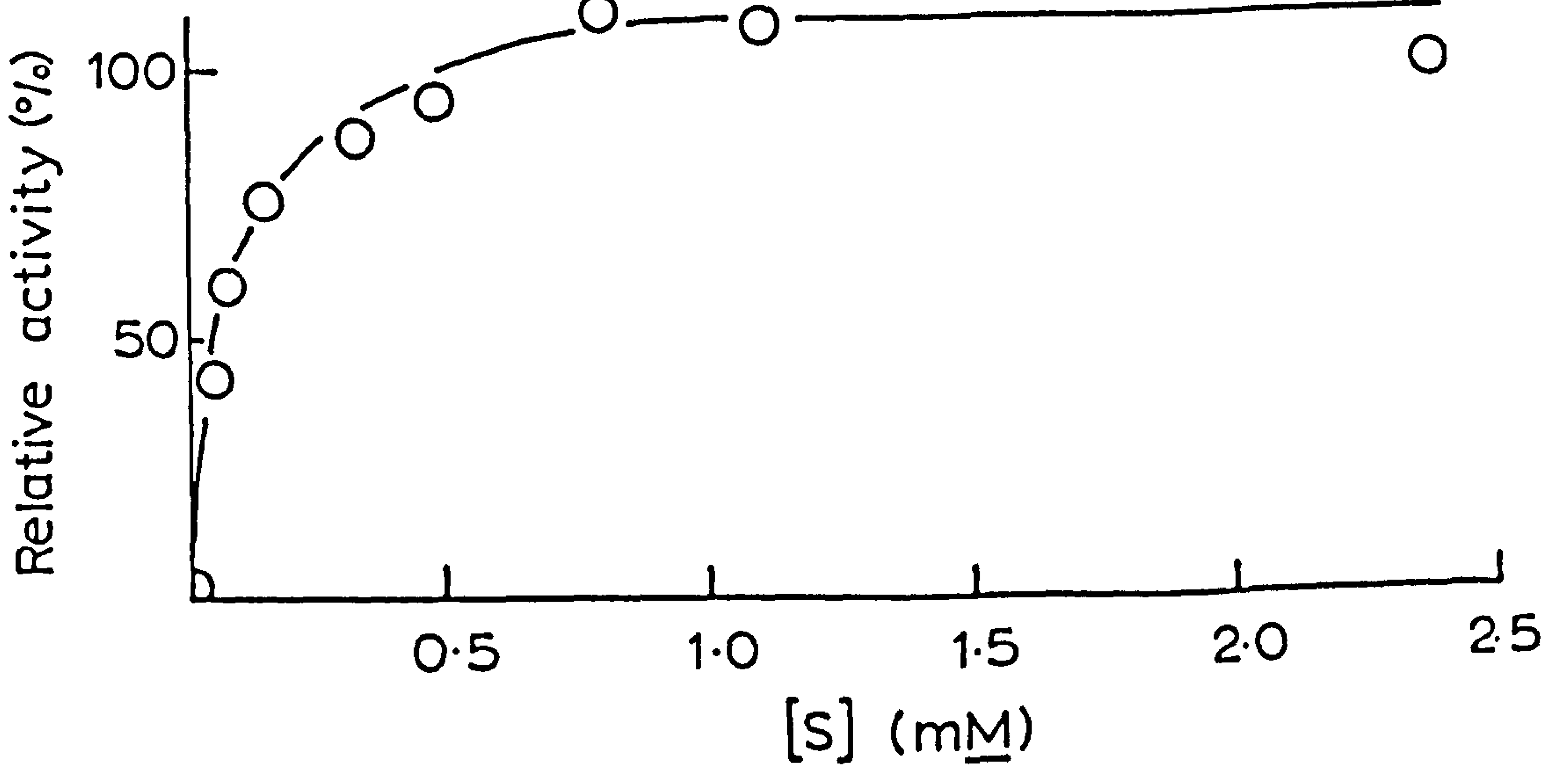

c) DC12
Relative activity (%)
100
50
0·5
1·0
1·5
2·0
2·5
[S] (mM)

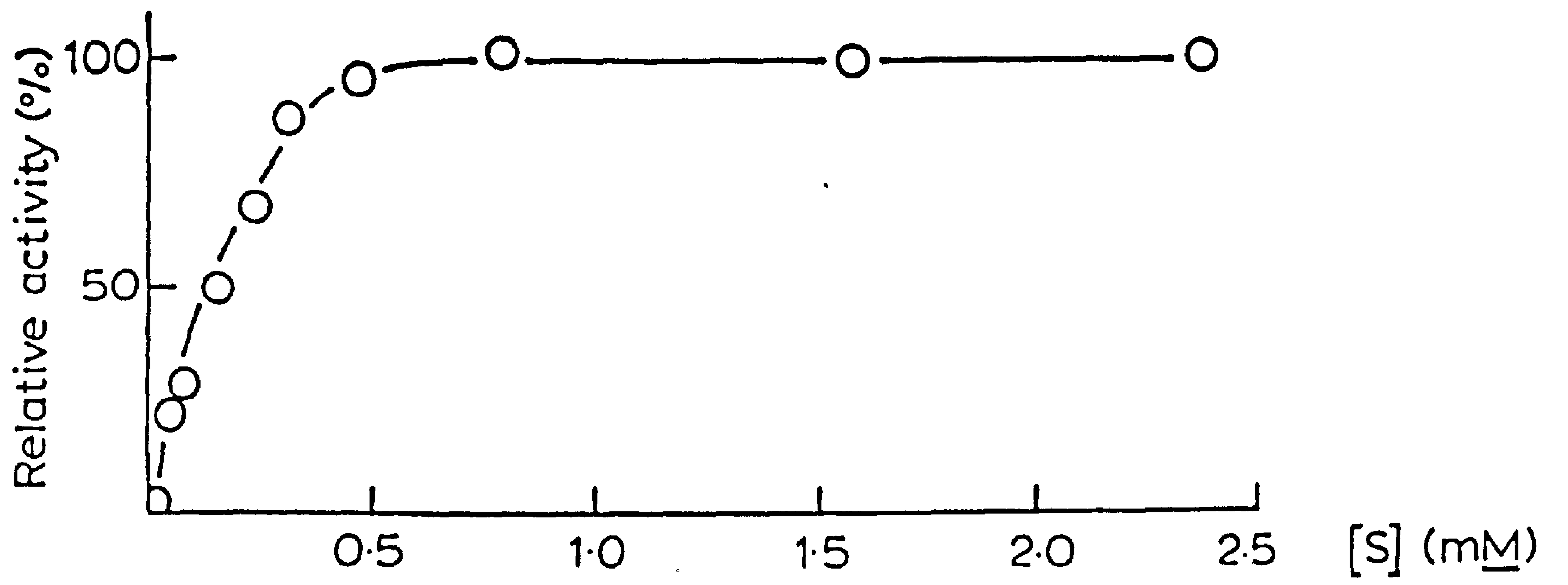
Figure 5.9.: Effect of substrate concentration on palmitoyl-, ω-hydroxypalmitoyl- hexadecanedioyl-CoA synthetase activity in crude cell-free extracts of succinate-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0).

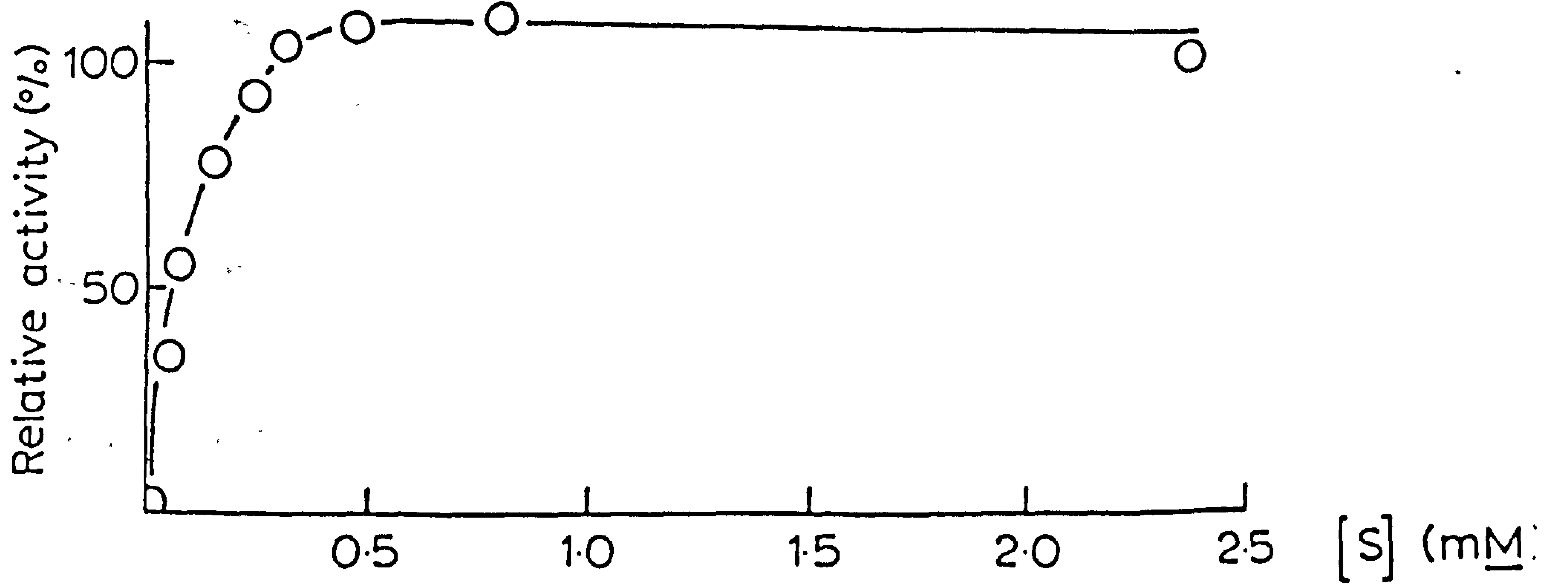
[CoASH] = 0.7mM, [ATP] = 4mM

100% ≡ 28.1 nmol/min/mg protein

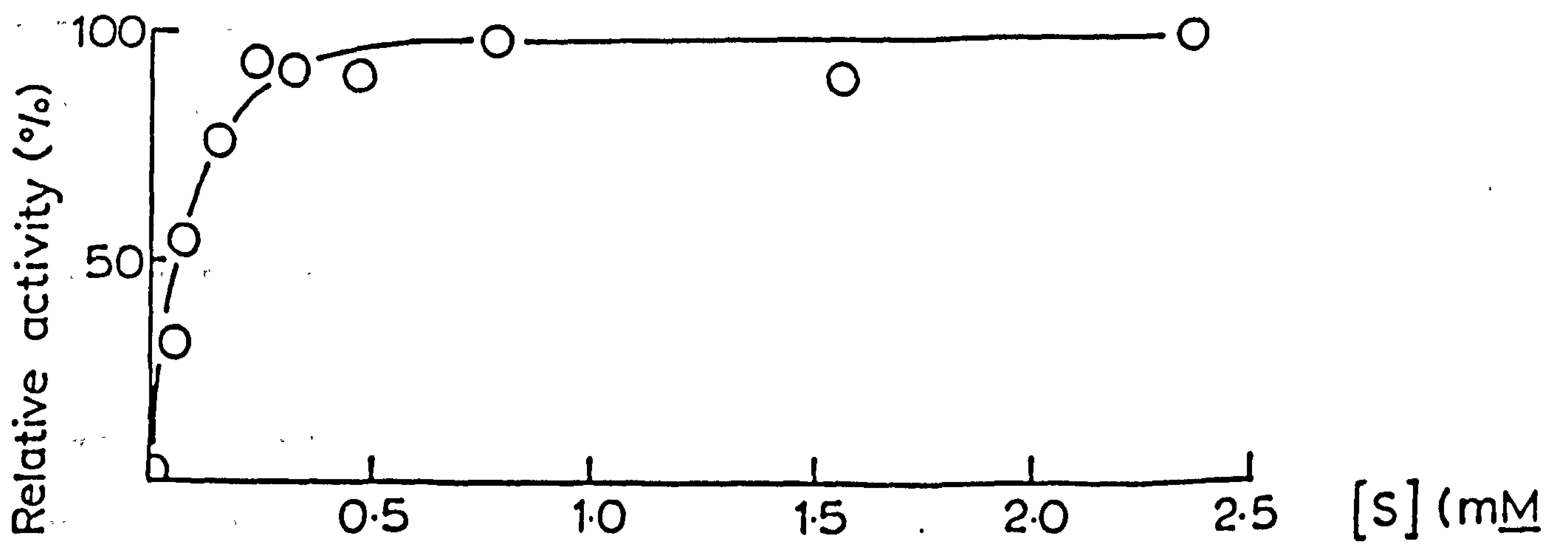
a) Palmitate



b) 16 - Hydroxypalmitate



c) DC_{16}



5.6.2. REACTION PRODUCT OF THE DICARBOXYL-CoA SYNTHETASE

With a dicarboxylic acid as substrate two reaction products are possible. Either one or both carboxylate groups could be esterified to COASH to give either the mono-CoA ester (DC_n-$SCoA_1$) or the di-CoA ester (DC_n-$SCoA_2$), respectively. An acyl-CoA synthetase assay with DC_{14} as substrate was allowed to run for 60 min. then the acyl-CoA esters were extracted and analysed by HPLC (see Methods). Crude cell-free extract from succinate-grown cells was used since such extracts contain less endogenous fatty acid than those from hexadecanol-grown cells.

The reaction product is DC_{14}-$SCoA_1$ (ie: the mono-CoA ester)(Fig. 5.10). No DC_{14}-CoA_2 could be detected. Thus once a CoASH molecule is esterified to one of the carboxyl-groups the other is no longer recognized as an acyl-CoA synthetase substrate.

5.6.3. NUCLEOSIDE TRIPHOSPHATE SPECIFICITY

With either laurate or dodecanedioate as fatty acid substrate the only effective nucleoside triphosphate substrate was ATP (Table 5.7). Essentially no activity was observed with CTP, GTP, UTP or ITP. This is in agreement with the specificity reported for other long-chain acyl-CoA synthetases: bacteria (Massaro and Lennarz 1965; Samuel *et al* 1970; Calmes and Deal 1973; Shimizu *et al* 1980), yeast (Hosaka *et al* 1979), rat liver (Tanaka *et al* 1979). Vamecq *et al* (1985) reported a similar specificity for the rat liver dicarboxyl-CoA synthetase. Rat liver contains a GTP-dependent acyl-CoA synthetase (Galzigna *et al* 1967). However, it should be pointed-out that such an activity has not been reported in bacteria.

The variation of lauroyl-CoA synthetase activity with ATP concentration is shown in Fig 5.11. $V_{max}/2$ is achieved at approx. 0.4mM ATP. The

Figure 5.10.: Reaction product of the dicarboxyl-CoA synthetase

Acyl-CoA synthetase assayed in potassium phosphate buffer (130mM, pH 8)

$[DC_{14}]$ = 1 mM, [CoASH] = 0.7mM, [ATP] = 4mM

Acyl-CoA esters separated by H.P.L.C. on a 5µ Lichrosorb RP C18 column using an acetonitrile-potassium phosphate gradient

A = CoASH

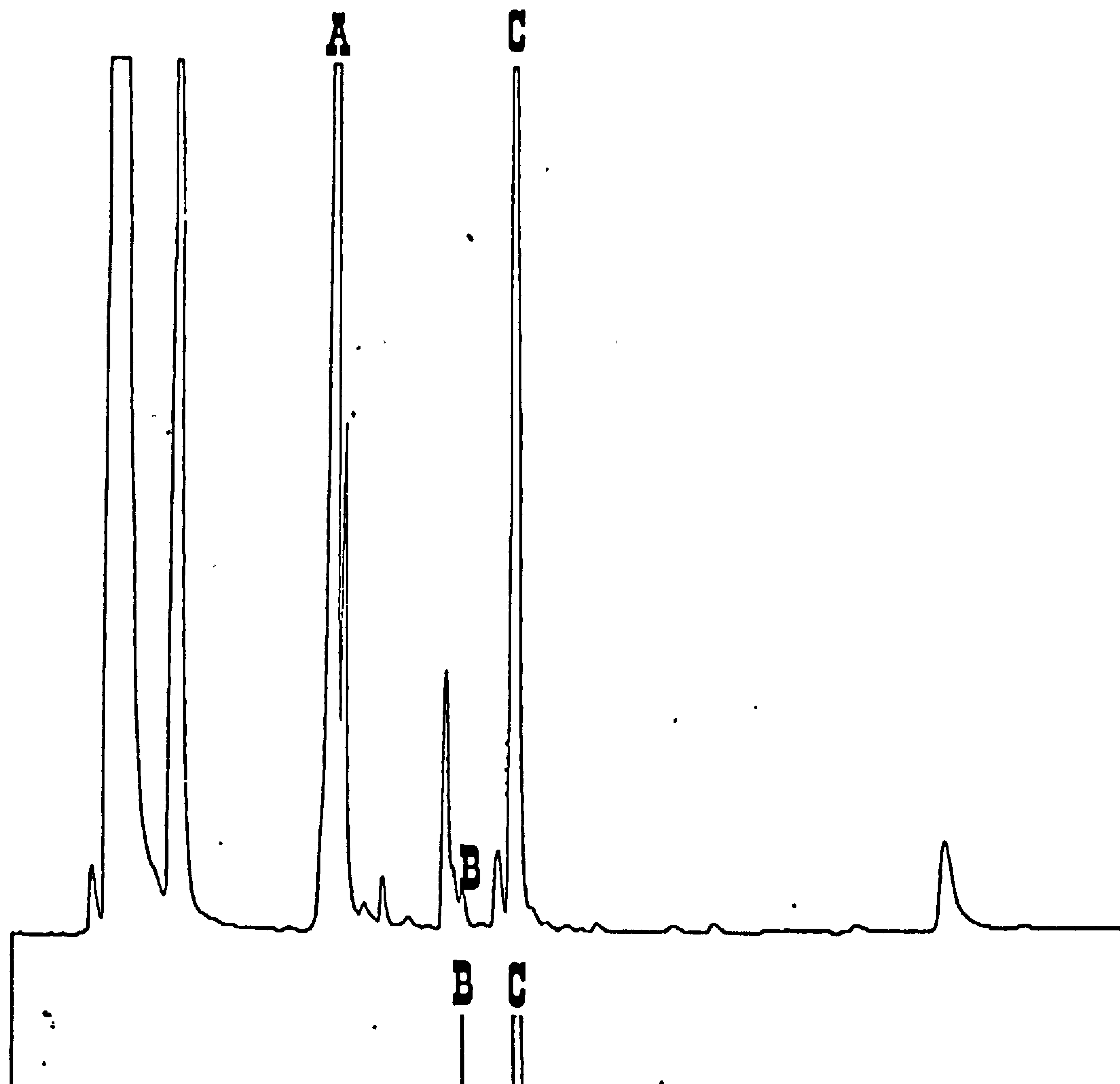
B = DC_{14}-CoA_2

C = DC_{14}-CoA_1

1 = reaction product of dicarboxyl-CoA synthetase

2 = standards

1.



2.

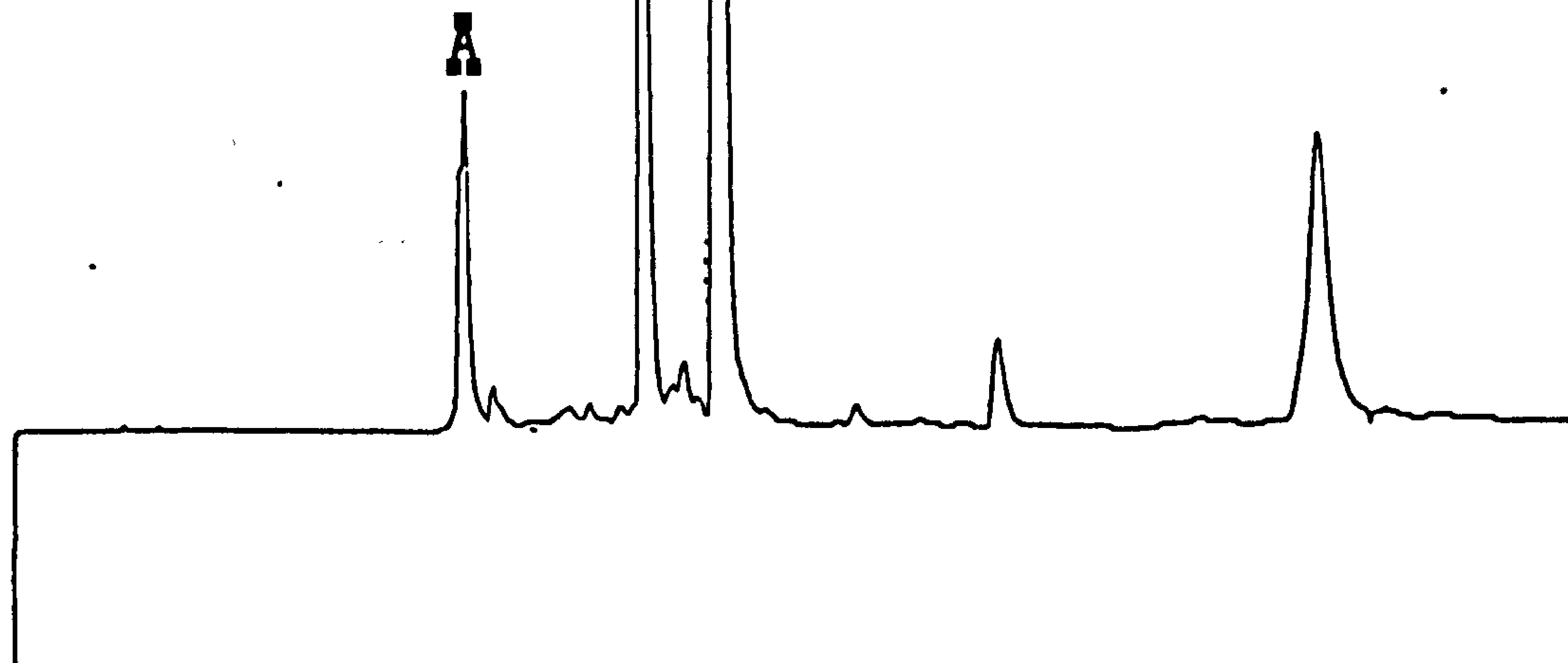


Table 5.7: Nucleoside triphosphate substrate specificity of acyl-CoA synthetase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C

Fatty Acid (1.4mM)	Nucleoside Triphosphate (4mM)	Activity (nmol/min/mg)	% of activity with ATP
	ATP	25.5	100
	GTP	2.5	9.9
laurate	CTP	1.8	7.1
	UTP	3.4	13.3
	ITP	4.0	15.8
	none	2.9	11.4
	ATP	17.3	100
	GTP	1.5	8.6
DC_{12}	CTP	0.5	2.9
	UTP	1.1	6.4
	ITP	2.7	15.8
	none	1.8	10.2

Assayed in potassium phosphate buffer (130mM, pH 8)

[fatty acid] = 2.4mM, [CoASH] = 0.7mM

Figure 5.11.: Effect of ATP concentration on lauroyl-CoA synthetase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C.

Assayed in potassium phosphate buffer (130mM, pH 8.0)

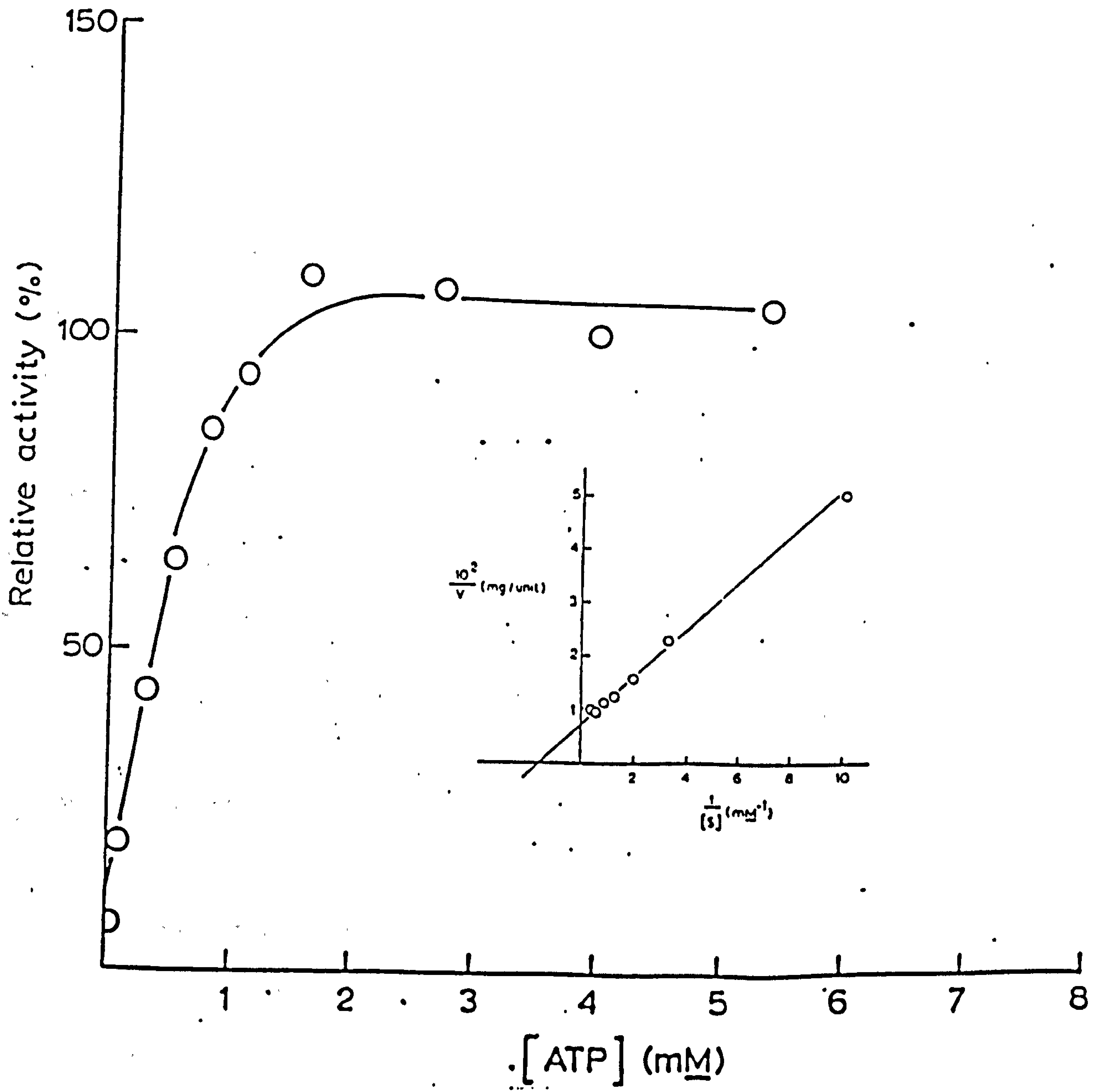
[laurate] = 2.4mM, [CoASH] = 0.7mM

100% ≡ 28.1 nmol/min/mg protein

Kinetic parameters calculated from Lineweaver-Burk plot (inset)

V_{max} = 40.1 nmol/min/mg protein

K_m = 0.63mM


150
100
50
Relative activity (%)
1
2
3
4
5
6
7
8
[ATP] (mM)
10²/v (mg/unit)
5
4
3
2
1
2
4
6
8
10
1/[S] (mM⁻¹)

Lineweaver-Burk plot of this date gives a K_m for ATP of 0.63mM (Fig. 5.11, inset). This is in good agreement with the K_m for ATP of other long-chain acyl-CoA synthetases eg: *E.coli* 3mM (Samuel *et al* 1970), *N.asteroides* 0.64mM (Calmes and Deal 1973), *C.lipolytica* 0.46mM (Hosaka *et al* 1979), rat liver microsomes and mitochondria 2.4mM (Tanaka *et al* 1979).

5.7. pH OPTIMUM OF ACYL-CoA SYNTHETASE

The pH profile of acyl-CoA synthetase activity is shown in Fig. 5.12. The pH optimum is approx. pH 9.0. Since the acyl-CoA synthetase assay used in these studies measures the NET rate of acyl-CoA formation (ie: acyl-CoA synthetase - thioesterase) it should be noted that the pH optimum reported here will be affected by the pH optimum of the thioesterase reaction (which was not determined). Futhermore, the rate of non-enzymic alkaline hydrolysis of lauroyl-CoA will increase with increasing pH, which will have the effect of shifting the pH optimum to a lower pH value than would otherwise have been the case.

5.8. SUBCELLULAR LOCATION OF ACYL-CoA SYNTHETASE

Crude cell-free extract from succinate-grown cells was ultracentrifuged at 100 000g for 90 min. at 4°C (Table 5.8). With laurate as substrate 67.6% of the original activity (≡ 85.4% of recovered activity) was found in the supernatant. With dodecanedioate as substrate 73.3% (≡ 91.5% of recovered activity) was found in the supernatant. Thus long-chain acyl-CoA synthetase activity is predominantly soluble (ie: cytoplasmic) in *Corynebacterium* 7E1C. Long-chain acyl-CoA synthetase is particulate in rat liver (Tanaka *et al* 1979) and in the yeasts *Candida lipolytica* (Mishina *et al* 1978a) and *Candida tropicalis* (Yamada *et al* 1980). The

Figure 5.12.: pH-activity profile of lauroyl-CoA synthetase activity in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C.

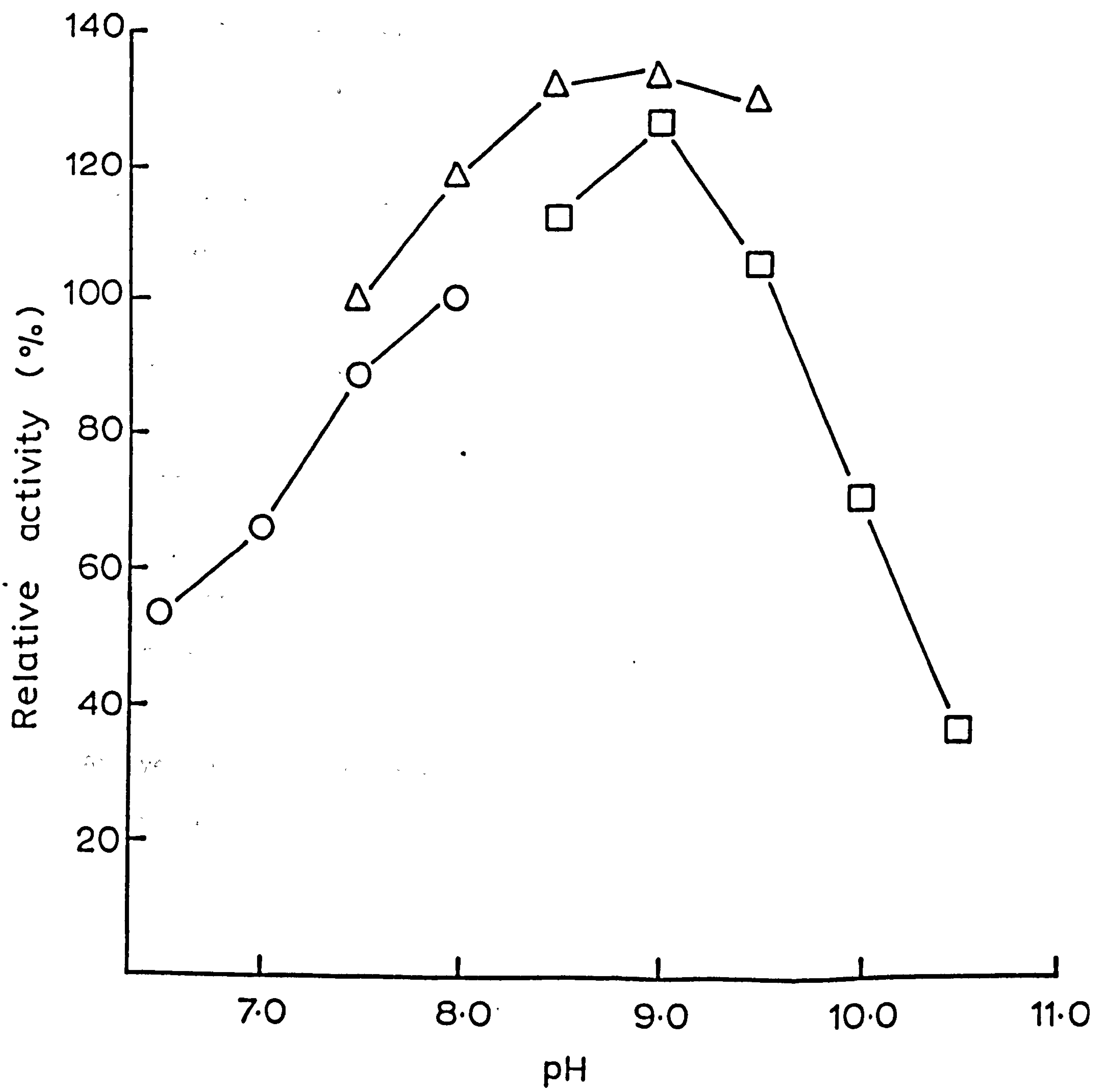
○ = potassium phosphate buffer (130mM)

△ = tris buffer (130mM)

□ = glycine-NaOH buffer (130mM)

[laurate] = 2.4mM, [CoASH] = 0.7mM, [ATP] = 4mM

100% ≡ 25.5 nmol/min/mg protein



140
120
100
80
60
40
20
Relative activity (%)
7·0
8·0
9·0
10·0
11·0
pH

Table 5.8: Subcellular location of acyl-CoA synthetase activity in succinate-grown *Corynebacterium* 7E1C

Fraction	Total Units		Specific activity*	
	C_{12}	DC_{12}	C_{12}	DC_{12}
Crude extract	568 (100)	251 (100)	46.8	20.9
Supernatant	384 (67.5)	184 (73.3)	47.2	22.6
Pellet	66 (11.5)	17 (6.9)	41.2	10.9

Crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C

Assayed in potassium phosphate buffer (130mM, pH 8)

[fatty acid] = 2.4mM, [CoASH] = 0.8mM, [ATP] = 4mM

constitutive acyl-CoA synthetases of *Nocardia asteroides* (Calmes and Deal 1973) and *Bacillus megaterium* (Massaro and Lennarz 1965) were repoted as being cytoplasmic. The situation in *E.coli*, the most studied bacterium, is less clear. Overath *et al* (1969) reported acyl-CoA synthetase to be membrane bound as did O'Brian and Frereman (1977); whilst Klein *et al* (1971) suggested a role in fatty acid uptake which presumably necessitates some form of membrane interaction. However, Samuel *et al* (1970) and Kameda and Nunn (1981) found *E.coli* acyl-CoA synthetase to be soluble, although they did not rule out a weak membrane interaction.

5.9. SUBCELLULAR LOCATION OF THIOESTERASE ACTIVITY

Crude cell-free extract from hexadecanol-grown cells was ultracentrifuged at 100 000g for 90min. at 4°C (Table 5.9). Thioesterase activity (lauroyl-CoA substrate) was predominantly soluble with 83.1% of the original activity (≡ 87.2% of recovered activity) in the supernatant. Thioesterases in the other bacteria that have been investigated, *E.coli* (Barnes and Wakil 1968; Barnes *et al* 1970) and *Rhodopseudomonas sphaeroides* (Boyce and Lueking 1984; Seay and Lueking 1986) are also soluble.

5.10. REGULATION OF THIOESTERASE ACTIVITY

The effect on thioesterase activity of a number of potential metabolites was investigated with a view to gaining more infomation as to its *in vivo* function(s)(Table 5.10). Also, such infomation will allow an estimate to be made of thioesterase activity in other assays ie: β-oxidation and acyl-CoA synthetase.

Only CoASH inhibited activity to any extent (40% inhibition at

Table 5.9: Subcellular location of lauroyl-CoA thioesterase activity in hexadecanol-grown *Corynebacterium* 7E1C

Fraction	Activity (nmol/min/mg)	Total Units (nmol/min)	Total Units (%)
Crude extract	43.0	433	100
Supernatant	57.1	360	83.1
Pellet	16.0	53	12.2

Crude cell-free extract was ultracentrifuged at 100 000g for 90 minutes at 4°C.

Assayed in potassium phosphate buffer (130mM, pH 8) with lauroyl-CoA (80µM).

Table 5.10: Effect of various metabolites on lauroyl-CoA thioesterase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Addition to standard assay	Relative activity (%)
None	100
ATP (4mM)	102
ADP (4mM)	103
AMP (4mM)	120
NAD^+ (1mM)	90
NADH (1mM)	104
CoASH (0.3mM)	59*

Assayed continuously by reaction with DTNB in potassium phosphate buffer (130mM, pH 8.0)

* = assayed discontinuously (see Methods)

Substrate = lauroyl-CoA (80μM)

100% ≡ 44nmol/min/mg protein

300μM).The effect of nucleotides on the thioesterases of *E.coli* and *Rhodopseudomonas sphaeroides* has not been investigated. However, the mitochondrial and peroxisomal thioesterases of rat brown adipose tissue are both inhibited by certain nucleotides (Alexson *et al* 1989). These authers demonstrated the mitochondrial activity to be inhibited by NADH (40% inhibition at 0.75mM), CoASH (73% inhibition at 75μM) and ATP (41% inhibition at 1.8mM). The peroxisomal activity was inhibited by CoASH (74% inhibition at 75μM) and ATP (41% inhibition at 1.6mM) but was unaffected by NADH. Thus lauroyl-CoA thioesterase activity of *Corynebacterium* 7E1C is relatively insensitive to nucleotide regulation compared to these enzymes.

5.11. FUNCTION AND SIGNIFICANCE OF THIOESTERASE ACTIVITY

The functions of thioesterases in any organism or tissue are unknown although various functions have been proposed. These are: (1) incorporation of exogenous fatty acids into lipids (Seay and Lueking 1986), (2) regulation of the chain-length of the fatty acid product of the fatty acid synthase (Libertini and Smith 1978; Knudson 1979; de Renobles *et al* 1980), (3) regulation of intracellular long-chain acyl-CoA concentrations (Barnes and Wakil 1968; Spencer *et al* 1978; Berge and Aarsland 1985), (4) Maintenance of the CoASH pool (Matsunaga *et al* 1985). It is of course quite conceivable that different thioesterases have different functions.

In view of the uncertainty as to the role of thioesterases it is difficult to ascribe a function to the thioesterase activity of *Corynebacterium* 7E1C.

The preference for long-chain acyl-CoA esters may indicate a role in regulating the intracellular acyl-CoA concentration since long-chain acyl-CoAs will have a strong detergent effect. The significantly higher

K_m values for thioesterase as compared to β-oxidation are consistent with this as a "safety-valve" only becoming fully active at high acyl-CoA concentrations. The inhibition by CoASH is also consistent with this role. Thioesterase activity would be maximal at low free CoASH concentrations which would occur when most intracellular CoA is acylated ie: when the acyl-CoA concentration is high. Furthermore, the futile cycle that thioesterase and acyl-CoA synthetase can potentially form (Fig.4.1) could be modulated by the CoASH concentration; thioesterase being maximally active at low CoASH concentrations whilst acyl-CoA synthetase, since CoASH is a substrate, is likely to be poorly active at low CoASH concentrations. It must be pointed-out that what constitute "high" and "low" CoASH concentrations for thioesterase and acyl-CoA synthetase are not presently known. Therefore until the inhibition constant (K_i) of CoASH towards the thioesterase and the K_m for CoASH of the acyl-CoA synthetase are known such a regulatory mechanism must remain conjecture. It is, however, an interesting possibility. Samuel and Alhaud (1969) reported that palmitoyl-CoA thioesterase activity in *E.coli* was induced by growth on oleate, although there was significant constitutive activity. This may indicate an involvement in regulating intracellular acyl-CoA concentration. Both *E.coli* thioesterases (I and II) are active with palmitoyl-CoA, so it is not known if one or both enzymes were induced by oleate; indeed it is important to note that the *E.coli* thioesterases were purified from cells grown on non-fatty acid containing media, ie: non-induced cells. Interestingly, an *E.coli* mutant lacking (10% of wild-type activity) the broad substrate specificity thioesterase II has been isolated for which no mutant phenotype could be found (Narasimhan *et al* 1986) when grown on non-fatty acid-containing media. This rather surprising result may indicate a role in regulating acyl-CoA concentrations which is only likely to be important during growth on fatty acid. It should be noted that thioesterase II is active

with β-hydroxmonocarboxyl-CoA esters, which are β-oxidation intermediates, although it was more active with the purified D-isomer (the isomer produced by the fatty acid synthase) than with a DL-racemate.

The activity of *Corynebacterium* 7E1C thioesterase towards ω-hydroxymonocarboxyl-CoA and dicarboxyl-CoA esters is not consistent with a function in phospholipid biosynthesis since the corresponding ω-hydroxyfatty acids are not found in phospholipids. Thioesterase activity towards such substrates could be envisaged to serve two functions. As with monocarboxylic acids a function could be the rgulation of acyl-CoA concentrations. Another posible function is related to dicarboxylic acid production. Dicarboxylic acids are accumulated (at least partially) extracellularly by *Corynebacterium* (du Pont 1973, 1974). Thus it would seem logical for the cell to remove the CoASH before the acyl-group is exported from the cell; the thioesterase(s) may conceivably play a role in the export mechanism (c.f. the proposed role of acyl-CoA synthetase in the uptake of exogenous fatty acids by *E.coli*, Klein *et al* 1971). However,in view of the apparent inability to produce DC_{16}, the high levels of ω-hydroxypalmitoyl-CoA and DC_{16} thioesterase activity are difficult to reconcile with such a function. Another observation that is difficult to explain, in view of the inability to synthesize di-CoA esters of dicarboxylic acids,is the similar activity with both the mono- and di-CoA esters of a given dicarboxylic acid; although perhaps this activity serves to prevent net synthesis of the di-CoA ester.

Despite the inability to ascribe any definite function(s) to the thioesterase activity of *Corynebacterium* 7E1C, the presence of such an activity requires that its effect on other assays be considered. Since the acyl-CoA synthetase assay is based on following the disappearance of free CoASH, on incorporation into acyl-CoA esters, the activity measured

in the presence of thioesterase is the NET rate of acyl-CoA synthesis (ie: acyl-CoA synthetase - thioesterase). Although, in view of the 40% inhibition of thioesterase by 0.3mM CoASH the activity under the conditions of the acyl-CoA synthetase assay (0.6mM CoASH) will be considerably lower than measured in the thioesterase assay, but probably still significant.

5.12. DETERMINATION OF THE NUMBER OF ACYL-CoA SYNTHETASES

In view of the very broad substrate specificity of acyl-CoA synthetase activity it was decided to investigate the number of acyl-CoA synthetase enzymes present in crude extracts. Of particular interest was the question of whether long-chain monocarboxylic and dicarboxylic acids were acted on by the same or different acyl-CoA synthetases.
The variation in the relative rates of activation of laurate and of DC_{12} by different batches of cell-free extract (Table 5.6) indicated that these substrates may be acted on by different enzymes although, as discused in section 5.6.1. this may be due to variations in the relative activities of lauroyl- and DC_{12}-CoA thioesterase.

5.12.1. THERMAL INACTIVATION OF ACYL-CoA SYNTHETASE

The rate of thermal inactivation of lauroyl-CoA synthetase and DC_{12}-CoA synthetase activity was determined with different batches of succinate-grown cells (Fig. 5.13). Extract from succinate-grown cells was used so that the endogenous rate was approx. zero. The absolute and the relative activities with laurate and DC_{12} are different in the two batches of extract. However, in both cases DC_{12}-CoA synthetase activity is more thermolabile than lauroyl-CoA synthetase activity. This

Figure 5.13.: Thermal inactivation of acyl-CoA synthetase activity in crude cell-free extracts of succinate-grown *Corynebacterium* 7E1C.

Aliquots (0.5ml) of extract were heated at 40°C for the stated period then assayed for acyl-CoA synthetase activity in potassium phosphate buffer (130mM, pH 8.0)

Assay conditions: [fatty acid] = 2.4mM, [CoASH] = 0.7mM

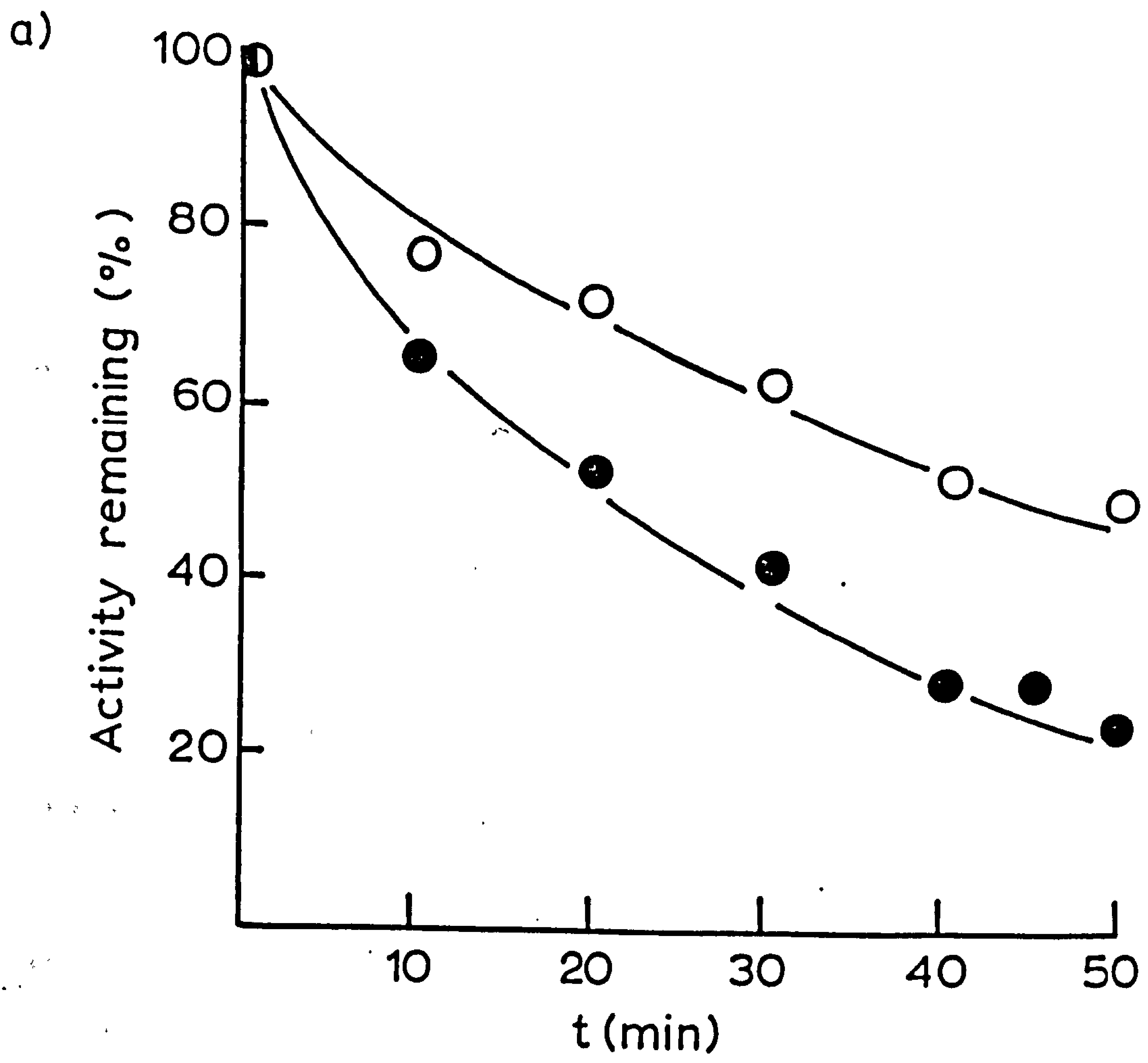
Initial activities:

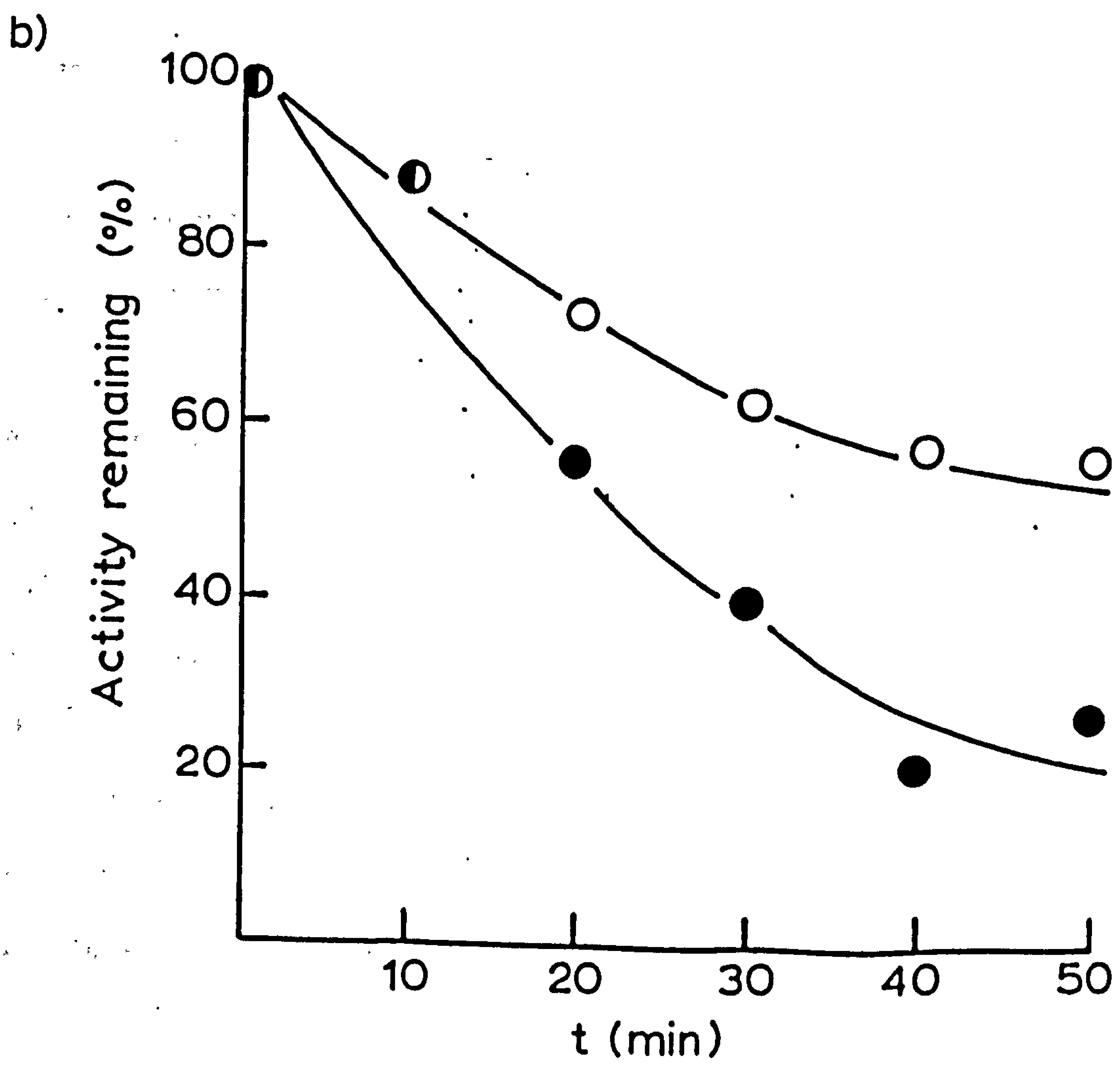
(a) laurate = 28.1 nmol/min/mg protein ○

DC_{12} = 28.1 nmol/min/mg protein ●

(b) laurate = 22.0 nmol/min/mg protein ○

DC_{12} = 11.5 nmol/min/mmg protein ●


a)
Activity remaining (%)
100
80
60
40
20
10
20
30
40
50
t (min)


b)
Activity remaining (%)
100
80
60
40
20
10
20
30
40
50
t (min)

seemingly implies that laurate and DC_{12} are acted on by different acyl-CoA synthetases. However, this differential inactivation may be due to a differential inactivation of lauroyl- and DC_{12}-CoA thioesterase activities rather than to a differential inactivation of different acyl-CoA synthetase activities.

5.12.2. ATTEMPTED PURIFICATION / SEPARATION OF ACYL-CoA SYNTHETASE ACTIVITIES

Thermal inactivation studies (see 5.12.1.) had indicated that crude cell-free extracts may contain more than one acyl-CoA synthetase. Therefore it was decided to try to separate the activities by FPLC using a polyethylene imine (PEI) ion-exchange column. This attempted separation/purification of acyl-CoA synthetase activities was not particularly successful. Recoveries of activity were usually in the range 25-50% (usually towards the lower end of this range) with recovery of DC_{12} activity being poorer than for activity with laurate. A problem encountered in assaying column fractions was that it was difficult to measure low activities accurately due to the high background absorbance since an absorbance decrease, rather than increase, is measured. Thus it is difficult to decide whether small peaks are due to changes in the background absorbance or whether they represent true activity peaks. The elution profile of a typical column run is shown in Fig. 5.14. and the recovery of activity summarized in Table 5.11.. There are 2 definite peaks of acyl-CoA synthetase activity. The first eluting peak (peak I) is more active with laurate than with DC_{12} whilst the second peak (peak II) is equally active with both substrates. The presence of 2 peaks of activity is reproducible but the relative activities with laurate and DC_{12} within each peak varied from one experiment to another. The presence of the other peaks was not

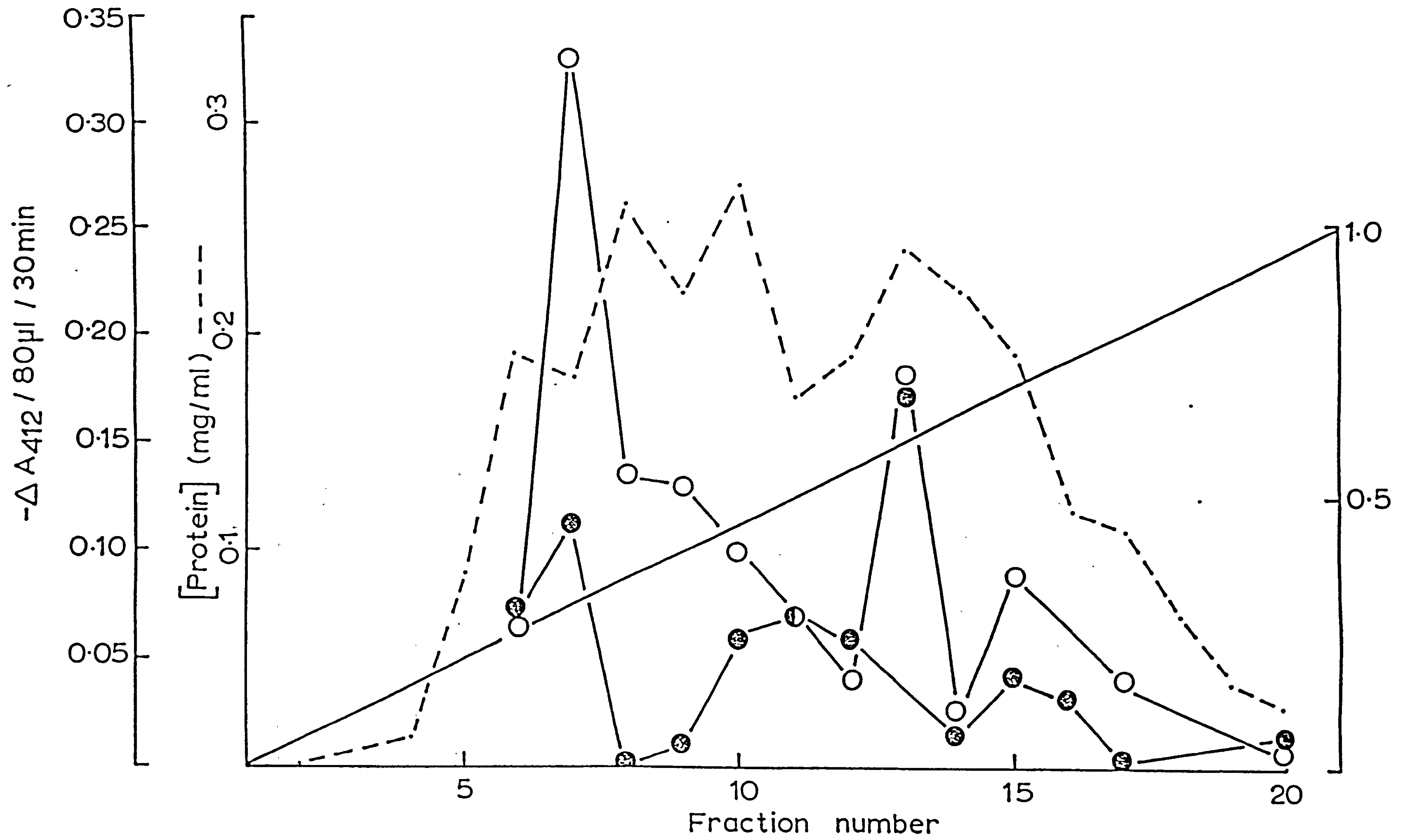
Figure 5.14: Purification profile of acyl-CoA synthetase activity in succinate-grown *Corynebacterium* 7E1C.

Chromatographed on a polyethylene imine ion-exchange column (6cm x 1.5cm)

Column fractions assayed for activity in potassium phosphate buffer (130mM, pH 8) with laurate (2.4mM) or DC_{12} (2.4mM) as substrate.

○ = activity with laurate

● = activity with DC_{12}



-Δ A412 / 80µl / 30min
0·35
0·30
0·25
0·20
0·15
0·10
0·05
[Protein] (mg/ml) - - - -
0·3
0·2
0·1
1·0
0·5
5
10
15
20
Fraction number

Table 5.11.: Attempted purification of the acyl-CoA synthetases of succinate-grown *Corynebacterium* 7E1C

Fraction	Units*			Specific activity**	
	C_{12}	DC_{12}		C_{12}	DC_{12}
Crude extract	47 (100)	31 (100)		25.4	15.6
PEI eluate	25 (54)	8 (26)	I	69.0	23.0
			II	28.0	27.0

* = nmol/min/mg protein

** = 1 unit ≡ 1 nmol/min

I = first eluting activity peak (see Fig.5.14)

II = second eluting activity peak

PEI = polyethylene imine

Assay conditions: [fatty acid] = 2.4 mM, [ATP] = 4mM, [CoASH] = 0.7mM

reproducible and they most likely represent variations in the background absorbance. The reason for the variation in the relative activities with laurate and DC_{12} within each peak is unknown. Peaks I and II were always clearly observable when assayed with laurate. However, when assayed with DC_{12} the activity varied from approx. zero (ie: not obviously greater than background variation) to 100% of that observed with laurate. The reason(s) for this variation is not known. It may indicate that peaks I and II both contain more than one acyl-CoA synthetase. Alternatively it may indicate the presence of differing levels of thioesterase activity in the acyl-CoA synthetase activity peaks. The column fractions were never assayed for acyl-CoA thioesterase activity.

Despite the problems encountered during the attempted separation of acyl-CoA synthetase activities it certainly seems that *Corynebacterium* 7E1C posses at least two acyl-CoA synthetase enzymes, although the relative activity of the two enzymes with monocarboxylic and dicarboxylic acids is unclear. The presence of more than one acyl-CoA synthetase would be consistent with the situation in the yeast *Candida lipolytica* where acyl-CoA synthetase I is active only with nonocarboxylic acids while acyl-CoA synthetase II is active with monocarboxylic, ω-hydroxymonocarboxlic and dicarboxylic acids (Mishina *et al* 1978a,b; Hosaka *et al* 1981). Similarly rat liver microsomes contain an acyl-CoA synthetase active with monocarboxylic and ω-hydroxymonocarboxylic but not dicarboxylic acids (Tanaka *et al* 1979); additionally they contain a dicarboxyl-CoA synthetase (Vamecq *et al* 1985). Shimizu *et al* (1980) demonstrated 2 peaks of palmitoyl-CoA synthetase activity upon chromatography of a cell-free extract of *Pseudomonas aeruginosa* on palmitate, whilst Trust and Millis (1971) found that various strains of palmitate-grown *Pseudomonas* possesses 3 acyl-CoA synthetases of different chain-length specificity

catalysing the activation of C_2 to C_{22} monocarboxylic acids.

6. β-OXIDATION

The β-oxidation system of *Corynebacterium* 7E1C was investigated with a view to determining its specificity towards monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids. The acyl-CoA synthetases of this organism display a vey broad substrate specificity and do not appear to discriminate greatly between the various classes of fatty acid (ie: monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic). Furthermore, within a class there is little discrimination with respect to acyl- group chain-length. Therefore, it seemed important to determine the specificity of the β-oxidation system to see how this is correlated with the spectrum of dicarboxylic acids produced by this organism.

6.1. β-OXIDATION ASSAYS

Two β-oxidation assays were used. One was a linked assay in which the acyl-CoA esters were generated *in situ* by the acyl-CoA synthetase(s) present in crude extracts. The other was a direct assay in which preformed acyl-CoA esters were added to the assay and was independent of the activity of the acyl-CoA synthetase(s).

In both assays activity could be measured either by following NAD^+ reduction at the 3-hydroxyacyl-CoA dehydrogenase step (this was done routinely) or by following acetyl-CoA production.

The cofactor requirements of the linked system (Table 6.1) are those expected of an acyl-CoA synthetase except for the additional requirement for NAD^+. The direct assay was independent of both ATP and CoASH, the only requirement being for acyl-CoA (which could not be substituted by free fatty acid) and NAD^+.

With the direct assay the endogenous activity was essentially zero regardless of the growth-substrate of the cells from which the extract was prepared. However, with the linked assay a very high endogenous rate (not, or poorly stimulated by addition of fatty acid substrate) was

Table 6.1: Cofactor requirements of the linked β-oxidation assay in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C.

Deletion from standard assay	Relative activity (%) NAD$^+$ reduction	Acetyl-CoA formation
None	100	100
-ATP	13	6
-CoASH	2	2
-$MgCl_2$	80	n.d.
-NAD$^+$	24*	24
-NAD$^+$,+NADP$^+$	3	n.d.

* rate rapidly fell to zero (within 2 min.)

Assayed with laurate (0.75 mM) in potassium phosphate (130 mM, pH 8.0)

[ATP] = 4 mM

[CoASH]= 0.6 mM

100% ≡ 30 nmol/min/mg protein

observed with extracts from cells grown on alkyl-containing substrates (ie: dodecanediol, hexadecanol or methyl palmitate). This high endogenous rate (upto 20nmol/min/mg) is presumably due to activation of endogenous fatty acid derived from the growth substrate. Therefore the linked assay could only be used with extracts from succinate-grown cells since these displayed an approx. zero endogenous rate.

It is important to note that cell-free extracts contain a number of activities that will interfere with the rates measured. These activities are NADH "oxidase", acetyl-CoA thioesterase, long-chain acyl-CoA thioesterase (see Chapter 5) and ω-hydroxyfatty acid dehydrogenase (only a possible problem when assaying the β-oxidation of ω-hydroxyfatty acids by measuring NAD^+ reduction). Typical values of these activities are shown in Table 6.2. The acetyl-CoA thioesterase and NADH "oxidase" activities will tend to result in an underestimation of the activity of the activity of the β-oxidation system as measured by acetyl-CoA and NADH production respectively. The long-chain thioesterase activity has the effect of rapidly reducing the acyl-CoA substrate concentration so that assays are not linear for prolonged periods of time (no more than 3-4 minutes). The presence of this activity and of NADH "oxidase" also means that it is not possible to determine the number of cycles of β-oxidation achieved as both substrate and product are degraded to an unknown extent. The presence of ω-hydroxyfatty acid dehydrogenase activity requires that rates of NAD^+ reduction measured for the β-oxidation of these substrates be corrected for NAD^+ reduction due to the ω-hydroxyfatty acid dehydrogenase activity. Fortuitously, ω-hydroxyfatty acid dehydrogenase (see Chapter 4) is maximally active at higher salt concentrations than obtain in the β-oxidation assays so this activity is not as much of a problem as it might otherwise be.

Table 6.2: Activities present in crude cell-free extracts of hexadecanol-grown *Corynebacterium* 7E1C which interere with the measurement of β-oxidatiion

Enzyme	Substrate	Activity (nmol/min/mg)
β-oxidation*	lauroyl-CoA (80μM)	37.4
	12-hydroxylauroyl-CoA (80 μM)	4.1
	16-hydroxypalmitoyl-CoA (80 μM)	31.7
Thioesterase	acetyl-CoA (80 μM)	10.6
	acetyl-CoA (37 μM)	4.1
	lauroyl-CoA (80μM)	43.0
ω-hydroxyfatty acid dehydrogenase	12-hydroxylaurate (80 μM)	0.8
	16-hydroxypalmitate (80 μM)	0
"NADH oxidase"	NADH (37 μM)	17.1
	NADH (18.5 μM)	14.6

* = measured as rate of NAD^+ reduction.

All activities assayed in potassium phosphate buffer (130 mM, pH 8.0)

6.1.1. LAG IN β-OXIDATION ASSAYS

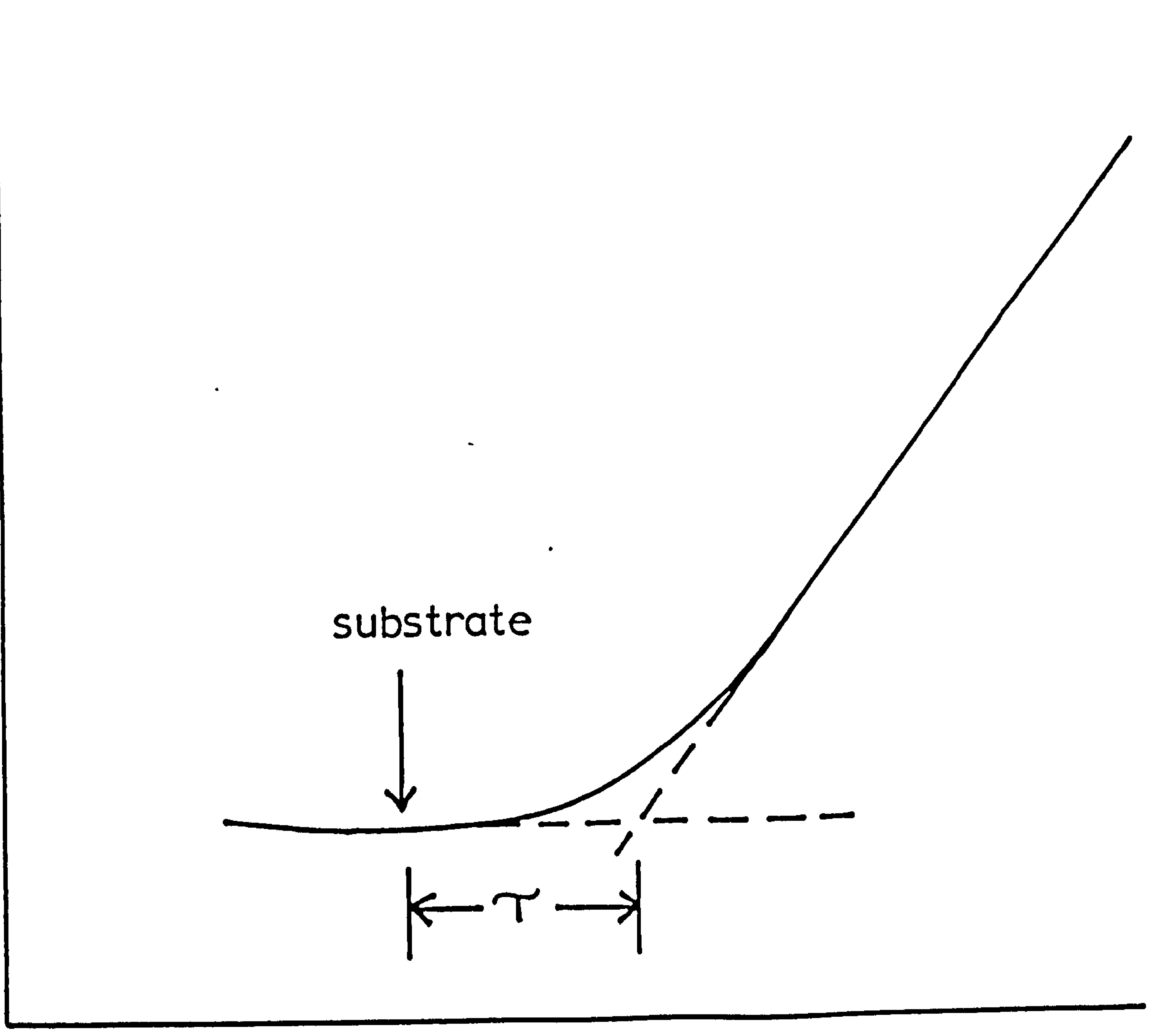
It was observed that β-oxidation assays displayed a lag phase during which the rate of NAD^+ reduction (or acetyl-CoA production) increased with time until a constant linear rate was obtained (Fig. 6.1). The lag was more pronounced in the linked than in the direct assay. The effect of the amount of extract added to assays on the length of lag (or transition-time, τ) was investigated. The transition time was inversely proportional to the amount of extract added in both the direct assay (Fig. 6.2) and the linked assay (Fig. 6.3). This is the expected behaviour of a series of consecutive reactions in which the concentration of the product of the first reaction must build-up to its steady-state concentration before the next reaction can proceed maximally and so on down the series of reactions (Easterby 1981 ; Kuchel 1985). The more extract is added the quicker the steady-state is reached and hence the shorter is τ. The transition time is longer (for a given volume of extract) with the linked assay (Figs. 6.2 and 6.3).

Vamecq and Draye (1987), when assaying peroxisomal acyl-CoA oxidase using a linked assay (ie: acyl-CoA generated *in situ*) observed a lag in H_2O_2 production that was inversely proportional to the amount of extract added. No lag was observed when the oxidase was assayed directly with acyl-CoA.

The lag observed when assaying β-oxidation in *Corynebacterium* 7E1C implies a pool of free intermdiates of the reaction pathway (Easterby 1981 ; Kuchel 1985). The implications of this behaviour with respect to the stuctural organization of β-oxidation in *Corynebacterium* 7E1C are discussed in section 6.7..

Figure 6.1: Lag in β-oxidation assays

τ = transition-time


A340
substrate
τ
t (min)

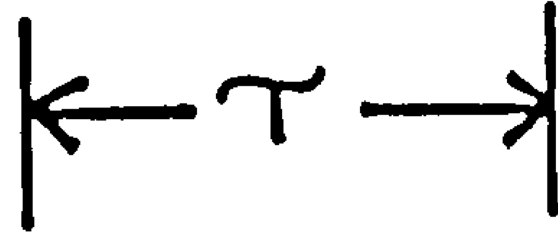
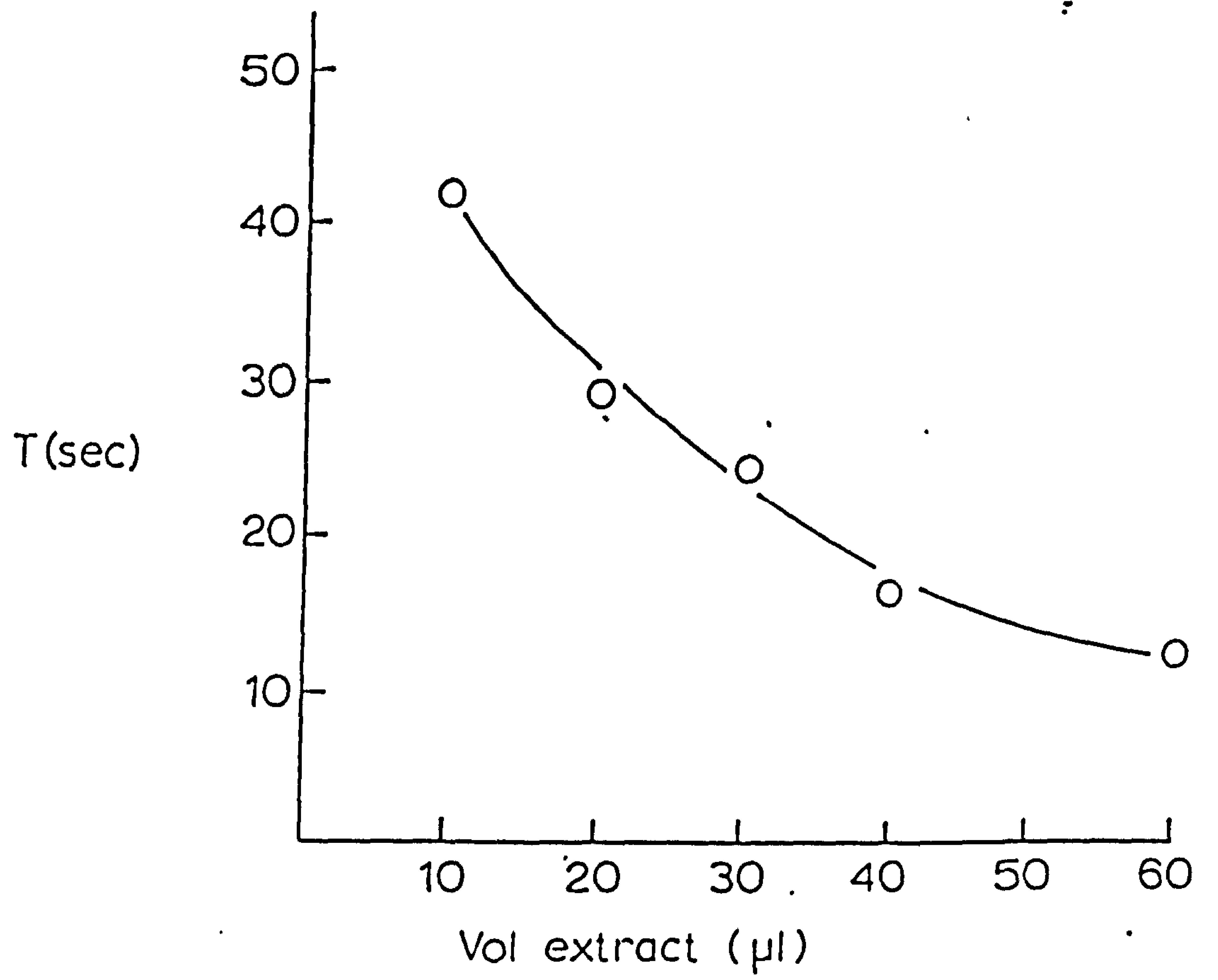


Figure 6.2: Variation of τ with volume of extract in the direct β-oxidation assay

Assayed in potassium phosphate buffer (130mM, pH 8) with lauroyl-CoA (80μM) as substrate. Cell-free extract from succinate-grown cells


T (sec)
50
40
30
20
10
10
20
30
40
50
60
Vol extract (μl)

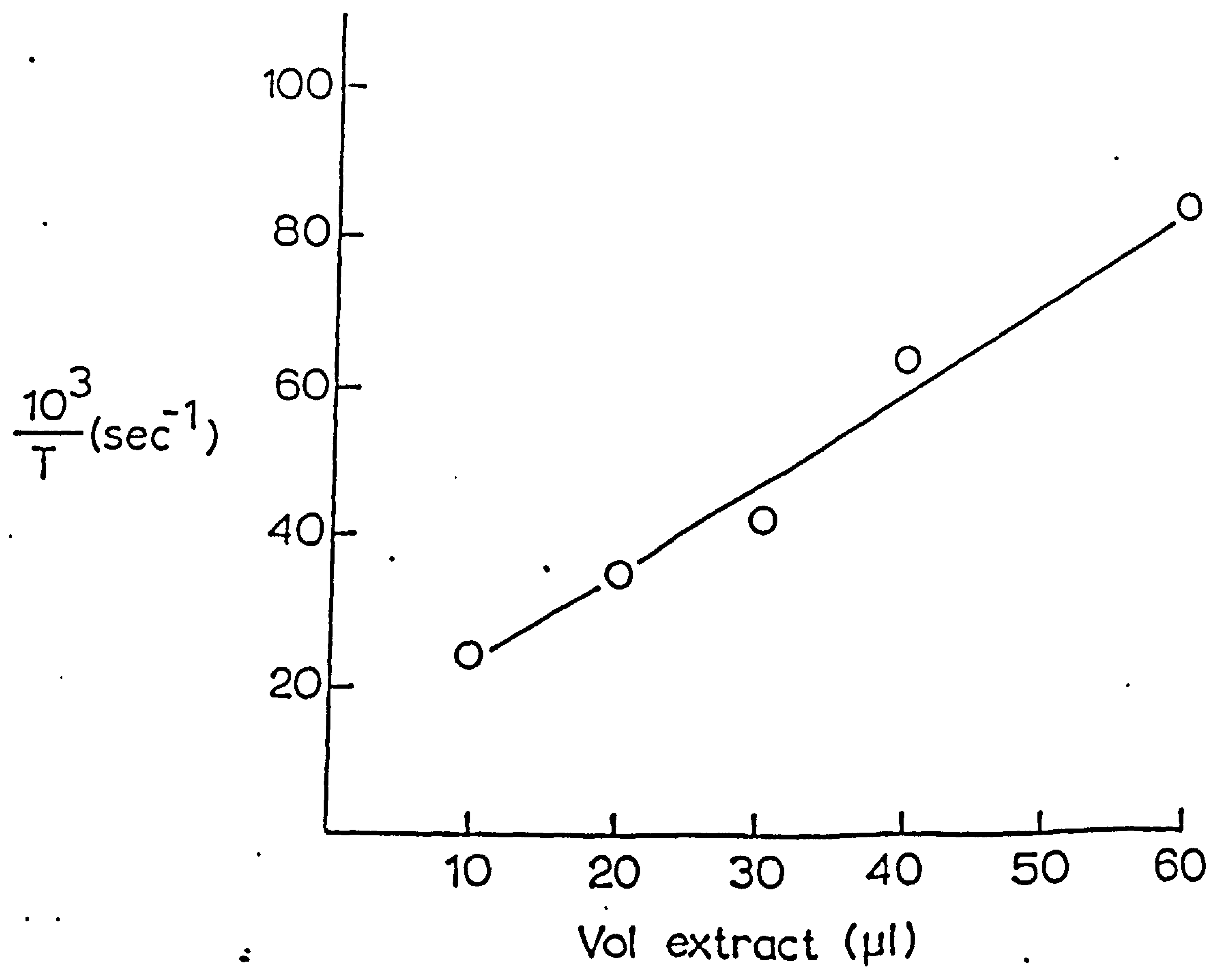
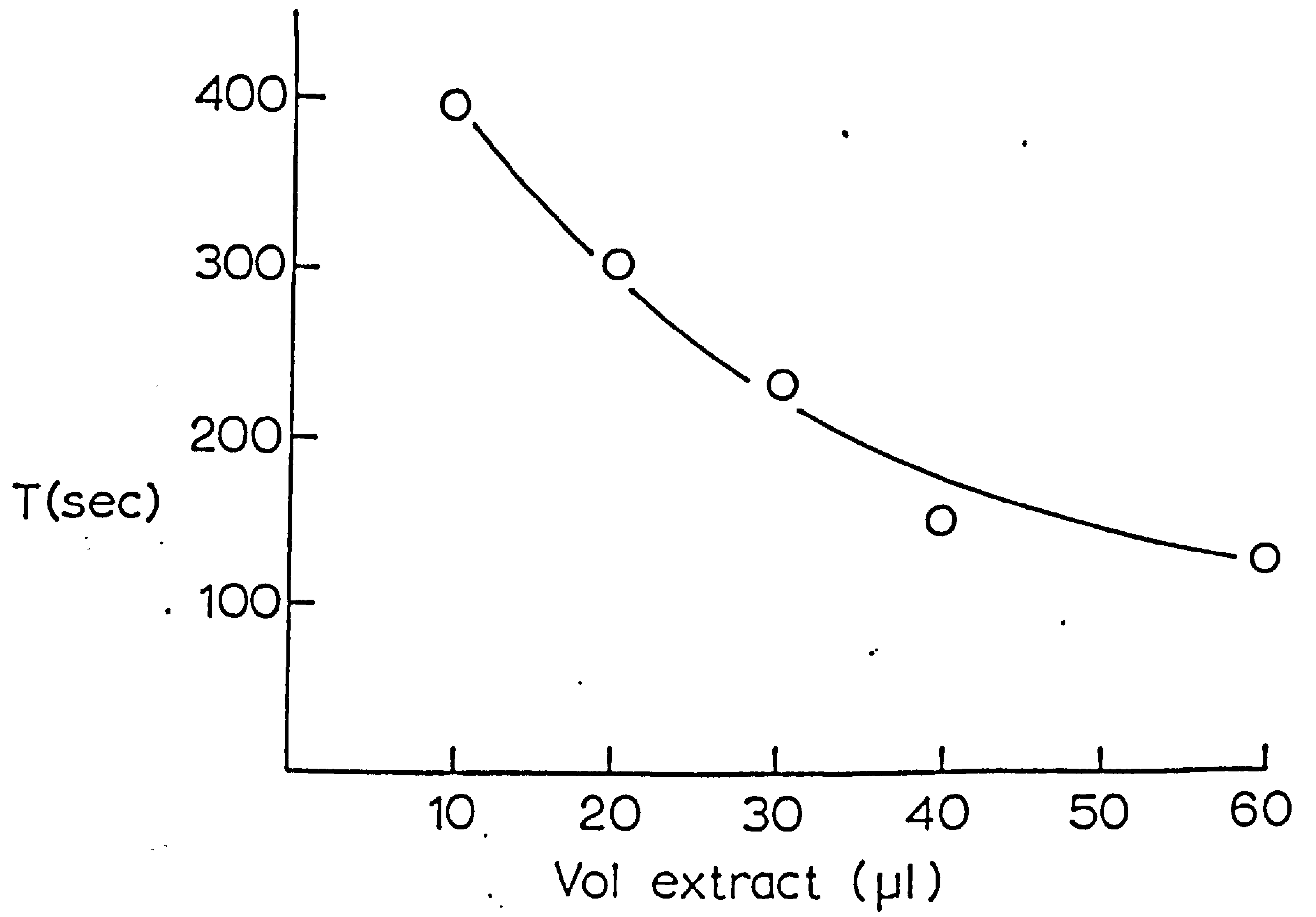
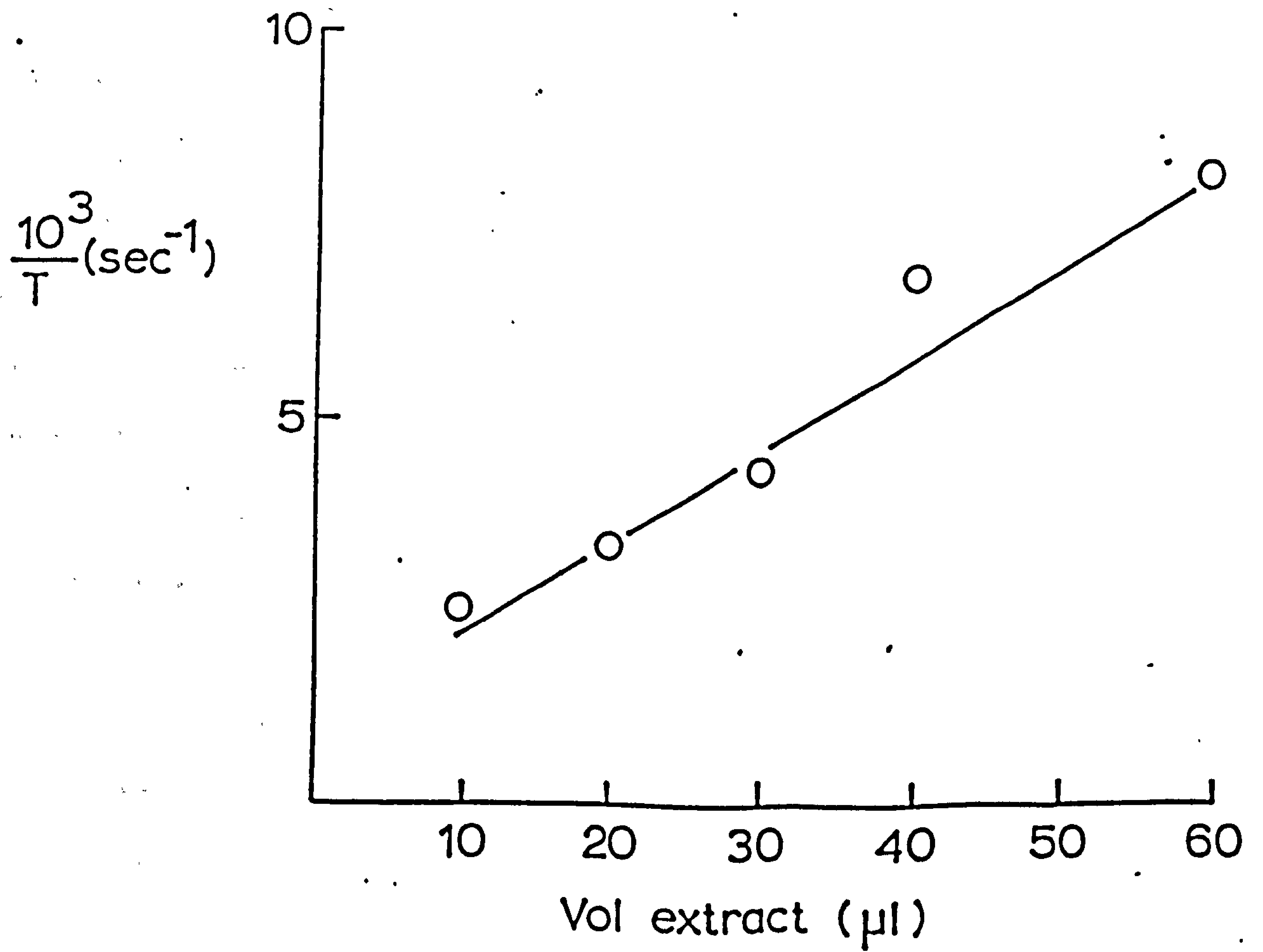

$\frac{10^3}{T}$ (sec^{-1})
100
80
60
40
20
10
20
30
40
50
60
Vol extract (μl)

Figure 6.3: Variation of τ with volume of extract in the 'linked' β-oxidation assay

Assayed in potassium phosphate buffer (130mM, pH 8) with laurate (0.75mM) as substrate. Cell-free extract from succinate-grown cells


400
300
200
100
T(sec)
10
20
30
40
50
60
Vol extract (µl)


10
5
$\frac{10^3}{T}$(sec^{-1})
10
20
30
40
50
60
Vol extract (µl)

6.2. INDUCTION OF β-OXIDATION

The specific activity of β-oxidation (assayed with myristoyl-CoA) after growth on a range of carbon-sources is shown in Table 6.3. Activity is essentially independent of carbon-source. This is drastically different to the situation in *E.coli* where the enzymes of β-oxidation are induced by growth on long-chain fatty acids (Overath *et al* 1967, 1969; Weeks *et al* 1969; Ailaud *et al* 1969; Klein *et al* 1971; O'Connell *et al* 1986). In *E.coli* the low levels of activity present in cells grown on acetate or succinate are repressed still further by growth on glucose whereas glucose had no effect on the expression of the β-oxidation system in *Corynebacterium* 7E1C. In the present study with *Corynebacterium* 7E1C rather than measuring the activities of the individual β-oxidation enzymes the rate of flux through the pathway (starting from acyl-CoA) has been measured. Calculating the capacity of the *E.coli* β-oxidation system on the basis of the rate-limiting step (acyl-CoA dehydrogenase) the following degrees of induction can be calculated. Relative rates after growth on (amino acid + glucose), amino acid and oleate are 1:3.9:30.2 (data of Weeks *et al* 1969). Using the data of O'Connell *et al* (1986) the specific activity of the rate-limiting step of the *E.coli* β-oxidation system is increased 60-fold in cells grown on oleate as compared to cells grown on oleate plus glucose. The situation in *Caulobacter crescentus* (O'Connell *et al* 1986) is somewhat different. High constitutive levels of the β-oxidation enzymes are present in glucose-grown cells. These activities are induced up to 3-fold by growth on oleate. This induction is prevented by the addition of glucose to the growth medium. These workers did not report on activities in cells grown on acetate or succinate.

From these results it is clear that the regulation of expression of the β-oxidation system of *Corynebacterium* 7E1C is rather different to that

Table 6.3: β-oxidation in crude cell-free extracts of *Corynebacterium* 7E1C after growth on different carbon-sources

Carbon-source	*Specific activity (nmol/min/mg)	
Glucose	44	(1)
Succinate	45.1 ± 9.1	(4)
Dodecanediol	39.8 ± 1.0	(2)
Hexadecanol	47.7 ± 7.2	(7)
Methyl palmitate	43.4	(1)

* measured as rate of NAD^+ reduction with myristoyl-CoA (80 μM) as assay substrate in potassium phosphate buffer (130 mM, pH 8.0)

Values are mean ± S.D. for (n) independent determinations

reported for other bacteria. The specific activity of the *Corynebacterium* 7E1C β-oxidation system is high compared to that of other bacteria. For *E.coli* values of up to 9nmol/min/mg protein have been reported for palmitoyl-CoA dehydrogenase (O'Brian and Frerman 1977) whilst a value of 43nmol/min/mg protein was reported for *C.crescentus* palmitoyl-CoA dehtdrogenase (O'Connell *et al* 1986). However, this latter value should be treated with some caution. O'Connell *et al* (1986) reported that to remove a competing dehydrogenase activity, crude extracts were partially purified by ammonium sulphate precipitation but no mention is made of whether the specific activity they quote is that of the partially purified preparation or whether it corresponds to the crude extract allowing for the (unquoted) purification factor. However, the important point is that *Corynebacterium* 7E1C posseses a very active β-oxidation system.

6.3. pH OPTIMUM OF β-OXIDATION

The pH optimum was determined using an extract from hexadecanol-grown cells. Myristoyl-CoA was used as substrate so that activity was independent of acyl-CoA synthetase. The pH optimum was approx. 8.0 (Fig. 6.4). No activity was observed in glycine-NaOH buffers, pH 9.0 to 10.5. The rapidly decreasing activity observed as the pH is increased above 8 may not solely be due to inactivation of the β-oxidation system but may also be due to increasingly rapid non-enzymic alkaline hydrolysis of the acyl-CoA substrate at elevated pH values. The contribution of thioesterase to acyl-CoA hydrolysis at elevated pHs has not been determined.

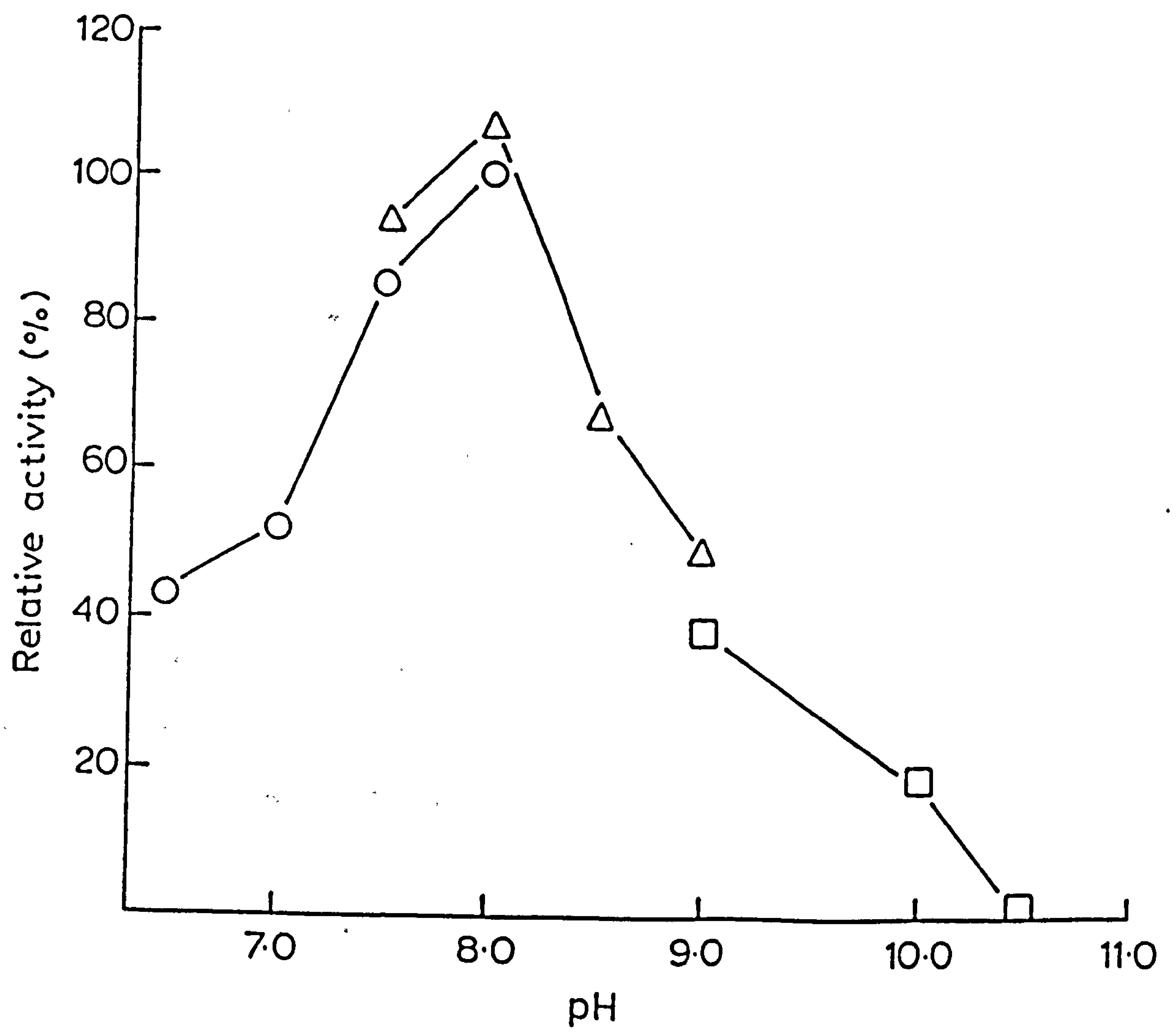
Figure 6.4: The pH optimum of β-oxidation in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed with myristoyl-CoA (80μM)

○= potassium phosphate buffer (130mM)

△= tris buffer (130mM)

□= sodium carbonate-sodium hydrogencarbonate buffer (130mM)


120
100
80
60
40
20
Relative activity (%)
7·0
8·0
9·0
10·0
11·0
pH

6.4. SUBCELLULAR LOCATION OF β-OXIDATION

Crude cell-free extract was ultracentrifuged at 100 000g for 90 minutes at 4°C (Table 6.4). Myristoyl-CoA was used as substrate so that the distribution could be measured independently of that of acyl-CoA synthetase. Of the original activity 105 % (98.8 ≡ % of recovered activity) was found in the supernatant. Thus β-oxidation was predominantly soluble ie: cytosolic.

This is in agreement with the situation in *E.coli* where acyl-CoA dehydrogenase (O'Brian and Frerman 1977), and the β-oxidation complex (O'Brian and Frerman 1977 ; Binstock and Schulz 1977) which contains the other 4 enzymes of β-oxidation are soluble ie: cytosolic. It should be noted, however, that the long-chain specific enoyl-CoA hydratase of *E.coli* showed a slight membrane association (O'Brian and Frerman 1977). The β-oxidation enzymes of *Caulobacter crescentus* are also soluble (O'Connell *etal* 1986). Similarly, eukaryotic β-oxidation systems, although located in organelles, are located in the matrix compartment. However, Sumegi and Srere (1984) reported an association of some mitochondrial β-oxidation enzymes with the inner mitochondrial membrane and a distinct membrane-bound 3-hydroxyacyl-CoA dehydrogenase has been described in mitochondria (El-Fakhri and Middleton 1982).

6.5. SUBSTRATE SPECIFICITY OF β-OXIDATION

6.5.1. MONOCARBOXYLIC ACIDS

The specificity of β-oxidation of saturated monocarboxylic acids was investigated using both the linked and direct assay systems. The specificity of β-oxidation of C_{10} to C_{16} monocarboxylic acids with a cell-free extract from succinate-grown cells, as measured by both the

Table 6.4.: Subcellular location of β-oxidation in succinate-grown *Corynebacterium* 7E1C

Fraction	Units* (%)	Specific activity** (nmol/min/mg)
Crude extract	158 (100)	19.5
Supernatant	166 (105)	22.5
Pellet	2 (1)	3.8

Crude cell-free extract was ultracentrifuged at 100 000 g for 90 minutes at 4°C

Assayed in potassium phosphate buffer (130mM, pH 8) with lauroyl-CoA (80μM) as substrate

* = 1 unit ≡ 1 nmol/min

** = measured as rate of NAD^+ reduction

linked and direct assay, is shown in Table 6.5. The two assays give similar but not identical results. With both assays a preference for long-chain substrates is seen. With the linked assay C_{16} is optimal whereas C_{14} is optimal with the direct assay. Activity is somewhat lower in the linked assay and is presumably due to the acyl-CoA synthetase being rate-limiting (specific activities of the acyl-CoA synthetase, typically ≈ 30 nmol/min/mg are lower than those of the β-oxidation system as measured in the direct assay, typically ≈ 45 nmol/min/mg).

The full-range (ie:C_4 to C_{16}) substrate specificity was determined using the direct assay with 80μM acyl-CoA as substate. The substrate specificity profile is essentially the same in both succinate-grown and hexadecanol-grown cells (Fig. 6.5). In both cases β-oxidation is most active with long-chain acyl-CoA esters, myristoyl-CoA (C_{14}) being optimal. Interestingly, this specificity is remarkably simmilar to that reported for rat liver peroxisomal β-oxidation (Lazarow 1978 ; Hyrb and Hogg 1979). To confirm that long-chain acyl-CoA esters are indeed the best β-oxidation substrates the kinetic parameters for the β-oxidation of decanoyl-, lauroyl-, myristoyl- and palmitoyl-CoA were determined using a crude cell-free extract from hexadecanol-grown cells (Figs. 6.6, 6.7, 6.8 and 6.9). There was considerable scatter of the points, especially with palmitoyl-CoA. The line of best fit for each plot was drawn to give the best fit (on visual inspection) giving greater "weight" to points obtained at high substrate concentration. The values obtained are sumarized in Table 6.6. V_{max} is highest with myristoyl-CoA but K_m increases substantially with decreasing chain-length. This confirms that long-chain acyl-CoA esters are indeed the preferred β-oxidation substrates in *Corynebacterium* 7E1C.

Table 6.5: Comparison of β-oxidation activities in a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C as measured with the linked and with the direct assays.

Acyl-chain length	Activity (nmol/min/mg)	
	Linked[1]	Direct[2]
C_{10}	8.3	12.2
C_{12}	28.9	37.3
C_{14}	30.9	60.0
C_{16}	38.9	45.6

1 = Free acid (0.75mM) as substrate [ATP] = 4mM, [CoASH] = 0.7mM

2 = Acyl-CoA ester (80µM) as substrate

Assayed in potassium phosphate buffer (130 mM, pH 8.0)

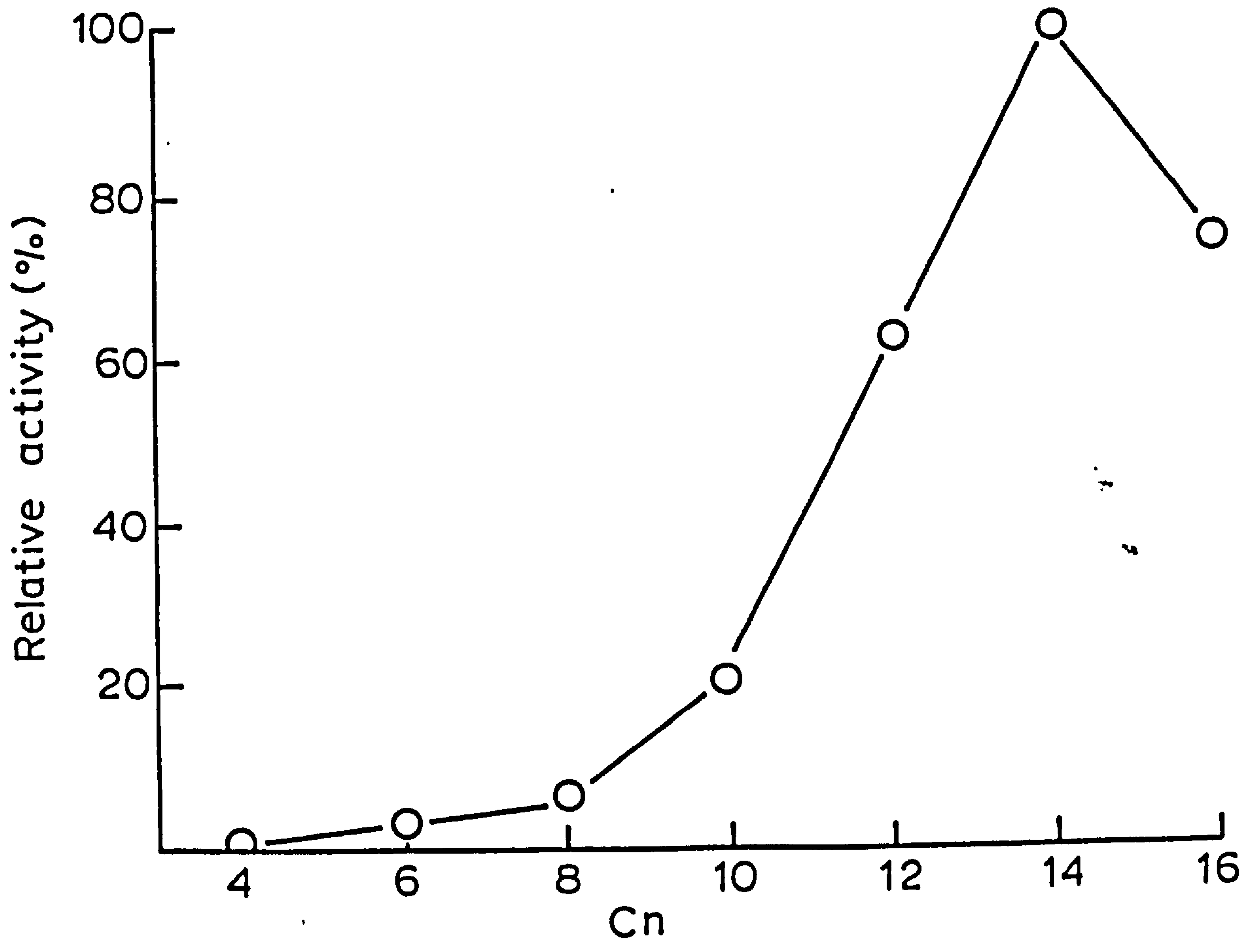
Figure 6.5: Substrate specificity of β-oxidation in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C with respect to acyl chain length in the direct β-oxidation assay

Assayed with saturated acyl-CoA (80μM) in potassium phosphate buffer (130mM, pH 8)

Specific activities:

Succinate-grown cells, 100% ≡ 60.0 nmol/min/mg protein

Hexadecanol-grown cells 100% ≡ 52.0 nmol/min/mg protein


a) Succinate-grown
Relative activity (%)
100
80
60
40
20
4
6
8
10
12
14
16
Cn

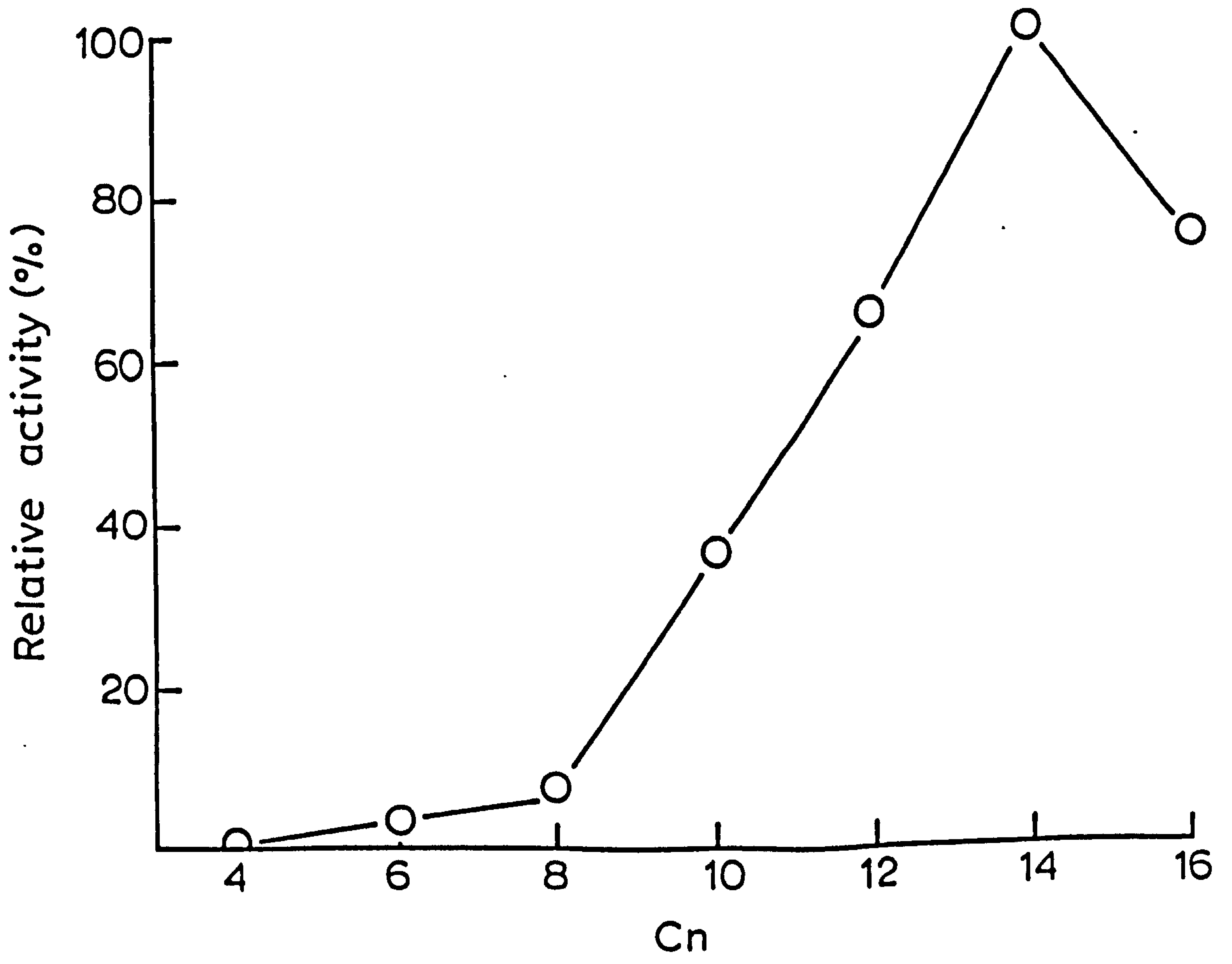
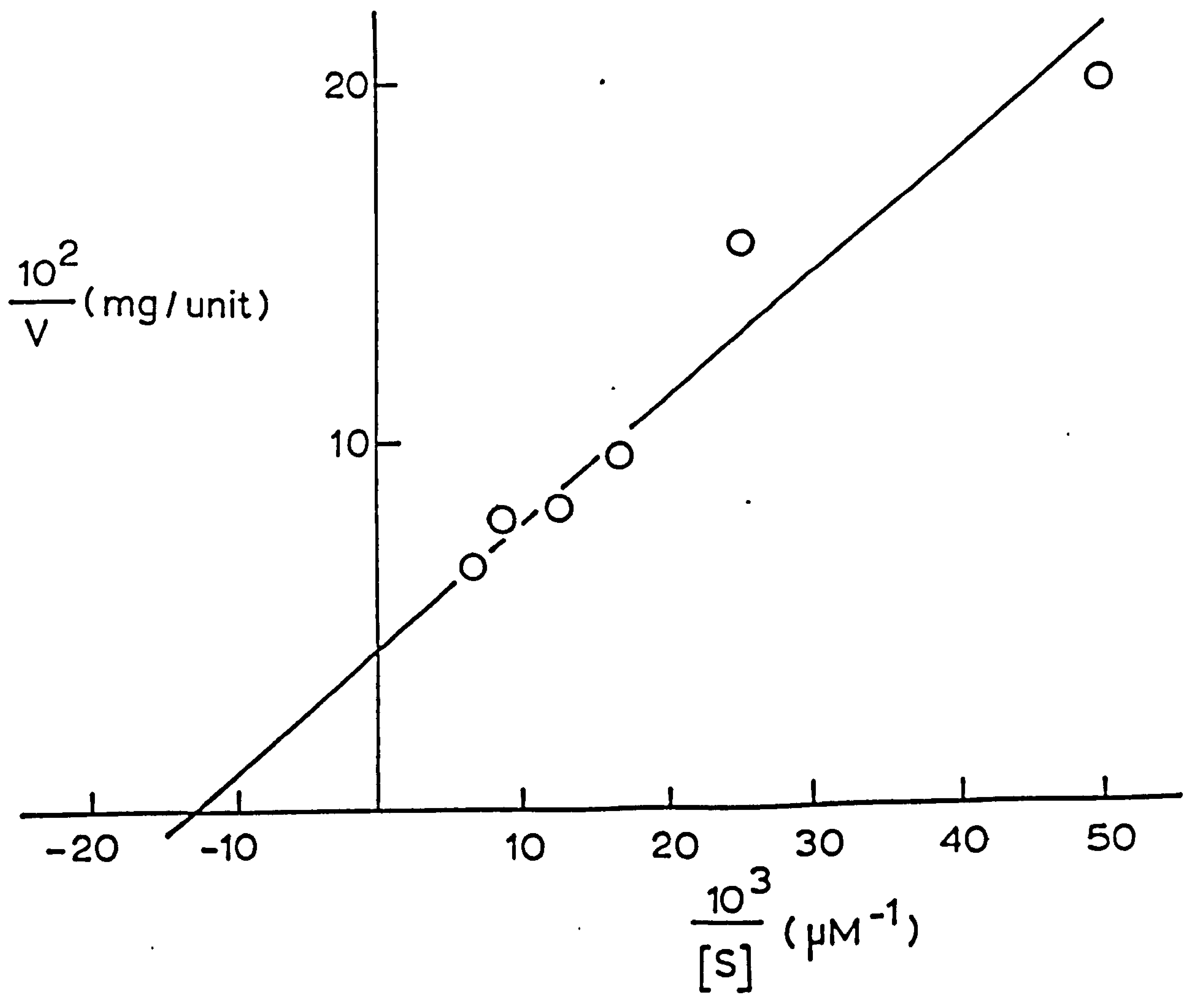

b) Hexadecanol-grown
Relative activity (%)
100
80
60
40
20
4
6
8
10
12
14
16
Cn

Figure 6.6: Lineweaver-Burk plot for the β-oxidation of decanoyl-CoA by a cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (130mM, pH 8) using the direct assay

V_{max} = 23 nmol/min/mg protein

K_m = 77 μM



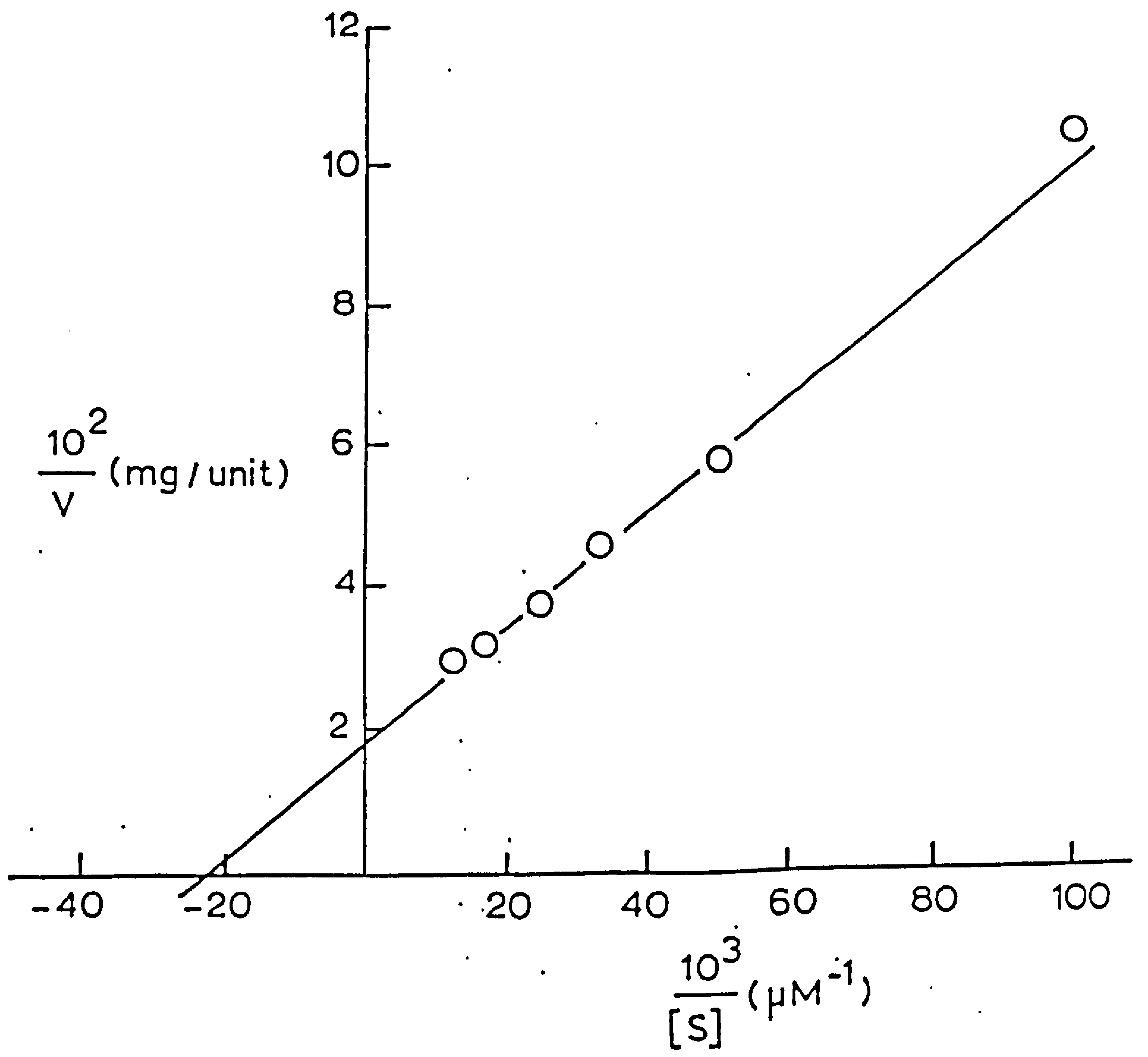
20
10
$\frac{10^2}{V}$ (mg / unit)
-20
-10
10
20
30
40
50
$\frac{10^3}{[S]}$ (μM^{-1})

Figure 6.7: Lineweaver-Burk plot for the β-oxidation of lauroyl-CoA by a cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (130mM, pH 8) using the direct β-oxidation assay

V_{max} = 56 nmol/min/mg protein

K_m = 43 μM



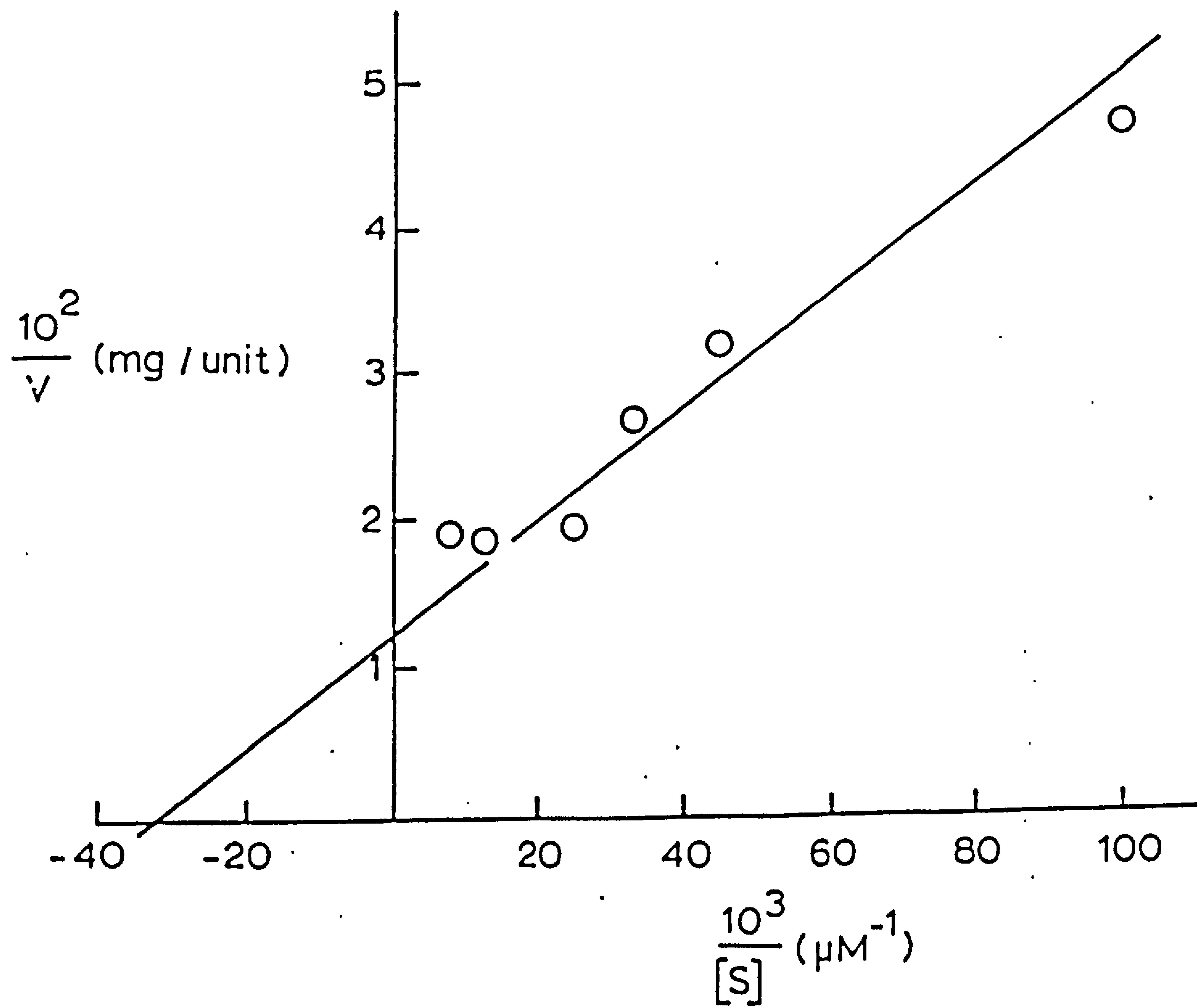
12
10
8
6
4
2
$\frac{10^2}{V}$ (mg / unit)
-40
-20
20
40
60
80
100
$\frac{10^3}{[S]}$ (μM^{-1})

Figure 6.8: Lineweaver-Burk plot for the β-oxidation of myristoyl-CoA by a cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (130mM, pH 8) using the direct β-oxidation assay

V_{max} = 83 nmol/min/mg protein

K_m = 31 µM



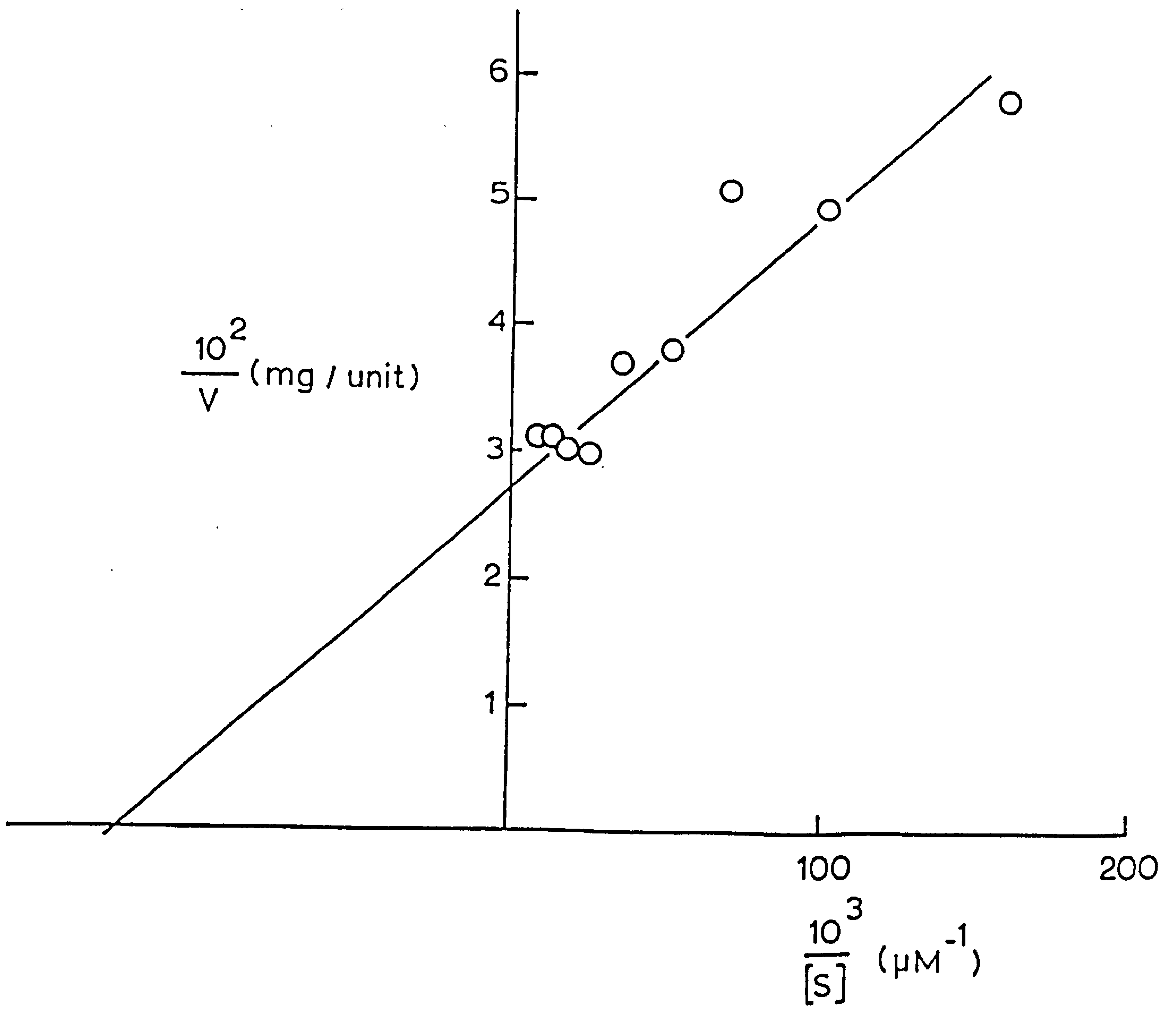
$\frac{10^2}{V}$ (mg / unit)
5
4
3
2
1
-40
-20
20
40
60
80
100
$\frac{10^3}{[S]}$ (μM^{-1})

Figure 6.9: Lineweaver-Burk plot for the β-oxidation of palmitoyl-CoA by cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phosphate buffer (130mM, pH 8) using the direct β-oxidation assay

V_{max} = 37 nmol/min/mg protein

K_m = 8 μM



6
5
4
3
2
1
$\frac{10^2}{V}$ (mg / unit)
100
200
$\frac{10^3}{[S]}$ (μM^{-1})

Table 6.6: Summary of the kinetic parameters of β-oxidation in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Acyl-CoA	V_{max} (nmol/min/mg)	K_m (μM)
C_{10}	23	77
C_{12}	56	43
C_{14}	83	31
C_{16}	37	8

Assayed in potassium phosphate buffer (130 mM, pH 8.0) using the direct assay

6.5.2. UNSATURATED FATTY ACIDS

The β-oxidation of unsaturated fatty acids was investigated using the direct assay (Table 6.7). Although the range of unsaturated acyl-CoA esters tested was rather limited it can be seen that *cis* unsaturated acyl-CoAs are β-oxidized very slowly compared to the corresponding saturated acyl-CoA. However, the only *trans* isomer tested, palmitelaidoyl-CoA (*trans* $\Delta^9C_{16:1}$-CoA) was β-oxidized at a comparable rate to palmitoyl-CoA ($C_{16:0}$-CoA). As a comparison, mitochondria β-oxidize *trans* unsaturated acyl-CoAs slowly compared to the corresponding *cis* unsaturated and saturated acyl-CoAs whereas, in general, peroxisomes β-oxidize *trans* unsaturated acyl-CoAs faster than the corresponding *cis* isomers and at least as fast as the corresponding saturated compound (Osmundsen 1982 a).

6.5.3 ω-HYDROXYMONOCARBOXYLIC AND DICARBOXYLIC ACIDS

Initially the β-oxidation of ω-substituted fatty acids was investigated using the liked assay in which the acyl-CoA esters were generated *in situ* by the acyl-CoA synthetase(s) present in crude cell-free extracts. With ω-hydroxymonocarboxylic acids as substrate the measured rate of NAD^+ reduction could be due to β-oxidation and/or ω-hydroxyfatty acid dehydrogenase activity shown to be present in crude extracts (see Chapter 4). Therefore, NAD^+ reduction due to ω-hydroxyfatty acid dehydrogenase was corrected for by performing assays in which CoASH and ATP were omitted so that the acyl-CoA synthetase(s) were inactive. The results of a typical experiment are shown in Table 6.8. Dicarboxylic acids are β-oxidized far more slowly than the corresponding monocarboxylic acids. However, with ω-hydroxymonocarboxylic acids the situation is somewhat different. 12-Hydroxylaurate is poorly β-oxidized

Table 6.7: β-oxidation of unsaturated acyl-CoA esters by a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C.

Acyl-CoA (80 μM)	Activity[a] (nmol/min/mg)	%[b]
C_{16}	26.1	100
trans Δ^9 C_{16}	25.4	97.3
cis Δ^9 C_{16}	9.9	37.9
C_{14}	45.1	100
cis Δ^9 C_{14}	6.3	14.0

a = measured as NAD^+ reduction

b = 100% corresponds to the activity with the saturated acyl-CoA of that chain length

Assayed in potassium phosphate buffer (130 mM, pH 8.0) using the direct assay

Table 6.8: β-oxidation of monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids by a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C.

Substrate	Activity* (nmol/min/mg)
Palmitate	38.9
Myristate	30.9
Laurate	28.9
Decanoate	8.3
DC_{16}	<1.0
DC_{14}	2.9
DC_{12}	1.9
DC_{10}	1.1
16-hydroxypalmitate	45.0
12-hydroxylaurate	8.4

* = NAD^+ reduction in the linked assay system

Assayed in potassium phosphate buffer (130 mM, pH 8.0)

(comparable to DC_{12}) whereas 16-hydroxypalmitate is rapidly β-oxidized (comparable to palmitate). Since these are very important results with respect to dicarboxylic acid accumulation it was necessary to confirm that the poor rate of β-oxidation of dicarboxylic acids and of 12-hydroxylaurate was due to the specificity of the β-oxidation system and not due to that of the acyl-CoA synthetase(s). Assays were performed in which the total CoASH consumption and acetyl-CoA were measured (and hence by difference the amount of long-chain acyl-CoA determined)(see Methods). The results of a typical experiment with palmitate, 16-hydroxypalmitate, DC_{14} and 12-hydroxylaurate are shown in Figs. 6.10 and 6.11. With all 4 substrates there is significant accumulation of long-chain acyl-CoA. However, the rates of acetyl-CoA production were very different. Palmitate (33 nmol/min/mg protein) and 16-hydroxypalmitate (22 nmol/min/mg protein) were rapidly β-oxidized whereas DC_{14} (3 nmol/min/mg protein) and 12-hydroxylaurate (3 nmol/min/mg protein) were slowly β-oxidized. With this batch of extract the activities measured in terms of NAD^+ reduction were (in nmol/min/mg protein): palmitate 25.3, 16-hydroxypalmitate 18.6, 12-hydroxylaurate 2.2, DC_{14} 1.7. These results are in good agreement with those obtained by measuring NAD^+ reduction. These results also demonstrate that activation of dicarboxylic acids and of 12-hydroxylaurate to their corresponding acyl-CoA esters is not a limiting factor to their subsequent β-oxidation. With all 4 substrates long-chain acyl-CoA accumulates at a comparable rate but comparable rates of acetyl-CoA production are not observed. Thus the specificity of β-oxidation is conferred by a step after the acyl-CoA synthetase reaction. Since the same specificity is observed whether, the parameter measured is NAD^+ reduction or acetyl-CoA production, the step conferring this specificity must be no further down the reaction pathway than 3-hydroxyacyl-CoA dehydrogenase ie: either acyl-CoA dehydrogenase, enoyl-CoA hydratase or

Figure 6.10: Acyl-CoA and acetyl-CoA formation during the β-oxidation of palmitate and of DC_{14} in the linked β-oxidation assay

β-oxidation assay performed in potassium phosphate buffer (130mM. pH 8)

Acetyl-CoA measured enzymically with citrate synthase (see Methods)

Cell-free extract from succinate-grown *Corynebacterium* 7E1C

[fatty acid] = 0.75mM, [CoASH] = 0.7mM, [ATP] = 4mM

○ = acyl-CoA

▲ = acetyl-CoA

β-Oxidation of DC14

nmol acetyl-CoA or acyl-CoA / 200μl

50
40
30
20
10

5 10 15 20 25 30

t (min)

β-Oxidation of palmitate

nmol acetyl-CoA or acyl-CoA / 200μl

50
40
30
20
10

5 10 15 20 25 30

t (min)

Figure 6.11: Acyl-CoA and acetyl-CoA formation during the β-oxidation of 16-hydroxypalmitate and of 12-hydroxylaurate in the linked β-oxidation assay

β-oxidation assay performed in potassium phosphate buffer (130mM, pH 8)

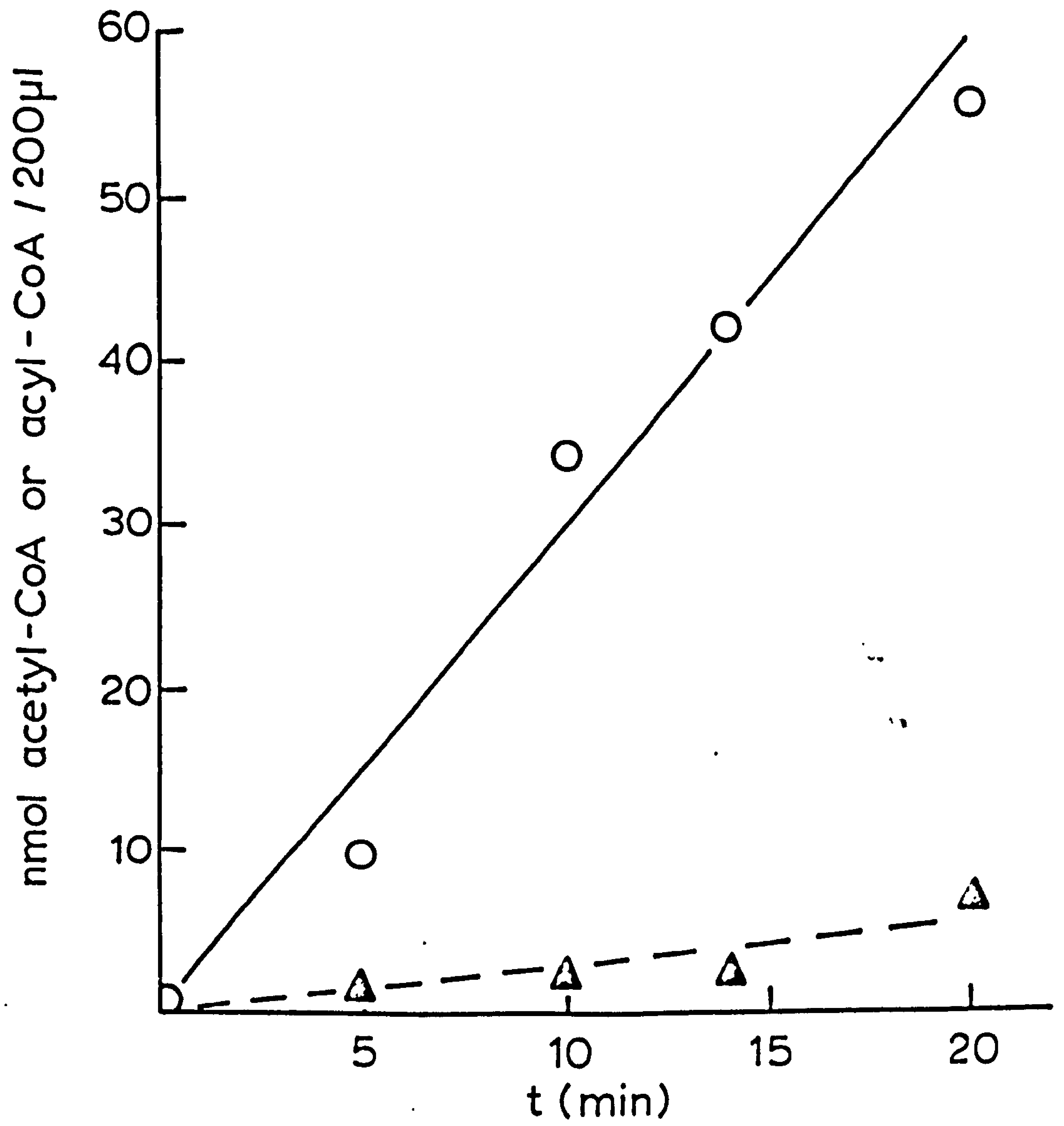
Acetyl-CoA measured enzymically with citrate synthase (see Methods)

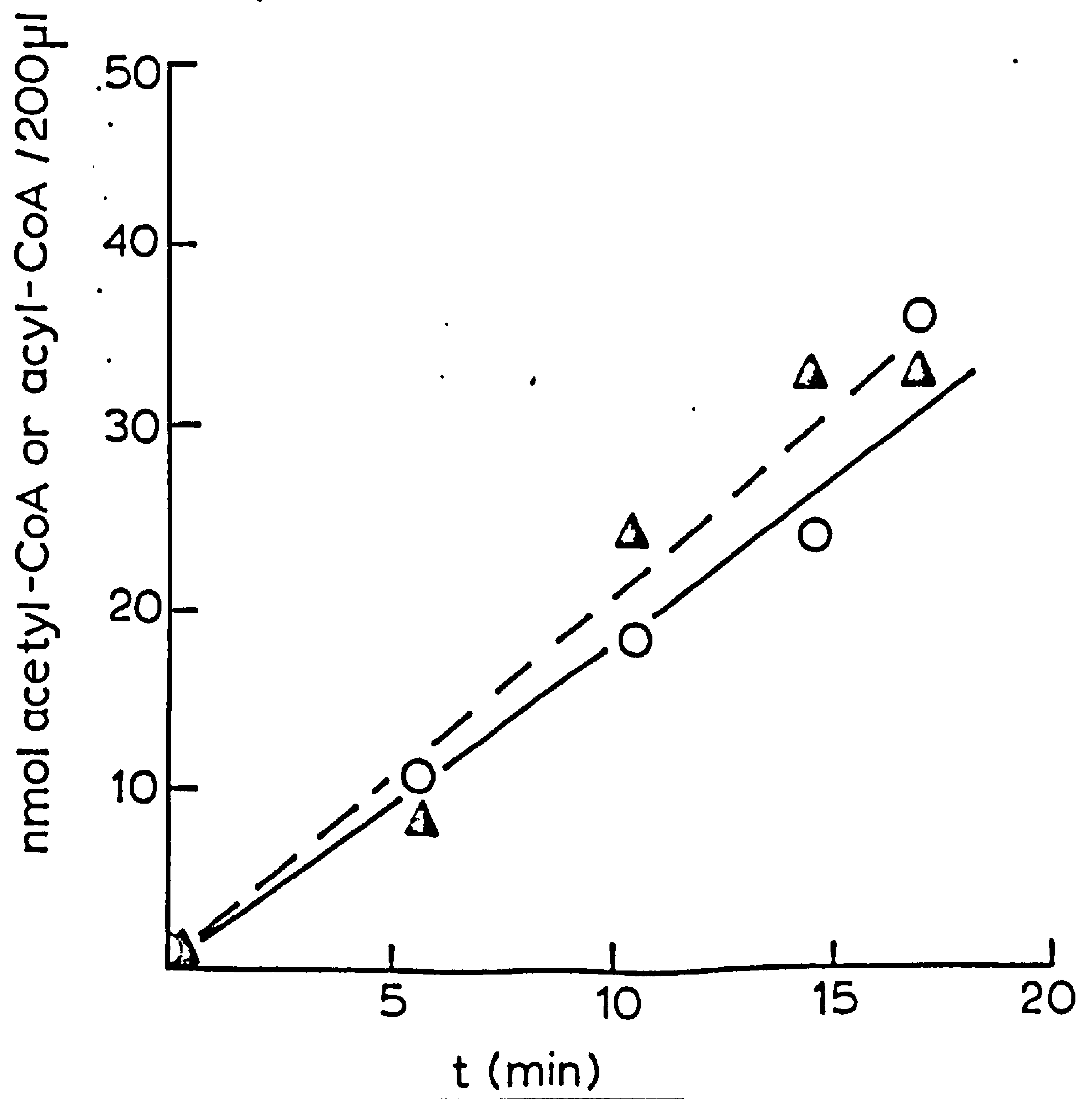
Cell-free extract from succinate-grown *Corynebacterium* 7E1C

[fatty acid] = 0.75mM, [CoASH] = 0.7mM, [ATP] = 4mM

○ = acyl-CoA

▲ = acetyl-CoA


β-Oxidation of 12-hydroxylaurate
nmol acetyl-CoA or acyl-CoA /200µl
60
50
40
30
20
10
5
10
15
20
t (min)


β-Oxidation of 16-hydroxypalmitate
nmol acetyl-CoA or acyl-CoA /200µl
50
40
30
20
10
5
10
15
20
t (min)

3-hydroxyacyl-CoA dehydrogenase.

H.P.L.C. analysis of the accumulated acyl-CoA esters in the DC_{14} assay showed that the main acyl-CoA accumulated was DC_{14}-CoA_1 (plus small amounts of DC_{12}-CoA_1). No Δ^2 enoyl-CoA or 3-hydroxyacyl-CoA derivatives could be detected (Fig. 6.14). This indicates that acyl-CoA dehydrogenase is the rate limiting step in the β-oxidation of DC_{14}. Similarly, H.P.L.C. analysis of the accumulated acyl-CoA in palmitate assays demonstrated the presence only of saturated acyl-CoA esters indicating that acyl-CoA dehydrogenase is the rate-limiting enzyme of the β-oxidation spiral. This is in agreement with the situation in *E.coli* where the specific activity of acyl-CoA dehydrogenase is significantly lower than that of the other β-oxidation enzymes whether measured with long-chain (C_{16}) or short-chain (C_4) substrates (O'Brian and Frerman 1977). The situation is the same in *Caulobacter crescentus* (O'Connell *etal* 1986). Similarly, in mitochondrial and peroxisomal β-oxidation acyl-CoA dehydrogenase and acyl-CoA oxidase, respectively, are rate-limiting (Lazarow 1978; Hyrb and Hogg 1979).

In order to confirm this specificity of β-oxidation independently of the acyl-CoA synthetase reaction, the acyl-CoA esters of a number of ω-hydroxymonocarboxylic and dicarboxylic acids were chemically synthesized and subsequently purified by H.P.L.C.. This also allowed the specificity of β-oxidation to be examined in cells grown on carbon-sources other than succinate. This is important since although the specificity of β-oxidation of monocarboxyl-CoA esters is essentially the same in both succinate-grown and hexadecanol-grown cells the possibility remained that the carbon-source may affect the ability to β-oxidize ω-hydroxymonocarboxylic and dicarboxlic acids. In particular, growth on dodecanediol which is presumably oxidized to DC_{12} via 12-hydroxylaurate might result in an increased ability to β-oxidize ω-hydroxymonocarboxylic and dicarboxylic acids.

In order to confirm that the NAD^+ reduction observed with the ω-hydroxymonocarboxyl-CoA esters was due to β-oxidation (rather than due to ω-hydroxyfatty acid dehydrogenase activity) the rates of NAD^+ reduction and acetyl-CoA production with 12-hydroxylauroyl-CoA, 16-hydroxypalmitoyl-CoA and lauroyl-CoA were measured (Fig. 6.12). With all 3 substrates a 1:1 stoichiometry of NAD^+ reduction:acetyl-CoA production was observed thus indicating that the NAD^+ reduction observed with the ω-hydroxymonocarboxyl-CoA esters was indeed due to β-oxidation. The specificity of β-oxidation of C_{12} and C_{16} monoocarboxyl-, ω-hydroxymonocarboxyl- and dicarboxyl-CoA esters in crude cell-free extracts of cells grown on succinate, dodecanediol and hexadecanol are shown is shown in Table 6.9. The substrate specificity is remarkably similar after growth on these different substrates. Growth on dodecanediol does not markedly increase the capacity to β-oxidize ω-hydroxymonocarboxylic or dicarboxylic acids. This confirms the results obtained with the linked β-oxidation assay. Dicarboxyl-CoA esters are poor β-oxidation oxidation substrates as is 12-hydroxylauroyl-CoA whereas 16-hydroxypalmitoyl-CoA is a good β-oxidation substrate.

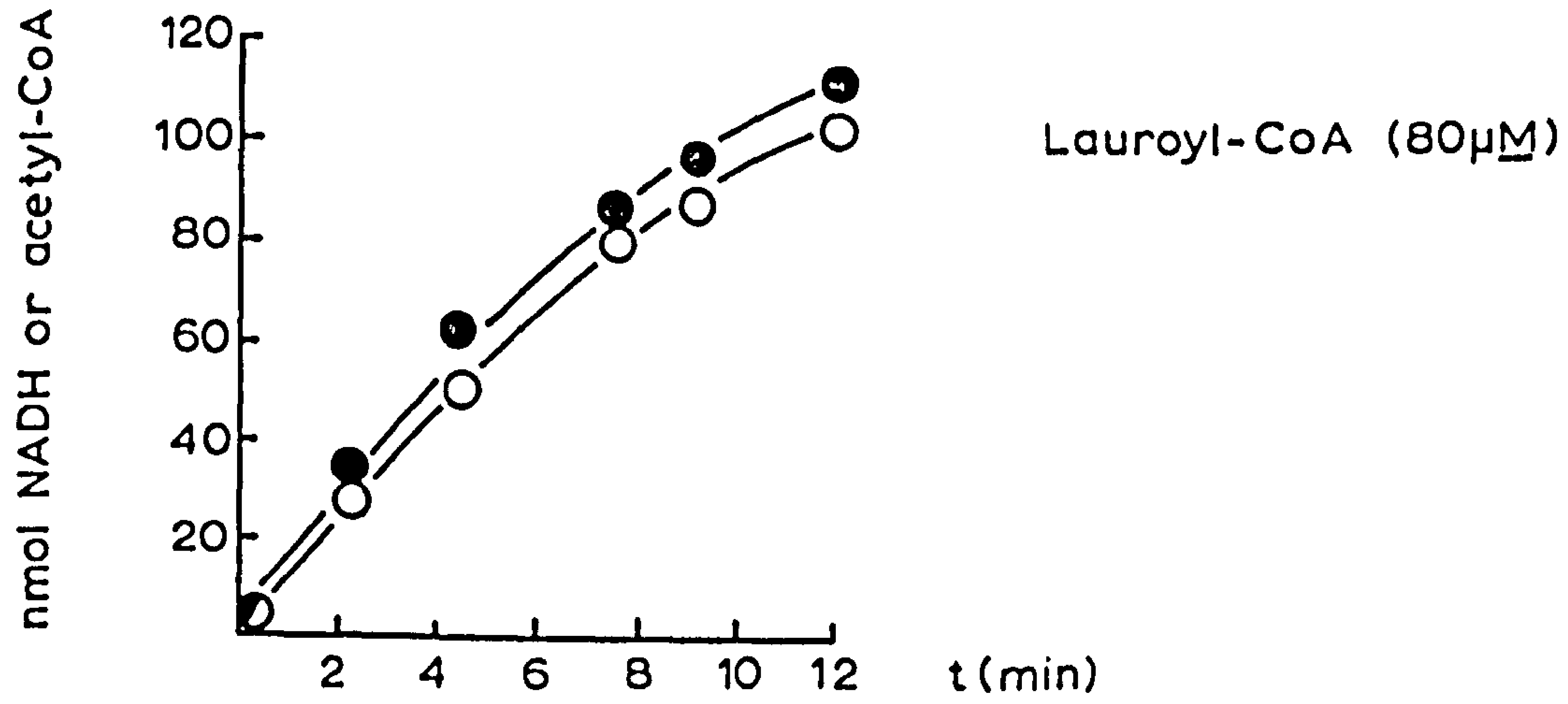
The specificity of the β-oxidation system of *Corynebacterium* 7E1C is very significant with respect to dicarboxylic acid accumulation by *Corynebacterium* 7E1C. The poor β-oxidation of dicarboxylic acids, irrespective of chain-length, is consistent with the accumulation of dicarboxylic acids. However, since DC_{12} and DC_{16} are both β-oxidized poorly one would predict accumulation of both these dicarboxylic acids. The critical difference is the specificity at the level of the ω-hydroxymonocarboxylic acids. The ω-hydroxymonocarboxylic acid is an obligatory intermediate in the production of α,ω-dicarboxylic acids (Yi and Rehm 1982b; see also Fig. 1.1). Thus not only is the rate of degradation of the dicarboxylic acid itself important in determining

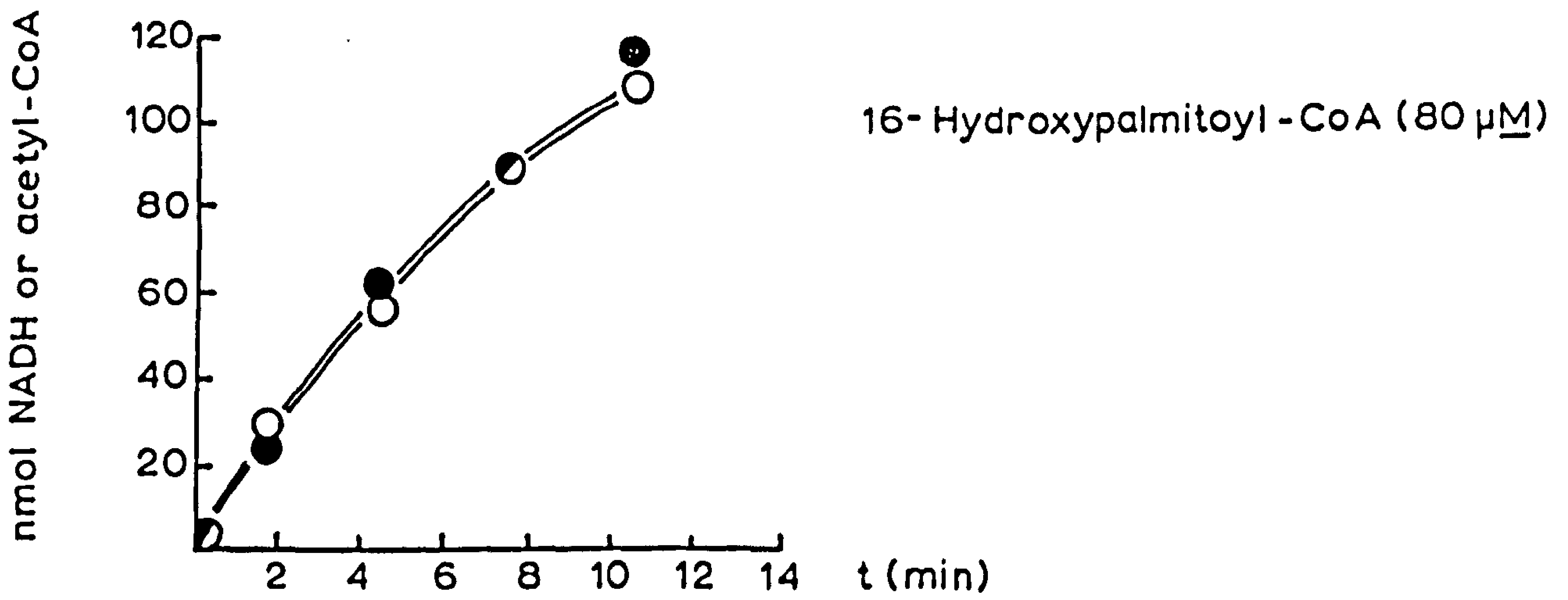
Figure 6.12: Formation of acetyl-CoA and NADH during the β-oxidation of laurate, 16-hydroxypalmitate and 12-hydroxylaurate by a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in potassium phoshate buffer (130mM, pH 8) with 80μM acyl-CoA

● = Acetyl-CoA

○ = NADH


a)
nmol NADH or acetyl-CoA
120
100
80
60
40
20
2
4
6
8
10
12
t (min)
Lauroyl-CoA (80µM)


b)
nmol NADH or acetyl-CoA
120
100
80
60
40
20
2
4
6
8
10
12
14
t (min)
16-Hydroxypalmitoyl-CoA (80 µM)

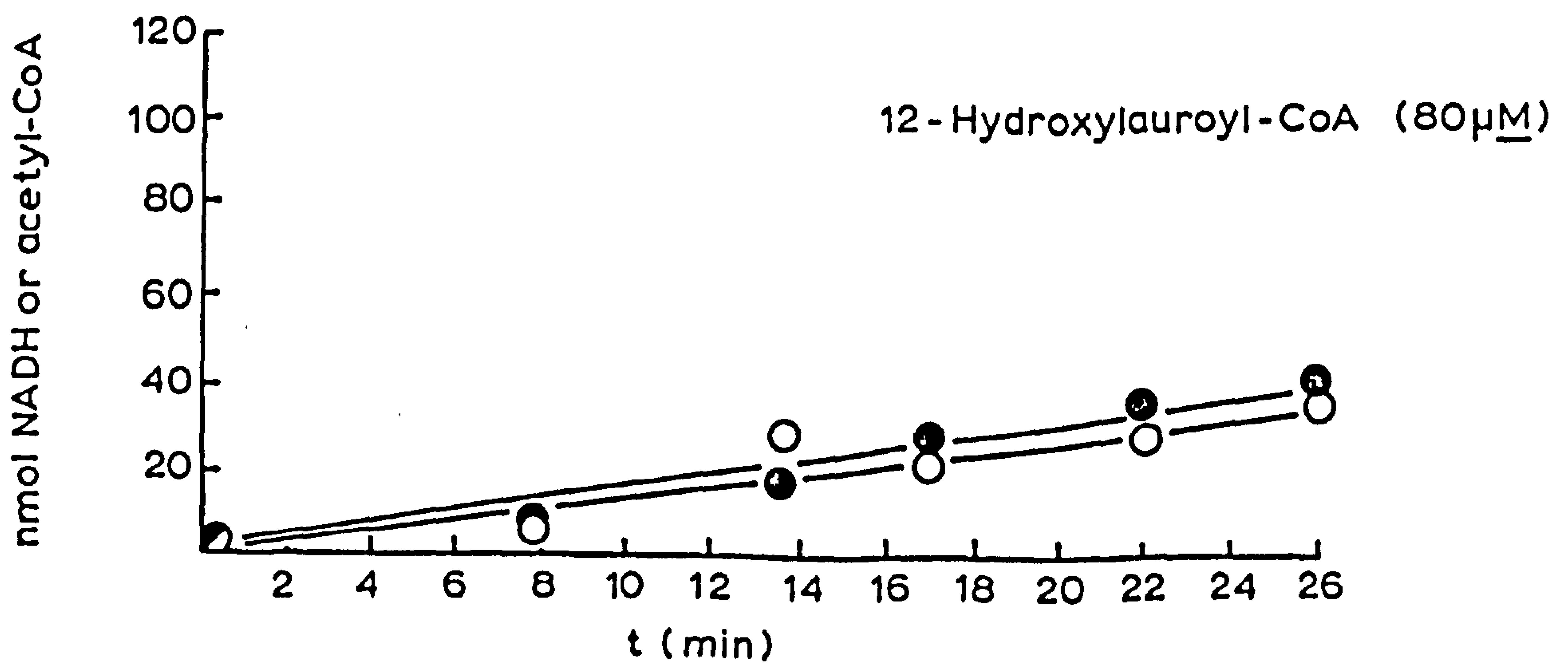

c)
nmol NADH or acetyl-CoA
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
22
24
26
t (min)
12-Hydroxylauroyl-CoA (80µM)

Table 6.9: β-oxidation of monocarboxyl-, ω-hydroxymonocarboxyl and dicarboxyl-CoA esters by cell-free extracts of *Corynebacterium* 7E1C grown on a range of carbon-sources

Assay Substrate (80μM)	Growth Substrate sp. activities in nmol/min/mg protein		
	succinate	dodecanediol	hexadecanol
C_{16}-SCoA	27.7	35.4	29.4
16HO C_{16}-SCoA	44.4	55.6	46.5
DC_{16}-$SCoA_1$	10.2	5.9	7.1
DC_{16}-$SCoA_2$	n.d.	n.d.	1.5
C_{12}-SCoA	38.6	29.5	22.3
12HO C_{12}-SCoA	5.2	3.8	4.0
DC_{12}-$SCoA_1$	3.2	1.5	2.3
DC_{12}-$SCoA_2$	n.d.	n.d.	0

n.d. = not done.

Assayed in potassium phosphate buffer (130 mM, pH 8.0) using the direct assay

C_{16}-SCoA = palmitoyl-CoA, 16HO C_{16}-SCoA = 16-hydroxypalmitoyl-CoA DC_{16}-$SCoA_1$ = mono CoA ester of hexadecanedioic acid, DC_{16}-$SCoA_2$ = di CoA ester of hexadecanedioic acid, C_{12}-SCoA = lauroyl-CoA, 12HO C_{12}-SCoA = 12-hydroxylauroyl-CoA, DC_{12}-$SCoA_1$ = mono CoA ester of dodecanedioic acid, DC_{12}-$SCoA_2$ = di CoA ester of dodecanedioic acid

whether or not accumulation occurs; the rate of degradation of the ω-hydroxymonocarboxylic acid will also be an important determining factor. Whereas with dicarboxylic acids the ability to act as β-oxidation substrates varies little with chain-length the rate of β-oxidation of ω-hydroxymonocarboxylic acids is highly dependent on chain-length and increases drastically on as the acyl- chain-length increases from C_{12} to C_{16}. The slow β-oxidation of 12-hydroxylaurate and DC_{12} will tend to favour accumulation of DC_{12} whereas the rapid β-oxidation of 16-hydroxypalmitate will tend to favour its degradation before it can be ω-oxidized to DC_{16}. This is consistent with the range of dicarboxylic acids produced by *Corynebacterium* 7E1C ie: $DC_{12} \gg DC_{14}$; no DC_{16}. This is also consistent with the inability to detect DC_{16} even during growth on 16-hydroxypalmitate.

Thus the metabolism of the ω-hydroxymonocarboxylic acid appears to be crucial in determining the dicarboxylic acid-producing capabilities of this organism.

Although the specificity of the *Corynebacterium* 7E1C system displays some notable similarities to that of the rat liver peroxisomal system with respect to saturated and unsaturated monocarboxylic acids, the specificity towards ω-hydroxymonocarboxylic and dicarboxylic acids is markedly different. Vamecq and Draye (1987) showed that C_{12} and C_{16} monocarboxyl-CoA, ω-hydroxymonocarboxyl-CoA and dicarboxyl-CoA esters were all good substrates for the rat liver peroxisomal acyl-CoA oxidase (with the C_{12} substrates being oxidized more rapidly than the corresponding C_{16} substrates). Similarly, when peroxisomal β-oxidation was assayed in terms of NAD^+ reduction lauroyl-CoA, 12-hydroxylauroyl-CoA and DC_{12}-CoA were β-oxidized at similar rates. Interestingly, rat liver mitochondria β-oxidized (polarographic measurement of O_2 consumption) lauroyl-CoA and 12-hydroxylauroyl-CoA at similar rates although DC_{12}-CoA was not oxidized as it was not a

substrate for the carnitine acyltransferases. However, it was demonstrated that the mitochondria contained dye-linked acyl-CoA dehydrogenase activity equally active with lauroyl-CoA, 12-hydroxylauroyl-CoA and DC_{12}-CoA. Thus the ability of the *Corynebacterium* 7E1C system to handle ω-substituted fatty acids is very restricted compared to the mammalian system.

6.6. REGULATION OF β-OXIDATION

The effect of a number of possible metabolites on the β-oxidation of myristoyl-CoA by a cell-free extract of hexadecanol-grown cells is shown in Table 6.10. CoASH had no effect on the rate of NAD^+ reduction. However, in the presence of CoASH assays stayed linear for longer which may be due to inhibition of thioesterase activity by CoASH (0.3mM CoASH inhibits lauroyl-CoA thioesterase activity approx. 40%, see Chapter 5). Only acetyl-CoA caused significant inhibition of myristoyl-CoA-dependent NAD^+ reduction. Malonyl-CoA did not inhibit β-oxidation. Malonyl-CoA inhibits mitochondrial β-oxidation by inhibiting carnitine palmitoyltransferase I (CPT I)(McGarry *et al* 1978) so its failure to inhibit the bacterial system is not surprising.

Only acetyl-CoA and acetoacetyl-CoA caused significant inhibition. The inhibition by acetoacetyl-CoA is interesting. On first inspection it appears to be a potent inhibitor of β-oxidation; 50% inhibition of myristoyl-CoA dependent reduction at 30 μM acetoacetyl-CoA. However, it was possible that this reduced rate of NAD^+ reduction was due to reoxidation of NADH by a short-chain specific 3-hydroxyacyl-CoA dehydrogenase reducing acetoacetyl-CoA to 3-hydroxybutyryl-CoA (Fig. 6.13). This possibility was investigated by measuring the rate of acetyl-CoA production, from myristoyl-CoA, in the presence and absence of acetoacetyl-CoA. In such experiments the total acetyl-CoA production

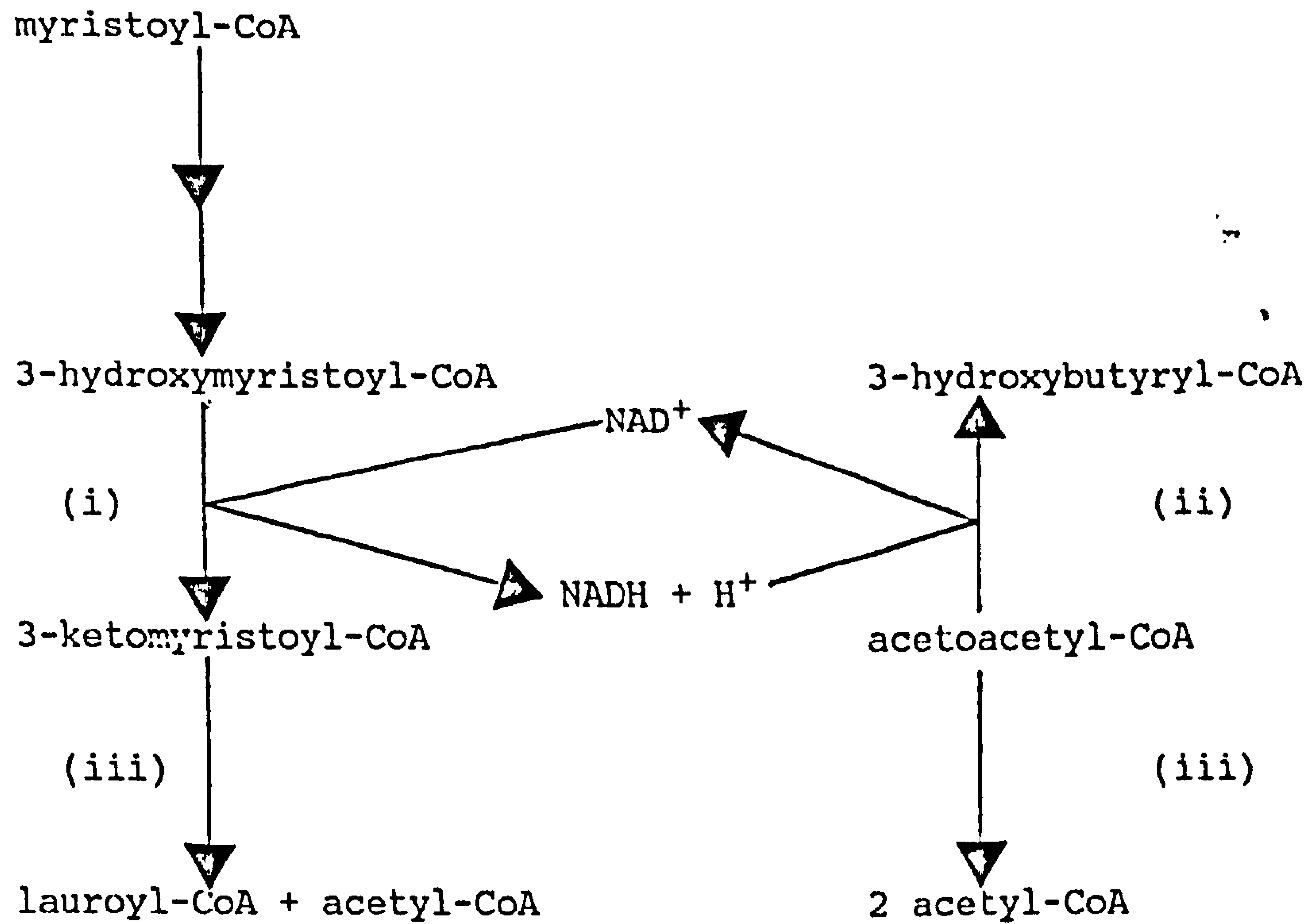
Table 6.10: Effect of potential metabolites on the β-oxidation of myristoyl-CoA by a cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C.

Addition to standard assay	*Activity (nmol/min/mg)	% of control activity
Experiment 1		
None	44	100
Acetyl-CoA (0.25 mM)	34.1	77.5
Acetyl-CoA (0.5 mM)	31.6	71.9
Acetyl-CoA (1.0 mM)	26.2	59.6
AMP (1.0 mM)	40.9	93.0
ADP (1.0 mM)	44	100
ATP (1.0 mM)	43.6	99
CoASH (1.0 mM)	43.6	99
Experiment 2.		
None	29.5	100
Acetoacetyl-CoA (0.03 mM)	14.5	49.2
Acetoacetyl-CoA (0.05 mM)	9.8	33.2
Acetoacetyl-CoA (0.10 mM)	6.5	22.0
Acetoacetyl-CoA (0.20 mM)	4.7	15.9
Acetoacetyl-CoA (1.0 mM)	3.4	11.5
Malonyl-CoA (1.0 mM)	32.5	110.2
Acetyl-CoA (1.0 mM)	16.3	55.3

* = Measured as rate of NAD^+ reduction in potassium phosphate buffer (130 mM, pH 8.0) using the direct assay

[myristoyl-CoA] = (80 μM)

Figure 6.13: Mechanism of reoxidation of NADH produced during the β-oxidation of myristoyl-CoA by a short-chain specific 3-hydroxyacyl-CoA dehydrogenase



(i) = long-chain specific 3-hydroxyacyl-CoA dehydrogenase

(ii) = short-chain specific 3-hydroxyacyl-CoA dehydrogenase

(iii) = thiolase(s)

had to be corrected for that produced by thiolytic cleavage of acetoacetyl-CoA itself. The result of a typical experiment is shown in Table 6.11. When β-oxidation is measured in terms of the rate of NAD^+ reduction acetoacetyl-CoA (0.3mM) inhibits β-oxidation approx. 92%. However, when β-oxidation is measured in terms of the net rate of acetyl-CoA production the same concentration of acetoacetyl-CoA inhibits β-oxidation approx. 26%. Thus acetoacetyl-CoA is not as potent an inhibitor of the β-oxidation of long-chain acyl-CoA as it first appeared to be. Furthermore, this result indicates the presence of two 3-hydroxyacyl-CoA dehydrogenases; a short-chain specific enzyme active with 3-hydroxbutyryl-CoA/acetoacetyl-CoA and a long-chain specific enzyme active with 3-hydroxymyristoyl-CoA. The approx. 26% inhibition of acetyl-CoA production in the presence of acetoacetyl-CoA may be due to some overlap in the specificity of the two enzymes, the acetoacetyl-CoA competing with 3-hydroxymyristoyl-CoA for the active site of the long-chain specific enzyme; acetoacetyl-CoA inhibits mitochondrial 3-hydroxyacyl-CoA dehydrogenase in such a manner (Schifferdech and Schulz 1974). Alternatively it may be due to inhibition of one of the other β-oxidation enzymes; enoyl-CoA hydratase is inhibited by acetoacetyl-CoA (Waterson and Hill 1972). This inhibition could also be due to the acetyl-CoA produced on thiolytic cleavage of acetoacetyl-CoA. However, the presence of acetoacetyl-CoA thiolase activity necessitates a reconsideration of the inhibition of β-oxidation by acetyl-CoA. This inhibition (measured in terms of the rate of NAD^+ reduction) could be due to acetyl-CoA itself or due to acetoacetyl-CoA produced by thiolase-catalysed condensation of acetyl-CoA. This possibility has not been examined.

The occurece of two 3-hydroxyacyl-CoA dehydrogenases is consistent with the situation in rat liver mitochondria from which have been purified two distinct 3-hydroxyacyl-CoA dehydrogenases, one short-chain specific

Table 6.11: Inhibition of the β-oxidation of myristoyl-CoA by acetoacetyl-CoA as measured by following NAD^+ reduction or acetoacetyl production.

	Parameter measured	Activity (nmol/min/mg)	% inhibition
Myristoyl-CoA*	NADH	37.0	0
	acetyl-CoA	38.0	0
Acetoacetyl-CoA**	NADH	0	-
	acetyl-CoA	21.7	-
Myristoyl-CoA + Acetoacetyl-CoA	NADH	3.0	91.9
	acetyl-CoA	49.9	-
(Myristoyl-CoA + Acetoacetyl-CoA) -(Acetoacetyl-CoA)	acetyl-CoA	28.2	25.8

* [Myristoyl-CoA] = 80 μM

** [Acetoacetyl-CoA] = 0.3 mM

Assayed in potassium phosphate buffer (130 mM, pH 8.0) using the direct assay

and the other long-chain specific (El-Fakhri and Middleton 1982). Overath *et al* (1969) proposed, on the basis of genetic evidence, that *E.coli* may posses both short-chain and long-chain specific versions of all the β-oxidation enzymes although to date this has only been confirmed, by purification, for enoyl-CoA hydratase (Beadle *et al* 1979) and thiolase (Binstock and Shultz 1975).It should be noted that O'Brian and Frerman (1977) reported differential loss of 3-hydroxyacyl-CoA dehydrogenase when purifying the *E.coli* β-oxidation complex (ie: C_4 97% recovery, C_{16} 21% recovery) which would indicate the presence of two 3-hydroxyacyl-CoA dehydrogenases.

The demonstration of thiolytic cleavage of acetoacetyl-CoA by *Corynebacterium* 7E1C (Table 6.11) is important. When measuring β-oxidation with saturated acyl-CoA esters little or no activity was observed with 80μM butyryl-CoA. However, the demonstration of thiolytic cleavage of acetoacetyl-CoA at a significant rate (21.3 nmol acetyl-CoA produced/min/mg protein which is equivalent to 10.7 nmol acetoacetyl-CoA cleaved/min/mg protein) at a high (0.3mM) concentration demonstrates that the final enzyme of β-oxidation system can handle short-chain substrates. However, the β-oxidation of butyryl-CoA at high (0.3mM) concentrations has not been investigated so it is not known if the first three β-oxidation enzymes can handle short-chain substrates at high concentrations.

6.7. INTERMEDIATES OF β-OXIDATION

During investigations of β-oxidation using the linked assay system it became apparent that that there was a significant accumulation of long-chain acyl-CoA (see Figs. 6.10 and 6.11). However the identity of this long-chain acyl-CoA was unknown. It was decided to investigate the nature of the accummulated acyl-CoA by H.P.L.C. of the intact acyl-CoA

esters (Bartlett *et al* 1988; Bartlett and Causey 1988; Watmough *et al* 1989).

6.7.1. β-OXIDATION OF DC_{14}

A standard linked β-oxidation assay (scaled-up to 7ml) was used. Duplicate aliquots were removed periodically. One aliquot was worked-up for H.P.L.C. analysis of the acyl-CoA esters present (see Methods). The other aliquot was used to determine total CoASH consumption and acety-CoA production and by difference, calculate the long-chain acyl-CoA accumulated (see Methods).

H.P.L.C. traces obtained with samples taken at 5 and 60 minutes are shown in Fig. 6.14. The time-course of DC_{14}-CoA production and of long-chain acyl-CoA as determined from measurments of total CoASH consumption is shown in Fig. 6.15. The determinations correlate well. The major long-chain acyl-CoA detected is DC_{14}-CoA_1. Small amounts of DC_{12}-CoA were detected after prolonged incubation. Although no authentic Δ^2 enoyl- or 3-hydroxy- derivatives were available there were no peaks that corresponded to their expected behaviour ie: the Δ^2 enoyl-derivative would be expected to have a retention time slightly shorter than the corresponding saturated compound whilst the 3-hydroxy-derivative would be expected to have a retention time slightly shorter than the corresponding C_{n-2} saturated compound (Watmough *et al* 1989). The accumulation of DC_{14}-CoA_1 and the absence of Δ^2 enoyl- and 3-hydroxy- derivatives indicates that the acyl-CoA dehydrogenase step is rate-limiting in the β-oxidation of DC_{14}.

Figure 6.14: H.P.L.C. analysis of the acyl-CoA intermediates of the β-oxidation of DC_{14} in the linked β-oxidation assay

The β-oxidation assay was performed in potassium phosphate buffer (130mM, pH 8)

[DC_{14}] = 0.75mM, [CoASH] = 0.7mM, [ATP] = 4mM

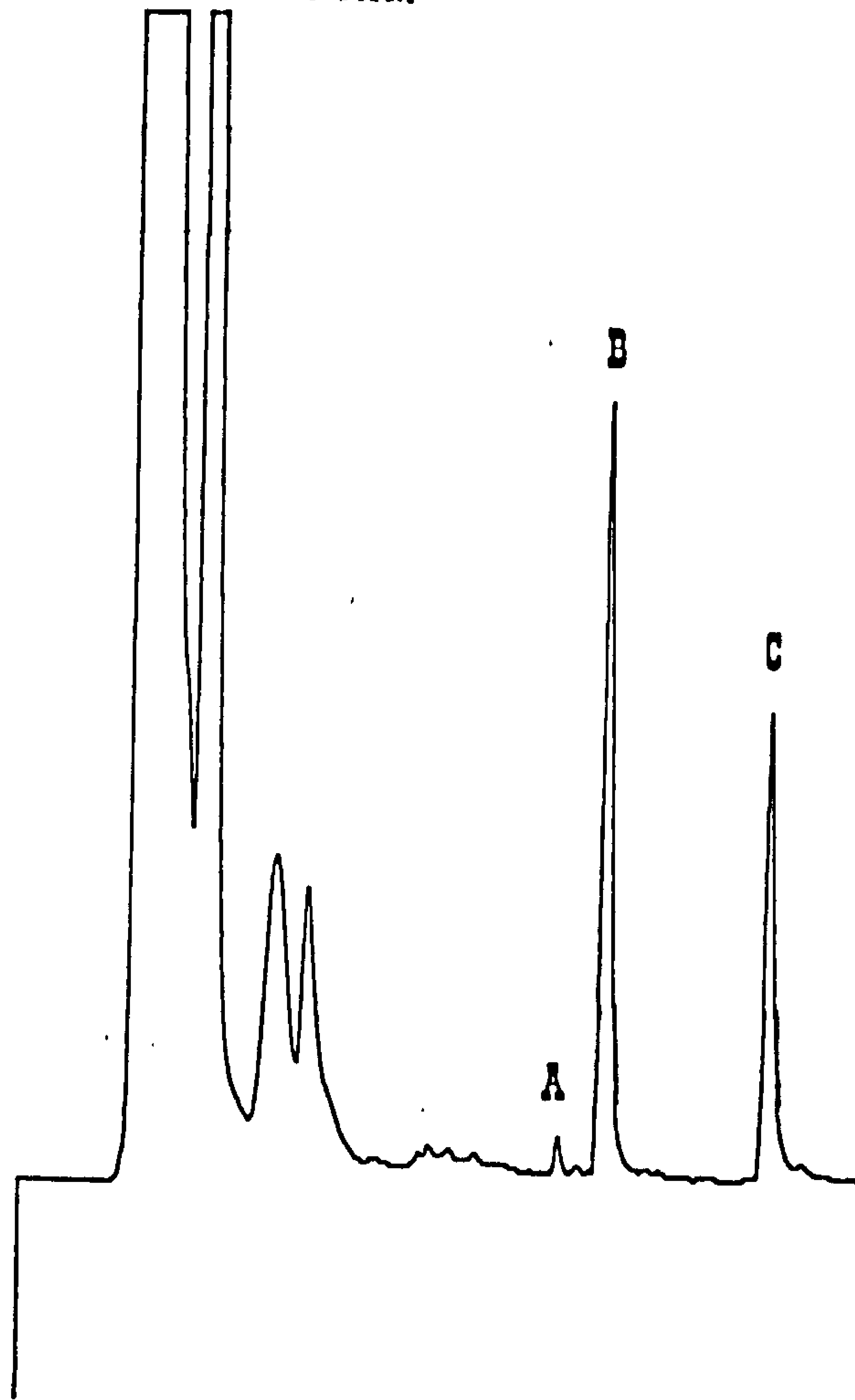
Crude cell-free extract from succinate-grown *Corynebacterium* 7E1C

The acyl-CoA esters were separated on a 10μ Lichrosorb RP C18 column using an acetonitrile-potassium phosphate (pH 5.3) gradient

A = DC_{12}-CoA_1

B = DC_{14}-CoA_1

C = lauroyl-CoA (internal standard; 100 μM)


30 MIN.
B
C
A

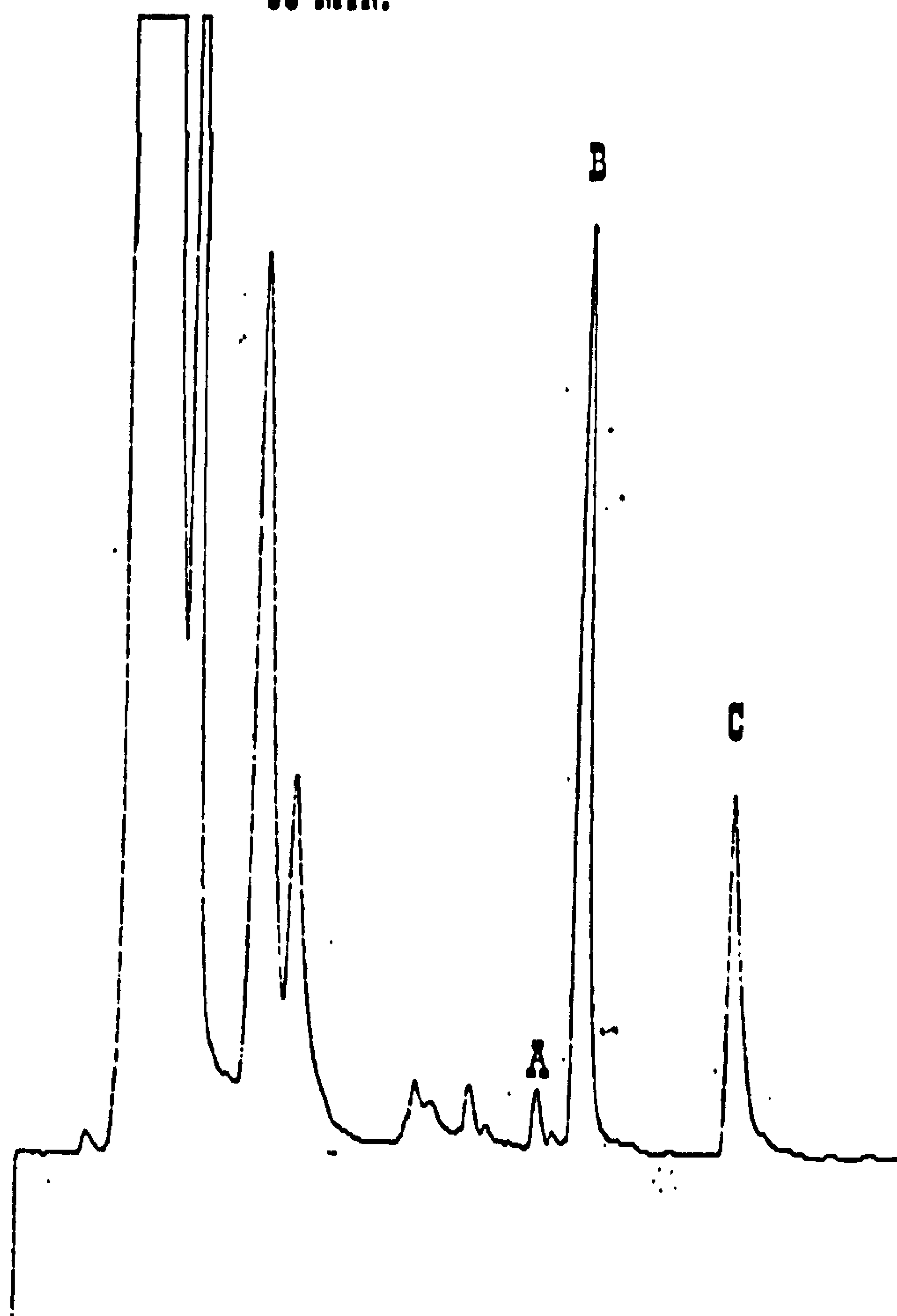

60 MIN.
B
C
A

Figure 6.15: Time-course of the production of acyl-CoA and acetyl-CoA during the β-oxidation of DC_{14} in the linked β-oxidation assay

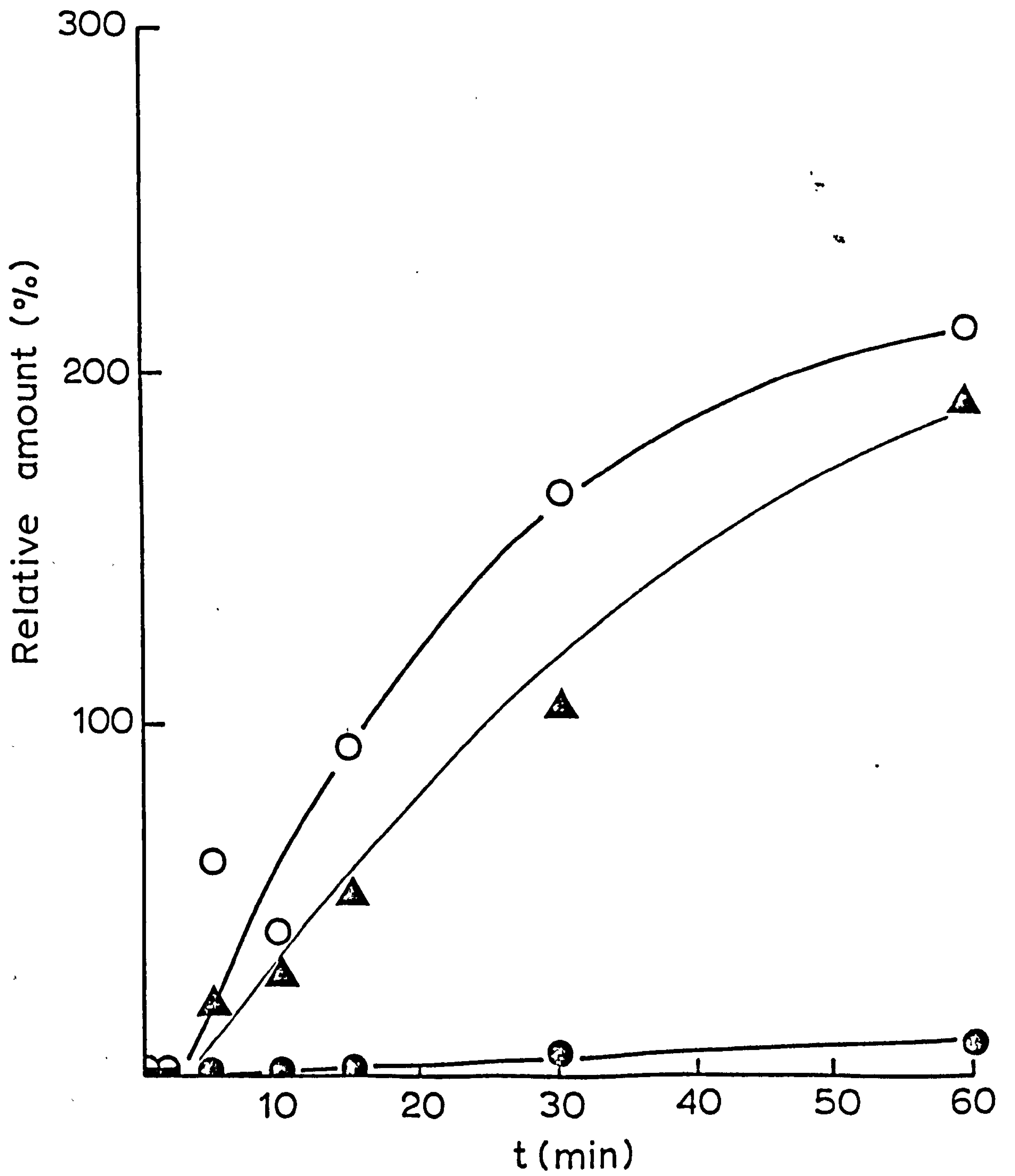
Assay conditions as for Figure 6.14

Acetyl-CoA measured enzymically with citrate synthase (see Methods)

● = acetyl-CoA

▲ = long-chain acyl-CoA (measured as total CoASH consumed - acetyl-CoA)

○ = DC_{14}-CoA as measured by H.P.L.C.



300
200
100
Relative amount (%)
10
20
30
40
50
60
t (min)

6.7.2. β-OXIDATION OF PALMITATE

6.7.2.1. LINKED ASSAY

This experiment was carried-out in the same manner as that described above (section 6.7.1.) except that palmitate replaced DC_{14}. H.P.L.C. traces of samples taken at 15 and 60 minutes are shown in Fig. 6.16. There is significant accumulation of acyl-CoA esters of chain-length C_{16} down to C_8. It is important to note that the major acyl-CoA of each chain-length is the SATURATED compound. In some cases small amounts of what may be the Δ^2 enoyl- (retention time slightly shorter than the corresponding saturated compound) and/or the 3-hydroxy- (retention time slightly shorter than the corresponding C_{n-2} saturated compound)(Watmough *et al* 1989) were detected. It should be noted that the C_{n-2} Δ^2 enoyl-CoA is will have a retention time almost indistiguishable from that of the C_n 3-hydroxy- compound, thus with acyl-CoA esters of 14 or less carbons these peaks will contain a mixture of C_n enoyl-CoA and C_{n-2} 3-hydroxyacyl-CoA. However, the peak with a slightly shorter retention time than palmitoyl-CoA would not be "contaminated" with 3-hydoxyoctadecanoyl-CoA. The retention time of this peak was approx.the same as that of authentic (enzymically synthesized with purified acyl-CoA oxidase) Δ^2 hexadecenoyl-CoA. However, in any case these peaks are very small compared to the saturated acyl-CoA peaks. This indicates that for C_8 to C_{16} chain lengths the acyl-CoA dehydrogenase is the rate limiting step in the β-oxidation spiral. The time-course of the production of the various chain-length acyl-CoA esters is shown in Fig. 6.17. This time-course indicates a precursor-product relationship between the the different chain-length acyl-CoA esters. Each acyl-CoA ester, in turn, builds-up to its maximum concentration and then decreases in concentration as the concentration

Figure 6.16: H.P.L.C. analysis of the acyl-CoA intermediates of the β-oxidation of palmitate in the "linked" β-oxidation assay

The β-oxidation assay was performed in potassium phosphate buffer (130mM, pH 8)

[palmitate] = 0.75mM, [CoASH] = 0.7mM, [ATP] = 4mM

Crude cell-free extract from succinate-grown *Corynebacterium* 7E1C

The acyl-CoA esters were separated on a 10M Lichrosorb RP C18 column using an acetonitrile-potassium phosphate (pH 5.3) gradient

A = butyryl-CoA (C_4)

B = hexanoyl-CoA (C_6)

C = octanoyl-CoA (C_8)

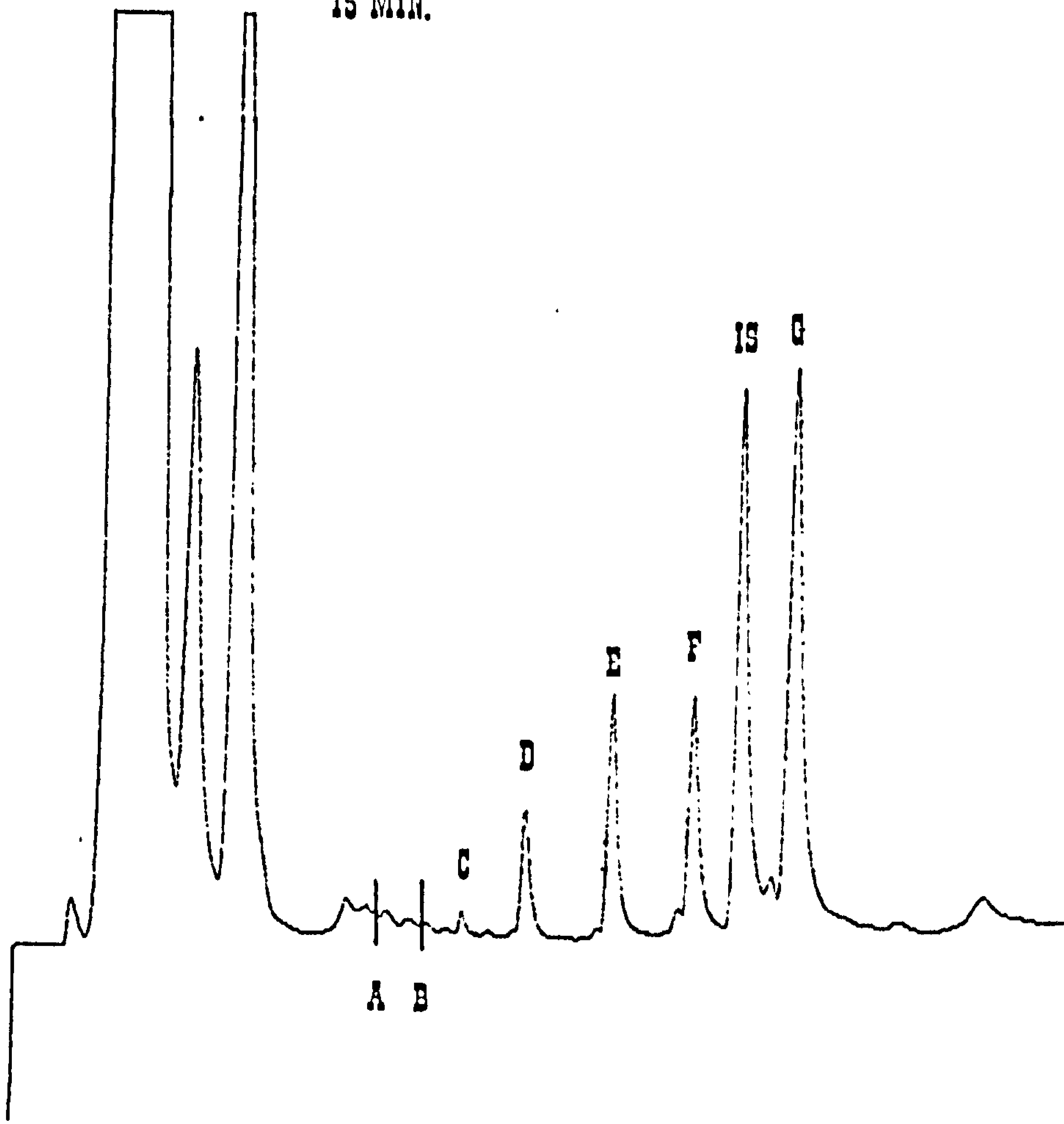
D = decanoyl-CoA (C_{10})

E = lauroyl-CoA (C_{12})

F = myristoyl-CoA (C_{14})

G = palmitoyl-CoA (C_{16})

IS = internal standard (pentadecanoyl-CoA; 50 μM)


15 MIN.
A
B
C
D
E
F
IS
G

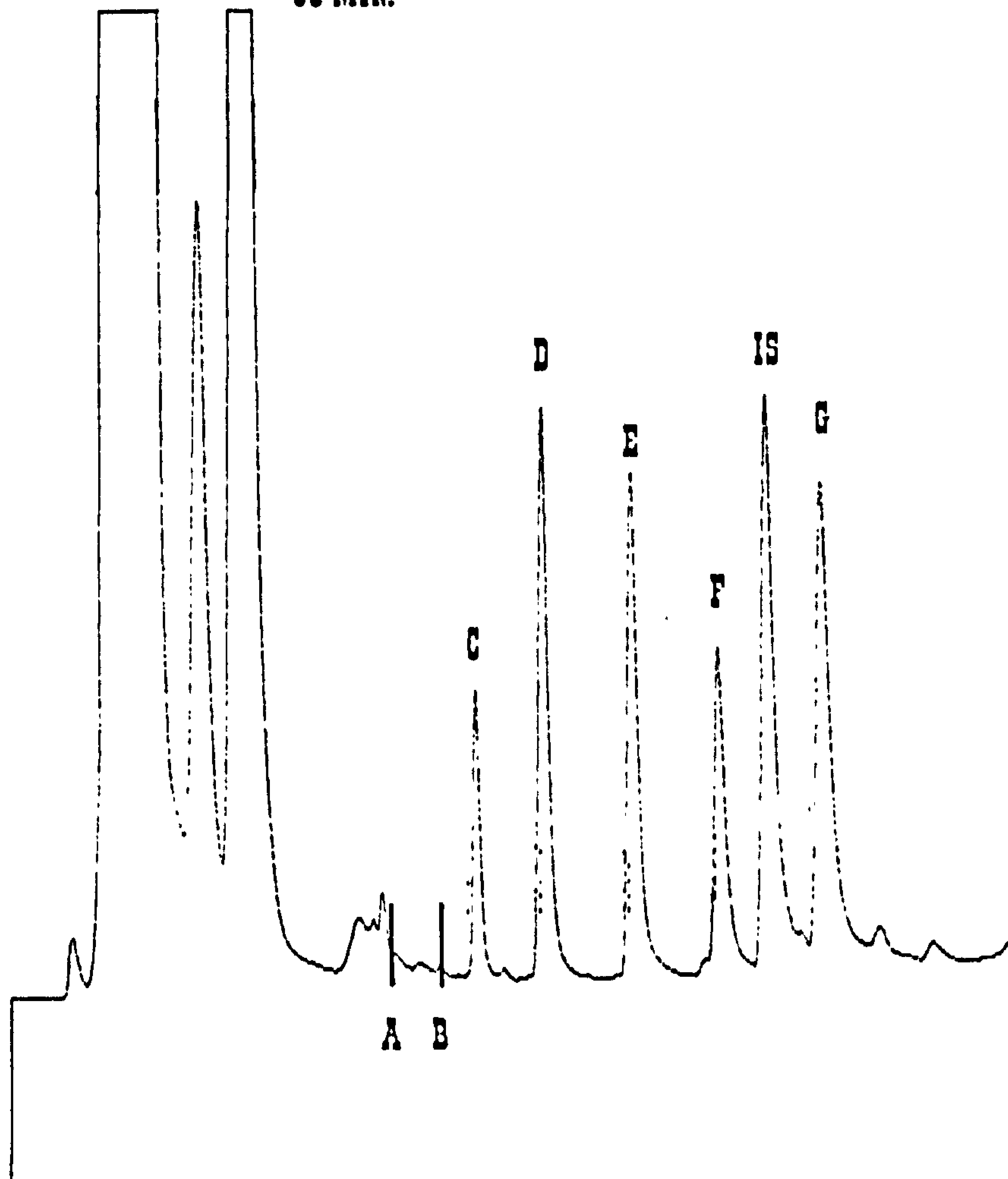

60 MIN.
A
B
C
D
E
F
IS
G

Figure 6.17: Time-course of acyl-CoA intermediate formation during the β-oxidation of palmitate in the 'linked' β-oxidation assay

Assay conditions as in Fig. 16

○ = palmotoyl-CoA

● = myristoyl-CoA

△ = lauroyl-CoA

▲ = decanoyl-CoA

□ = octanoyl-CoA

100% ≡ 50 µM acyl-CoA

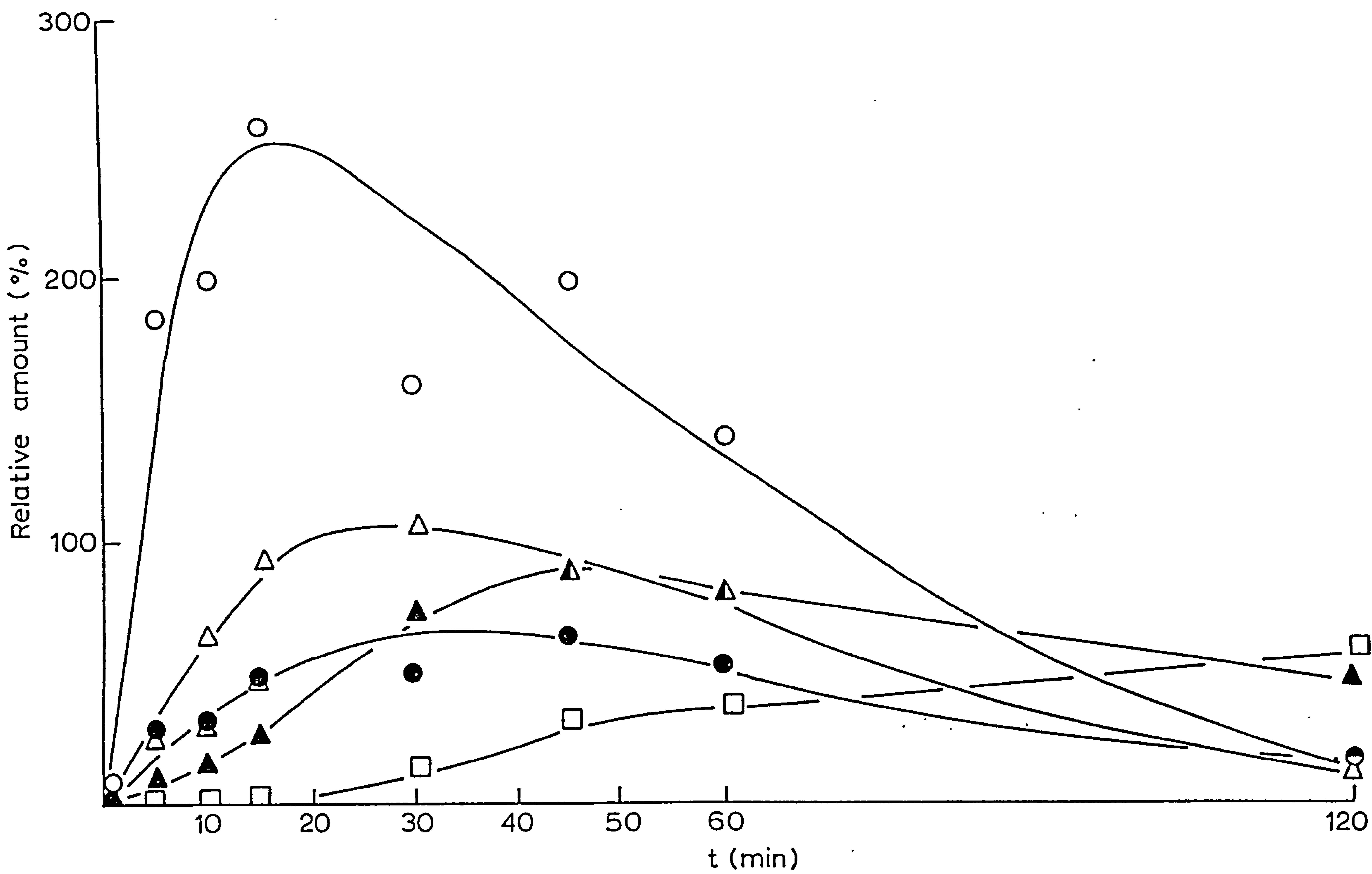

Relative amount (%)
300
200
100
10
20
30
40
50
60
120
t (min)

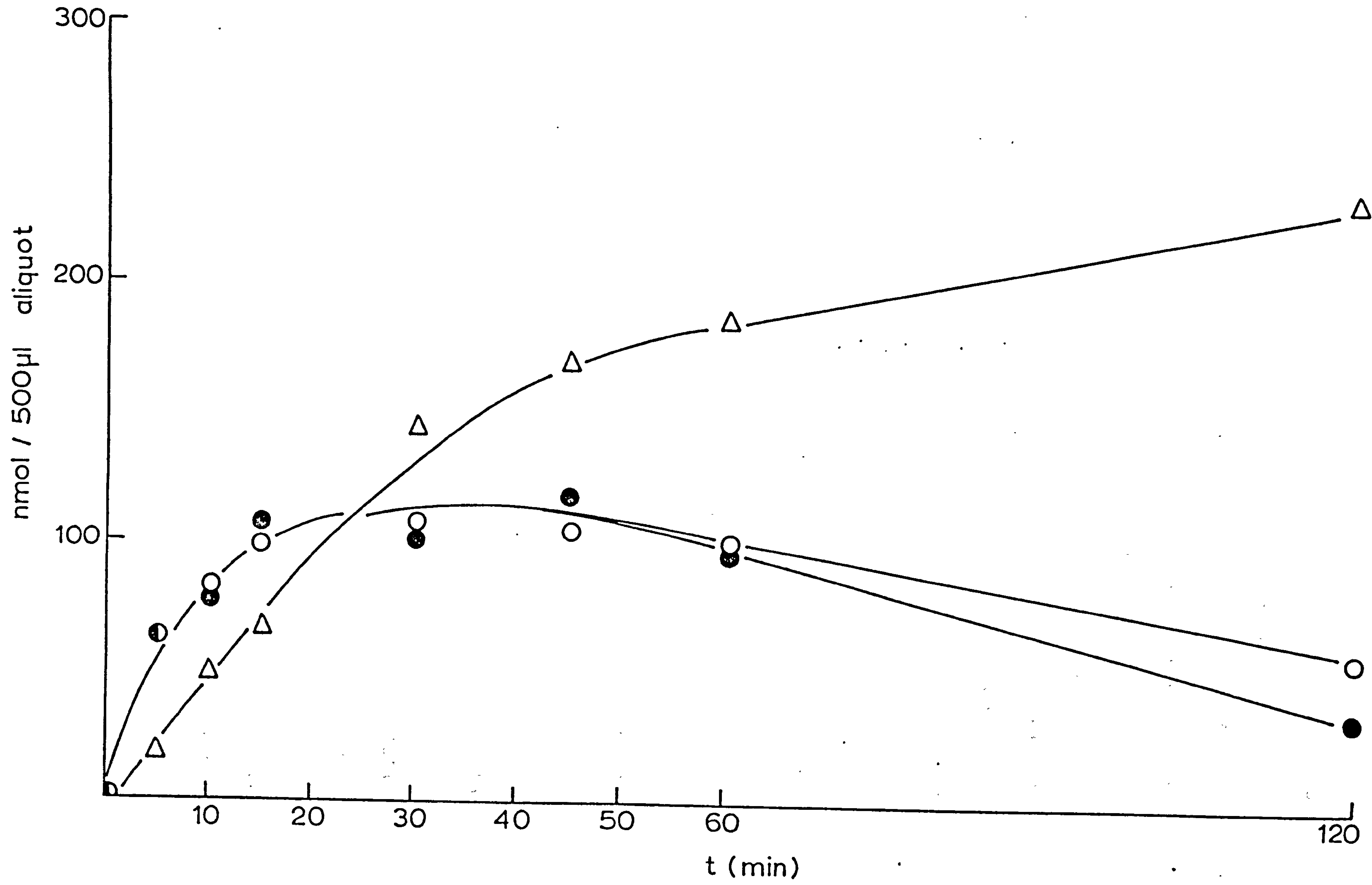
Figure 6.18: Time-course of acetyl-CoA and total acyl-CoA formation during the β-oxidation of palmitate in the "linked" β-oxidation assay

Assay conditions as in Fig. 16

△= acetyl-CoA

○= long-chain acyl-CoA (measured as total CoASH consumed - acetyl-CoA)

●= total acyl-CoA as determined by H.P.L.C.



nmol / 500µl aliquot
300
200
100
10
20
30
40
50
60
120
t (min)

of C_{n-2} acyl-CoA ester builds-up to is maximum concentration before in-turn decreasing and so on down the homologous series. This is the behaviour expected of a consecutive, linked reactions involving free intermediates (Stewart *et al* 1973; Easterby 1981; Kuchel 1985). By free intermediates is meant intermediates that are not so tightly bound to β-oxidation enzymes so as not to be in equilibrium with the bulk aqueous phase.

The time-course of long-chain acyl-CoA and acetyl-CoA formation, as determined enzymically, and of total long-chain acyl-CoA as determined by H.P.L.C. ia shown in Fig. 6.18. There is a very good correlation between the two sets of data. This indicates that the calculation of total long-chain acyl-CoA from (total CoASH consumption)-(acetyl-CoA production) is a good estimate of total long-chain acyl-CoA.

6.7.2.2. DIRECT ASSAY

The occurence of β-oxidation intermediates was also investigated using the direct assay with palmitoyl-CoA as substrate. A standard direct assay was scaled-up appropriately and aliquots sampled periodically as with the linked assay system. The time-course of intermediate production is shown in Fig. 6.19. The levels of intermediates in the direct assay are lower (≈ 4 μM myristoyl-CoA) than in the linked assay (≈ 52 μM lauroyl-CoA). This is probably due to two factors. Firstly, the total concentration of long-chain acyl-CoA is higher in the linked assay than in the direct assay due to the continual synthesis of palmitoyl-CoA by the acyl-CoA synthetase(s); the more acyl-CoA that is fed into the system the higher the concentration of intermediates is likely to be. Secondly, the presence of acyl-CoA thioesterase activity in crude extracts will affect the levels of intermediate accumulation. In the linked assay the acyl-CoA synthetases will be active and under such

Figure 6.19: Time-course of acyl-CoA intermediate formation during the β-oxidation of palmitoyl-CoA in the direct β-oxidation assay

Assay performed in potassium phosphate buffer (130mM, pH 8) with 80μM palmitoyl-CoA

Crude cell-free extract from succinate-grown *Corynebacterium* 7E1C

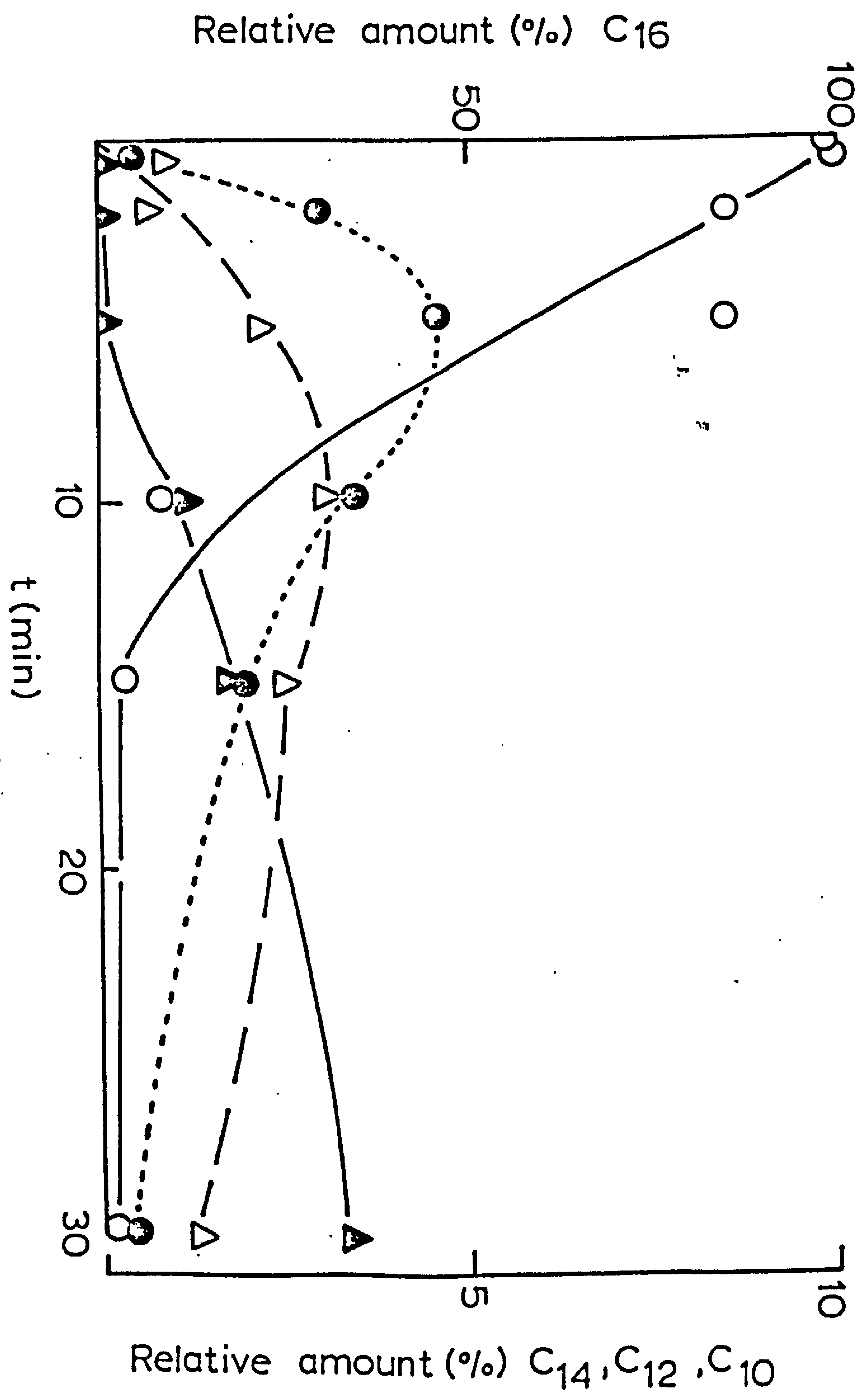
○ = palmitoyl-CoA

● = myristoyl-CoA

△ = lauroyl-CoA

▲ = decanoyl-CoA

100% ≡ 80 μM acyl-CoA


Relative amount (%) C16
100
50
10
20
30
t (min)
5
10
Relative amount (%) C14, C12, C10

conditions there will be net acyl-CoA synthesis so that in effect the thioesterase will be inoperative under the conditions of the linked assay. However, the acyl-CoA synthetases will not be operative under the conditions of the direct assay (ATP ommitted, CoASH concentration 80μM as opposed to 0.7mM in the linked assay) and under these conditions there will be a net hydrolysis of acyl-CoA which will tend to lower the concentration of accumulated acyl-CoA esters.

However, the important point is that chain-shortened acyl-CoA esters do accumulate and they display a precursor-product relationship to one another, just as in the linked assay. Furthermore, as in the linked assay, only saturated acyl-CoAs accumulate. Thus although there are certain quantitative differences between the linked and direct assays they are in very good qualitative agreement.

6.7.2.3. EFFECT OF INHIBITION OF 3-HYDROXYACYL-CoA DEHYDROGENASE ON INTERMEDIATE ACCUMULATION

The effects, on intermediate accumulation, of inhibition of the 3-hydroxyacyl-CoA dehydrogenase step of β-oxidation were investigated using palmitate in the linked β-oxidation assay. 3-Hydroxyacyl-CoA dehydrogenase was inhibited by preincubating the cell-free extract with NADase (cell-free extract (1ml, approx. 3 mg/ml protein) was incubated for 5 min. at room temperature with 0.1 units NADase) to remove traces of NAD^+ and not adding NAD^+ to the assay.

H.P.L.C. traces of samples taken at 5 and 60 minutes are shown in Fig. 6.20. These traces are very different to those obtained when 3-hydroxyacyl-CoA dehydrogenase is fully active (c.f. Fig. 6.16). There are 3 major acyl-CoA peaks (the 4th peak is the pentadecanoyl-CoA internal standard) corresponding to palmitoyl-CoA, *trans* Δ^2 hexadecenoyl-CoA and 3-hydroxypalmitoyl-CoA. Small amounts of shorter

Figure 6.20: H.P.L.C. analysis of the acyl-CoA intermediates of the β-oxidation of palmitate in the "linked" β-oxidation assay when 3-hydroxyacyl-CoA dehydrogenase is inhibited

Hydroxyacyl-CoA dehydrogenase was inhibited by ommiting NAD^+ from the reaction mixture and treating the cell-free extract with NADase

Assay performed in potassium phosphate buffer (130mM, pH 8)

[palmitate] = 0.75mM, [CoASH] = 0.7mM, [ATP] = 4mM

Crude cell-free extract from succinete-grown *Corynebacterium* 7E1C

A = butyryl-CoA (C_4)

B = hexanoyl-CoA (C_6)

C = octanoyl-CoA (C_8)

D = decanoyl-CoA (C_{10})

E = lauroyl-CoA (C_{12})

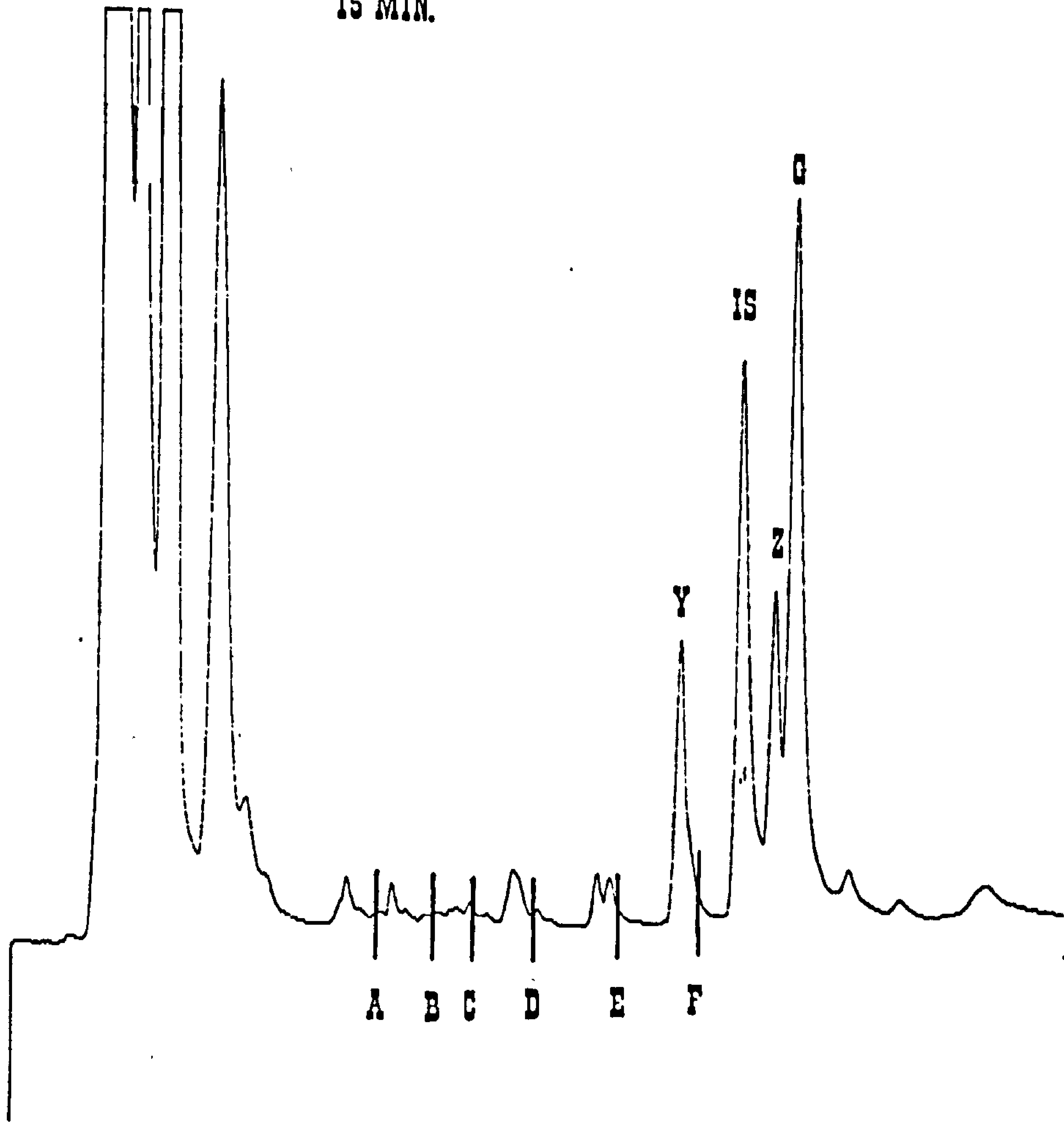
F = myristoyl-CoA (C_{14})

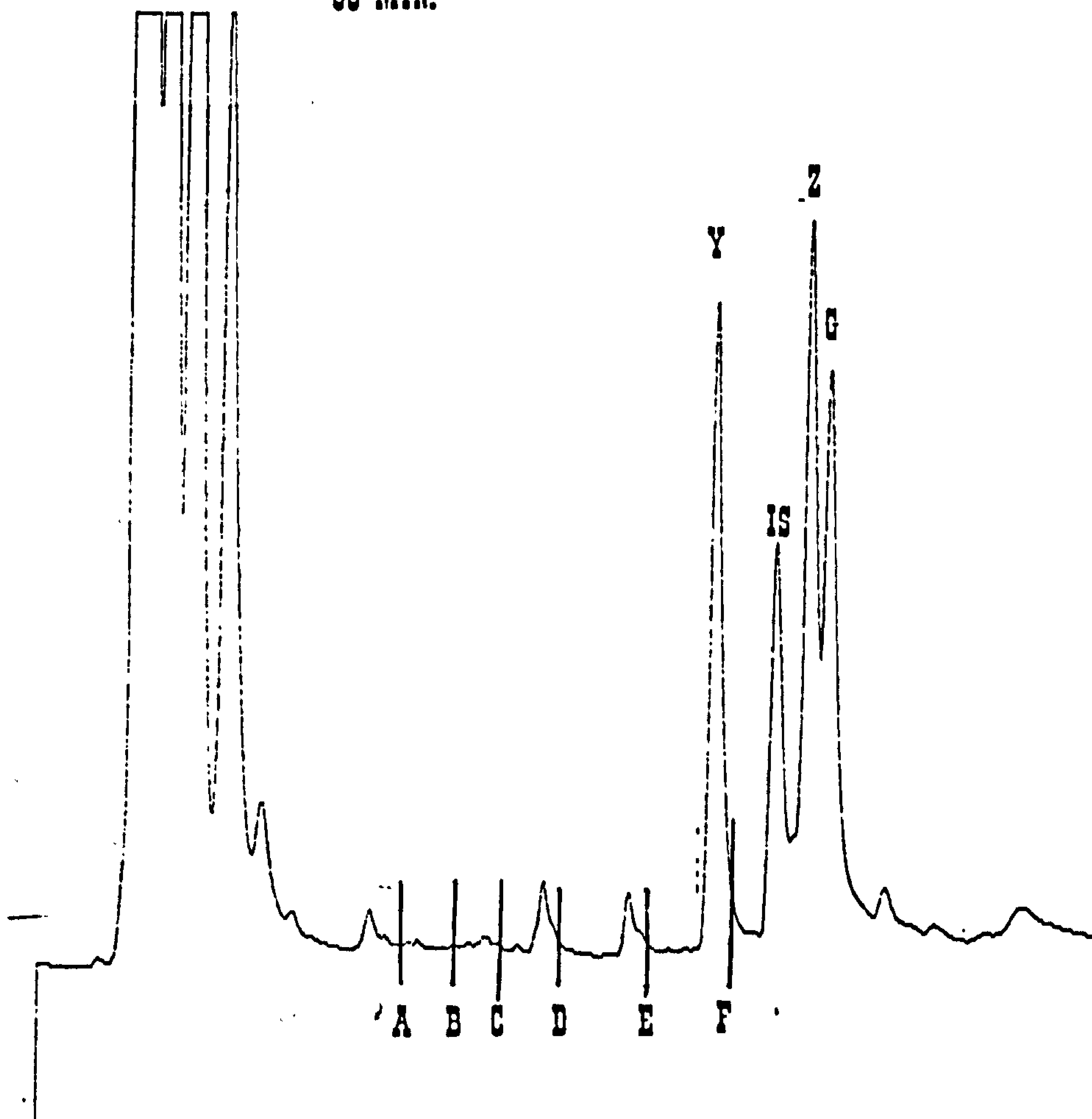
G = palmitoyl-CoA (C_{16})

Y = tetradecenoyl-CoA/3-hydroxypalmitoyl-CoA

Z = *trans* Δ^2 hexedecenoyl-CoA

IS = internal standard (pentadecanoyl-CoA; 50 μM)


15 MIN.
G
IS
Z
Y
A
B
C
D
E
F


60 MIN.
Z
Y
G
IS
A
B
C
D
E
F

chain acyl-CoA were also detected. They are lauroyl-CoA and decanoyl-CoA along with what may be their Δ^2 enoyl- and 3-hydroxy- derivatives. No acetyl-CoA production could be detected when assayed enzymically (with citrate synthase) which indicates that the 3-hydroxyacyl-CoA dehydrogenase was severely inhibited. However, 3-hydroxyacyl-CoA dehydrogenase may still have been able to turn over extremely slowly which would explain the small amounts of chain-shortened acyl-CoA detected. The lauroyl-CoA and decanoyl-CoA peaks were absent from the controls incubated in the absence of palmitate which implies they were derived from palmitate rather than endogenous laurate and decanoate.

The time-course of acyl-CoA intermediate production is shown in Fig. 6.21. These results demonstrate that when 3-hydroxyacyl-CoA dehydrogenase is inhibited Δ^2 enoyl-CoA and 3-hydroxyacyl-CoA esters accumulate significantly. The detection of Δ^2 unsaturated and 3-hydroxyacyl-CoA esters under these conditions shows that the inability to detect these compounds under normal conditions is not due to the present analytical system failing to resolve them from saturated acyl-CoAs. These results are in agreement with those obtained with the mitochondrial β-oxidation system where 3-hydroxyacyl-CoA esters only accumulate significantly when NADH dehydrogenase is inhibited by rotenone (Bremer and Wojtczak 1972; Stanley and Tubbs 1974, 1975; Bartlett *et al* 1988; Watmough *et al* 1989). Lopes-Cardoza *et al* (1978) detected the carnitine esters of 3-hydroxypalmitate, 3-hydroxymyristate and 3-hydroxylaurate during state 4 mitochondrial β-oxidation of palmitate; the high $NADH/NAD^+$ ratio pertaining under such conditions would inhibit the 3-hydroxyacyl-CoA dehydrogenase (Bremer and Wojtczak 1972). Similarly, Bartlett *et al* (1990) detected unsaturated intermediates during peroxisomal β-oxidation of palmitate only in the absence of a NAD^+ regenerating system.

The results obtained here illustrate the point made earlier that under

Figure 6.21: Time-course of intermediate formation during the β-oxidation of palmitate in the linked β-oxidation assay when 3-hydroxyacyl-CoA dehydrogenase is inhibited

Assay conditions as in Fig. 20

Acetyl-CoA was measured enzymically with citrate synthase (see Methods)

Other acyl-CoA esters determined by H.P.L.C.

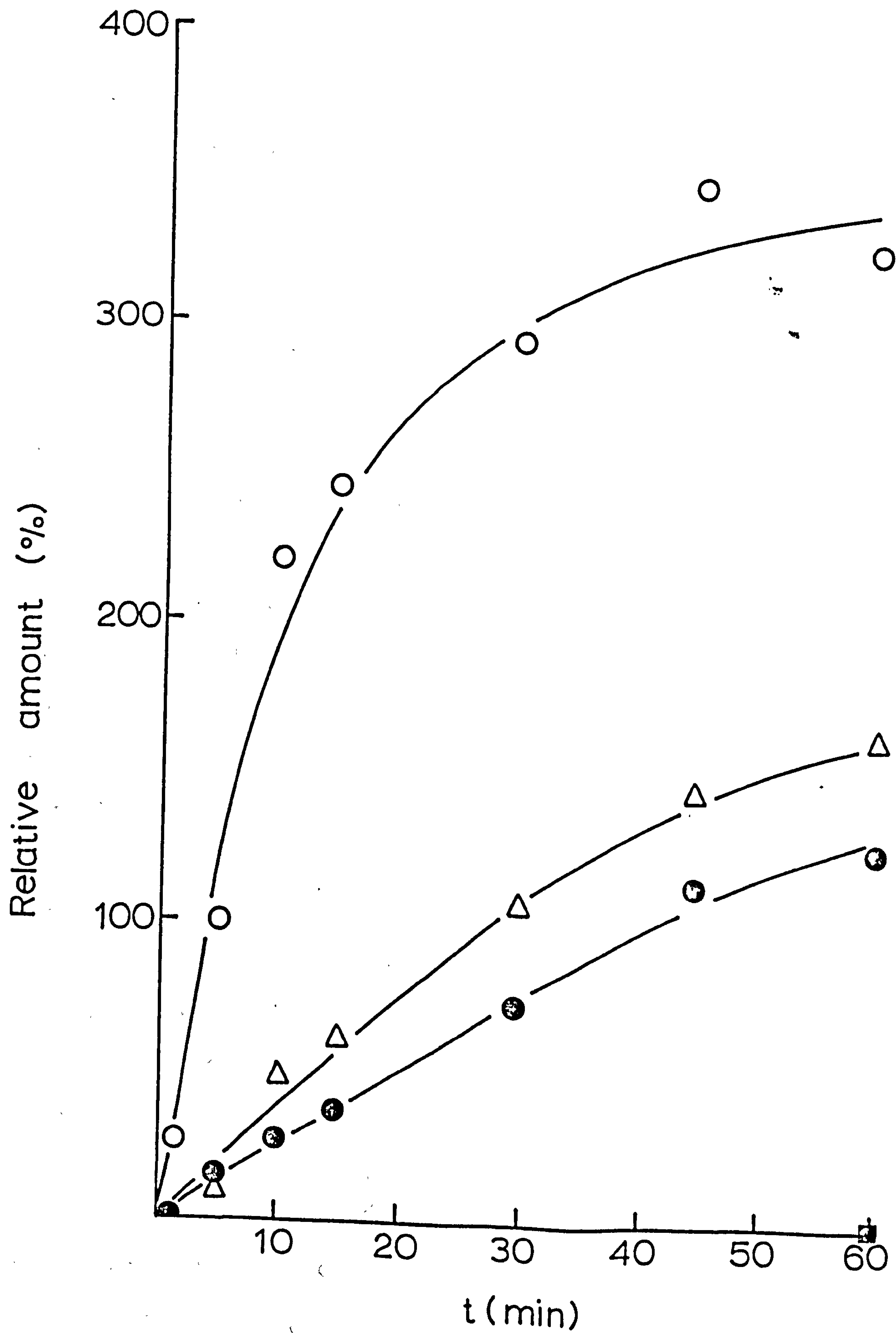
○ = palmitoyl-CoA

△ = *trans* Δ^2 hexadecenoyl-CoA

● = 3-hydroxypalmitoyl-CoA

■ = acetyl-CoA

100% ≡ 50 µM acyl-CoA


400
300
200
100
Relative amount (%)
10
20
30
40
50
60
t (min)

normal assay conditions (ie: in the presence of NAD^+ so that 3-hydroxyacyl-CoA dehydrogenase is fully active) the accumulation of acyl-CoA intemediates other than saturated acyl-CoAs is minimal, further emphasizing that under normal assay conditions acyl-CoA dehydrogenase is the rate-limiting enzyme of the β-oxidation spiral.

6.7.3. SIGNIFICANCE OF ACCMULATION OF β-OXIDATION INTERMEDIATES

The accumulation of β-oxidation intermediates by *Corynebacterium* 7E1C is very interesting. During mitochondrial β-oxidation of palmitate there is little or no accumulation of β-oxidation intermediates (Garland *et al* 1965) which led to the suggestion (Garland and Tubbs 1968) that the mitochondrial β-oxidation enzymes may be organized into some form of multienzyme complex so that there were in effect no free (free meaning in equilibrium with the bulk aqueous phase) intermediates of β-oxidation. It appeared that once an acyl-CoA ester entered the β-oxidation spiral it was completely degraded to acetyl-CoA without being released. Thus the significant accumulation of intermediates during palmitate oxidation by cell-free extracts of *Corynebacterium* 7E1C is strikingly different and may reflect a difference in the stuctural organization of β-oxidation. If a series of consecutive linked reactions proceeds via free intemediates then there should be a lag before the end product is produced at a maximum rate during which time the subsequent pathway intermediates should each, in turn rise and fall in concentration (Stanley *et al* 1973; Easterby 1981; Kuchel 1985). If however, the reaction proceeds via a multienzyme complex with no free intermediates then the final product of the pathway should appear without a lag and no intermediates should be detected .

The results obtained with the *Corynebacterium* 7E1C β-oxidation system can be summarized as follows. (1) Assays display a lag period during

which the rate of β-oxidation (as measured by following NAD^+ reduction) increases with time until a constant linear rate is achieved (see section 7.1.1.). (2) There is significant accumulation of (saturated) chain-shortened acyl-CoA esters which display a precursor-product relationship. (3) There is a prolonged lag, during the β-oxidation of palmitoyl-CoA (or when starting from the free acid) before decanoyl-CoA and octanoyl-CoA appear. These results are consistent with β-oxidation proceeding via free intermediates. Stewart *et al* (1973) working with an aqueous extract of acetone-dried mitochondria found that [1^{14}C]octanoate gave rise to acetyl-CoA with a higher specific radioactivity than did [8^{14}C]octanoate implying that the carboxyl- end of the molecule was preferentially oxidized. This meant that once an octanoate molecule entered the β-oxidation spiral it was NOT completely degraded to acetyl-CoA without release of intermediates and indeed 3-hydroxyoctanoate (in very small amounts) and hexanoate were detected. The accumulation of 3-hydroxyoctanoate was significantly increased by the addition of rotenone. These results are akin to those obtained in this investigation with *Corynebacterium* 7E1C. When intact mitochondria were used much lower levels of intermediates were detected. Stanley and Tubbs (1974,1975), detected small amounts of myristoyl-, lauroyl- and decanoyl-CoA (measured by radio-GLC of water soluble fatty acyl-groups) during oxidation of radiolabelled palmitate by intact mitochondria. However, there was no lag in their appearance and dilution of the radiolabel with unlabelled palmitate demonstrated them not to be on the main kinetic pathway. Thus it was concluded that they represented a slow leakage from the main pathway with which they were not in equilibrium. On the basis of these results these authors proposed a "leaky hosepipe" model of β-oxidation in which the intermediates they detected represented leakage. Recently, using H.P.L.C. to analyse the INTACT acyl-CoAs directly, Watmough *et al* (1989) demonstrated that in intact,

uncoupled mitochondria oxidizing palmitoyl-CoA there was a small acumulation of myristoyl-CoA and trace amounts of lauroyl-CoA were also detected. No unsaturated acyl-CoA was detected except when NADH dehydrogenase was inhibited by rotenone. This results again implied some degree of structural organization of the β-oxidation pathway and indeed an association of some of the β-oxidation enzymes with the inner mitochondrial membrane has been reported (Sumegi and Srere 1984). The bacterial β-oxidation as exemplified by *E.coli* displays a remarkable degree of structural organization. A β-oxidation complex has been isolated from this organism consisting of 2 subunits. One protein posseses 4 activities; enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase and *cis* Δ^3 *trans* Δ^2 enoyl-CoA isomerase. The other subunit contains 3-ketoacyl-CoA thiolase. Such a system would be expected to be highly efficient at catalysing the β-oxidation sequence of reactions and indeed it has been demonstrated (Yang *et al* 1986) that dec-2-enoyl-CoA is converted to 3-ketodecanoyl-CoA without any lag in product (NADH) formation when catalysed by the *E.coli* β-oxidation complex (or by the peroxisomal bifunctional enoyl-CoA hydratase-hydroxyacyl-CoA dehydrogenase) but when the *E.coli* complex was replaced with purified mitochondrial enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase a definite lag in NADH production was observed. This was explained in terms of "substrate-channeling" on the *E.coli* β-oxidation complex. Thus the behaviour of the *E.coli* β-oxidation appears to be significantly different to that of *Corynebacterium* 7E1C and more akin to the mitochondrial system. However, a few points should be considered. In the experiments performed with *Corynebacteium* 7E1C the acyl-CoA was used as substrate as opposed to the enoyl-CoA in the *E.coli* experiments. The *E.coli* acyl-CoA dehydrogenase is not associated with the β-oxidation complex and so, although substrate channelling occurs between the

enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase sites on the multifunctional protein such channelling may not occur between acyl-CoA dehydrogenase and enoyl-CoA hydratase or between thiolase and acyl-CoA dehydrogenase at the end of each turn of the spiral. If this is so then one would expect saturated esters (produced at the end of each turn of the β-oxidation spiral) and unsaturated acyl-CoA esters (produced by acyl-CoA dehydrogenase) to accumulate to some degree, although if the acyl-CoA dehydrogenase is rate-limiting the unsaturated acyl-CoAs would not accumulate to any great extent. Thus the results obtained with *Corynebacterium* 7E1C indicate that under *in vitro* conditions the saturated acyl-CoA esters behave as free intermediates although the possibility of substrate channelling of the other intermediates cannot be discounted. It is possible that *in vivo* there would not be such an accumulation of acyl-CoA as the amount of accumulation would be limited by the amount of cellular CoASH. However, the results obtained here still differ from those obtained with the mitochondrial system (Stanley and Tubbs 1975) in that the saturated acyl-CoAs displayed no precursor-product relationship in the mitochondrial system. This may imply that β-oxidation is less well structurally organized than in the mitochondrial system or it may be that in the process of breaking the bacterial cell the β-oxidation system is disrupted as appears to be the case with mitochondria. The mammalian (rat liver) peroxisomal β-oxidation system behaves somewhat differently to its mitochondrial counterpart. Bartlett *et al* (1988, 1990) demonstrated significant accumulation of β-oxidation intermediates. In their latter work (Bartlett *et al* 1990) they demonstrated the accumulation of all the saturated acyl-CoA ester intermediates (ie: C_{16} to C_2) during the peroxisomal β-oxidation of [U-^{14}C]palmitate. In the presence of a NAD^+ -regenerating system only saturated intermediates were detected whereas in the absence of such a system some unsaturated intermediates were

detected. This accumulation of β-oxidation intermediates by the peroxisomal system is more akin to the results obtained in this study with the (cell-free) bacterial system than it is to the mammalian mitochondrial system. An important point to be considered is whether the *in vitro* behaviour of the *Corynebacterium* 7E1C β-oxidation system corresponds to its *in vivo* behaviour. Stanley and Tubbs (1975), when considering the organization of the mitochondrial β-oxidation system suggested that the confined enviroment of the mitochondrion may behave rather like a multienzyme complex; the small intramitochondrial volume resulting in the steady-state concentrations of intermediates being achieved rapidly and the absolute amounts being very small. A similar situation can be envisaged for the bacterial system *in vivo*.

7. CONCLUSIONS

This research project has been concerned with an investigation of dicarboxylic acid production by the alkane-utilizing bacterium *Corynebacterium* 7E1C. Both the range of dicarboxylic acids produced by this organism and a number of enzyme systems involved in dicarboxylic acid metabolism have been studied. In previous chapters the individual enzyme systems have been described and their roles in dicarboxylic acid metabolism discussed. In this chapter the integration of the various enzyme systems is discussed in relation to how their combined activities result in the observed range of dicarboxylic acids produced by this organism from different chain-length alkanes (and fatty acids).

A problem encountered when considering the integration of the various enzyme systems is the observation that *in vivo* the various enzymes have markedly different optimum reaction conditions (eg: pH, ionic strength, buffer). However, this problem can be overcome by assuming that the *in vivo* conditions will be optimal for all the enzymes.
The pathways of dicarboxylic acid metabolism are outlined in Figure 7.1.. Whether a dicarboxylic acid of a particular chain-length accumulates or not will depend on the relative rates of its formation and degradation. If the rate of formation exceeds the rate of degradation it will accumulate. If the rates of formation and degradation are equal the dicarboxylic acid will not accumulate. Most of the enzyme systems investigated displayed marked specificities with respect to acyl-chain-length. Thus the relative rates of formation and degradation of dicarboxylic acids are likely to vary with the chain-length of the dicarboxylic acid in question.

The range of dicarboxylic acids accumulated by *Corynebacterium* 7E1C is rather limited. The best yields of dicarboxylic acid (DC_{12}) are obtained from dodecane. No dicarboxylic acids could be detected during growth on

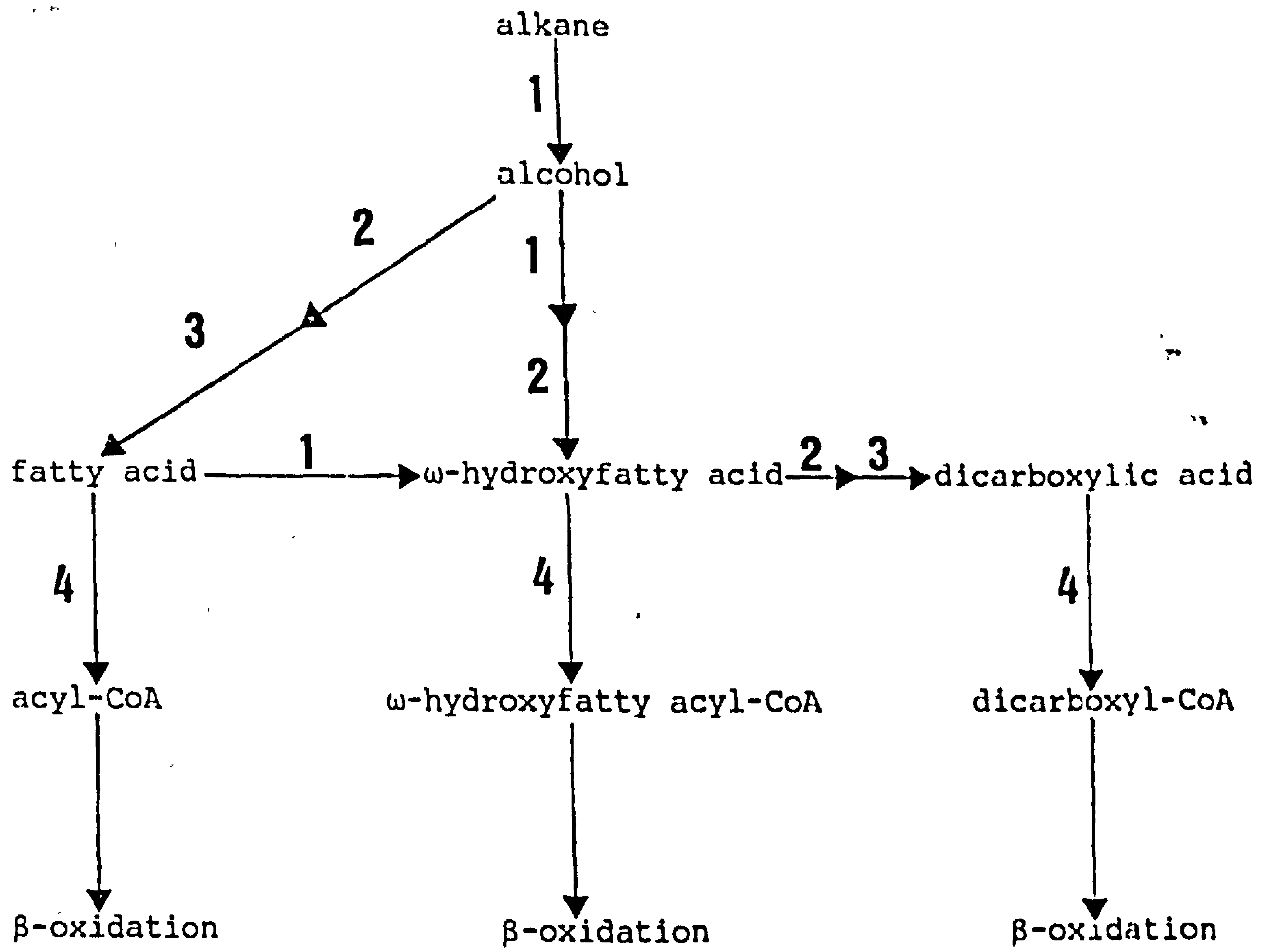
Figure 7.1: Pathways of dicarboxylic acid metabolism

1 = ω-hydroxylase

2 = alcohol dehydrogenase

3 = aldehyde dehydrogenase

4 = acyl-CoA synthetase



alkane
1
alcohol
2
1
3
2
fatty acid
1
ω-hydroxyfatty acid
2
3
dicarboxylic acid
4
4
4
acyl-CoA
ω-hydroxyfatty acyl-CoA
dicarboxyl-CoA
β-oxidation
β-oxidation
β-oxidation

hexadecane, methyl palmitate or 16-hydroxypalmitate.

As far as the formation of dicarboxylic acids is concerned only the alcohol dehydrogenases were investigated. With ω-hydroxyfatty acids C_{10}, C_{12} and C_{16} substrates displayed simmilar values of V_{max} (42-50 nmol/min/mg) whereas K_m decreased rapidly with increasing chain-length (C_{10} = 1520μM; C_{12} = 77μM; C_{16} = 22μM). Thus, at low substrate concentrations 16-hydroxypalmitate will be oxidized more rapidly than 12-hydroxylaurate but with increasing substrate concentration the respective rates will converge. With α,ω-diols 1,12-dodecanediol is oxidized far more rapidly than the corresponding C_{14} and C_{16} substrates at both high and low substrate concentrations. Thus if significant dicarboxylic acid is formed via the α,ω-diol (c.f. Bacchin *et al* 1974) then dicarboxylic acid production would be favoured from C_{12} rather than from C_{16} substrates. However, the specificity of the ω-hydroxylase must also be considered. Unfortunately the specifity of this reaction is currently unknown. It is, however, important to note than even when cells were grown on 16-hydroxypalmitate no DC_{16} could be detected. This implies that even when cells are supplied with 16-hydroxypalmitate any DC_{16} formed is degraded as rapidly as it is formed.

Activation to the corresponding acyl-CoA is a prerequisite for the subsequent β-oxidation of monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids. However, the acyl-CoA synthetases present in crude extracts have a remarkably broad substrate specificity and display similar activities with C_{10} to C_{16} monocarboxylic, ω-hydroxymonocarboxylic and dicarboxylic acids. Thus it seems that the specificity of the acyl-CoA synthetases does not determine the range of dicarboxylic acids accumulated by this organism.

In contrast the specificity of the β-oxidation system is far more

stringent with respect to both acyl chain-length and type of carboxylic acid. Dicarboxylic acids (C_{10} to C_{16}) are all β-oxidized slowly compared to the correspondind monocarboxylic acids. The rate of β-oxidation of ω-hydroxyfatty acids is highly dependent on acyl chain-length. 16-Hydroxypalmitate is β-oxidized rapidly (at a rate comparable to a monocarboxylic acid) whereas 12-hydroxylaurate is β-oxidized slowly (at a rate comparable to a dicarboxylic acid).

Comparison of the rates of conversion of ω-hydroxyfatty acid to dicarboxylic acid and of β-oxidation of the ω-hydroxyfatty acid are very interesting. With 12-hydroxylaurate values of V_{max} of approx. 50 nmol/min/mg have been measured for the ω-hydroxyfatty acid dehydrogenase reaction whilst values of up to 8.4 nmol/min/mg have been recorded (in the linked assay) for the β-oxidation of this substrate. The corresponding rates observed with 16-hydroxypalmitate are 42 nmol/min/mg (ω-hydroxyfatty acid dehydrogenase) and 45 nmol/min/mg (β-oxidation). Thus, the relative partition between conversion to dicarboxylic acid and β-oxidation to acetyl-CoA is likely to be very different for these two substrates. The slow β-oxidation of 12-hydroxylaurate favours its conversion to DC_{12} whilst the rapid β-oxidation of 16-hydroxypalmitate results in conversion to DC_{16} being less favoured. Thus one can envisage a situation where DC_{12} is β-oxidized more slowly than it is formed whereas DC_{16} is β-oxidized as rapidly as it is formed. Therefore, it appears that the ω-hydroxyfatty acid occupies a pivotal position in dicarboxylic acid metabolism in this organism; the relative partition of the ω-hydroxyfatty acid between degradation (β-oxidation) and oxidation to the dicarboxylic acid determining whether or not the dicaboxylic acid in question accumulates. These results indicate that that the specificity of the β-oxidation system is the major factor determinimg the range of dicarboxylic acids accumulated by *Corynebacterium* 7E1C. For

dicarboxylic acids to accumulate not only must the dicarboxylic acid itself be a poor β-oxidation substrate but the corresponding ω-hydroxyfatty acid must also be β-oxidized slowly.
Thus, for this organism to produce DC_{16} (commercially more valuable than DC_{12}) the β-oxidation system of this organism needs to be completely "blocked" otherwise the rapid β-oxidation of 16-hydroxypalmitate will tend to prevent accumulation of DC_{16}.

The specificities of the enzymes investigated are consistant with the range of dicarboxylic acids accumulated by this organism. The *Corynebacterium* 7E1C system appears to be be a good "model" system in which to study the biochemistry of dicarboxylic acid metabolism. In order to gain a better understanding of dicarboxylic acid metabolism further studies need to be carried out. For example the determination of the specificity of the ω-hydroxylase reaction is essential to a complete understanding of the conversion of alkanes to dicarboxylic acids whilst a kinetic study of the β-oxidation of ω-hydroxymonocarboxylic and dicarboxylic acids would give more information on the ability of the β-oxidation system to cope with these substrates.

In conclusion it may be stated that the major aims of the project have been achieved. Some key enzymes of dicarboxylic acid metabolism have been investigated and a biochemical explanation obtained for the range of dicarboxylic acids accumulated by *Corynebacterium* 7E1C.

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