

STUDIES ON CITRACONASE, A NEW ENZYME AND ON CERTAIN ASPECTS OF
BACTERIAL DEGRADATION OF C₅-DICARBOXYLIC ACIDS

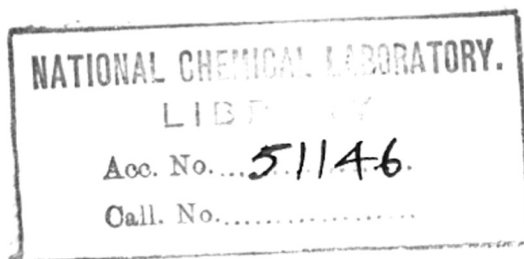
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A Thesis submitted to the University of Madras for the Degree of
DOCTOR OF PHILOSOPHY

by

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TH-723



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NATIONAL CHEMICAL LABORATORY

POONA

1967



ACKNOWLEDGMENT

The author wishes to express his deep sense of gratitude to Dr. M. R. Raghavendra Rao for his valuable advice and many useful discussions during the course of this work.

The author gratefully acknowledges the valuable advice given by Dr. P. K. Bhattacharyya in the studies on the stereochemical aspects of the hydration reactions.

The author would like to thank Dr. C. Sivaraman for suggestions particularly in carrying out the ultra-centrifugal studies.

The author also wishes to thank Dr. V. Jagannathan, Head of the Division of Biochemistry and Dr. J. C. Sadana for their keen interest in the work.

The author wishes to thank Mr. Iqbal Mulla and Mr. K. G. Deshpande of this Laboratory for the NMR and IR spectra respectively.

The author also wishes to thank all his colleagues for their encouragement and Mr. B. R. Narayanan for typing the thesis.

The author is indebted to the Director, National Chemical Laboratory for permission to submit this work in the form of a thesis.

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LIST OF ABBREVIATIONS AND TRIVIAL NAMES

Trivial names

Citraconic acid,	Methylmaleic acid
Citramalic acid,	α -Methylmalic acid
Citrane,	Citrate oxaloacetate-lyase (E.C.4.1.3.6)
Itaconic acid,	Methylenesuccinic acid
Mesaconic acid,	Methylfumaric acid
Tricarballic acid,	1,2,3-Propanetricarboxylic acid
TCA cycle,	Tricarboxylic acid cycle = Kreb's cycle = Citric acid cycle

Abbreviations

ATP	Adenosine triphosphate
CFE	Cell-free extract
CoA	Coenzyme A
CMP	Chloramphenicol
DCPIP	Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethyl-cellulose
2,4-DNPH	2,4-Dinitrophenylhydrazones(s)
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavineadenine dinucleotide
FMN	Flavin mononucleotide
x g	times normal gravitational force
g	gram
mg	milligram
μ g	microgram
GSH	Glutathione (reduced)
hr	hour
IR	Infra-red
MB	Methylene blue
min	minute
NAD	Nicotinamide-adenine dinucleotide
NADH	Reduced NAD
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced NADP
NMR	Nuclear (proton) magnetic resonance
PCMB	p-Chloromercuribenzoate (presently supposed to be p-hydroxymercuribenzoate)
PMS	Phenazine methosulfate
O.D.	Optical density
O.D. 435	Optical density at 435 m μ
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

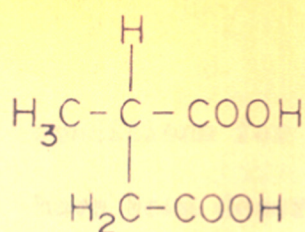
CHAPTER I

INTRODUCTION

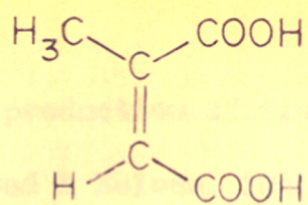
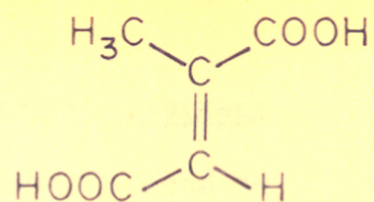
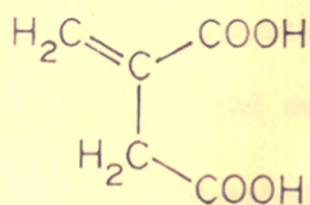
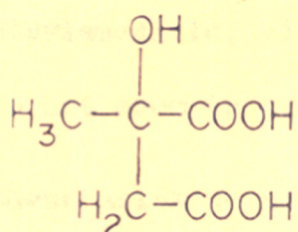
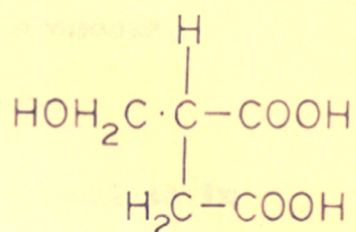
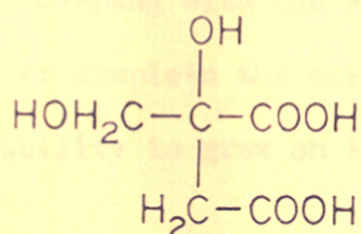
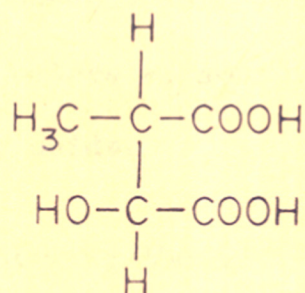
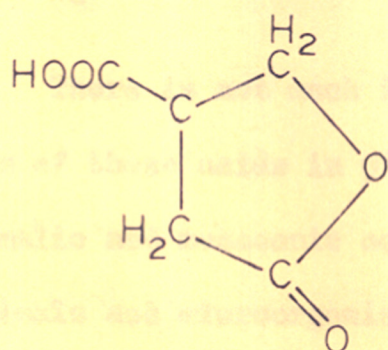
INTRODUCTION

The following structurally related branched C₅-dicarboxylic acids are known: methylsuccinic, citraconic, mesaconic, itaconic, citramalic, itatartaric, paraconic and aconic acids. The structural formulae of these acids are shown in Fig.1.

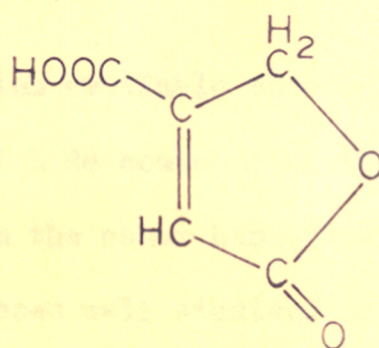
Occurrence: Among the above, mesaconic, itaconic, (+)- and (-)-citramalic and itatartaric acids are known to occur in nature. Mesaconic acid has been isolated from the plant Calabash curare (Karrer & Matter, 1946) and from cabbage leaves (Burton, 1928). It has also been detected in sugar cane (Saccharum officinarum) (Roberts & Martin, 1954) to the extent of 0.03 to 0.05 per cent of the dry solids of cane juice. Laevorotatory citramalate has been isolated from apple peels (Hulme, 1954a). It is one of the principal acids produced by respiration-deficient "petite" mutants of brewer's yeast (Sai & Amaha, 1965). (+)-Citramalic acid has been reported to be present in the shoots of lucerne (Medicago sativa) (Richardson & Hulme, 1937). It is formed from glutamate via mesaconate in Clostridium tetanomorphum (Munch-Peterson & Barker, 1958). It is also shown to be an intermediate in the biosynthesis of glutamate from acetate and pyruvate in Acetobacter suboxydans (Maragoudakis *et al.* 1966) which lacks the TCA cycle (Rao, 1957; Cheldelin, 1961). Some photosynthetic organisms synthesize it (Arnon *et al.* 1960; Losada *et al.* 1960). Itaconic acid does not seem to occur in plants or animals, but two species of Aspergillus, A.itaconicus (Kinoshita, 1932) and A.terreus (Calam, *et al.* 1939) accumulate it in sugar media. The



Methylsuccinic

Citraconic
(Methylmaleic)Mesaconic
(Methylfumaric)Itaconic
(Methylenesuccinic)Citramalic
(α -methylmalic)Itamalic
(Hydroxymethyl succinic)Itatartaric
(α -Hydroxymethyl malic) β -methylmalic

Paraconic



Aconic

FIG. 1 STRUCTURAL FORMULAE OF BRANCHED C_5 -DICARBOXYLIC ACIDS.

conditions for the optimal production of itaconic acid by A.terreus have been described (Lockwood & Nelson, 1946). Itatartaric acid is a metabolic product formed by an ultraviolet induced mutant of A.terreus (Stodola et al. 1945) in yields of 5.8 per cent of the total acids.

The other acids, methylsuccinic, citraconic, paraconic, itamalic and aconic acids do not seem to occur in nature.

Metabolism: While these acids do not seem to accumulate in appreciable quantities in the animal kingdom, plants and microorganisms are known to have the capacity to accumulate some of them (Burton, 1928; Richardson & Hulme, 1937; Karrer, 1946; Roberts, 1954; Crombie, 1960). In keeping with the expectation - because this would be essential to complete the carbon cycle in nature -, some microorganisms have the ability to grow on these peculiar acids.

Much information is available concerning the metabolism of the naturally occurring branched C_5 -dicarboxylic acids, viz. citramalic, mesaconic and itaconic acids. In contrast little is known of the metabolism of the "unnatural" (those which have not yet been found in nature) C_5 -dicarboxylic acids.

There is not much information available concerning the metabolism of these acids in plants which do contain varying amounts of citramalic and mesaconic acids. On the other hand, their degradation in animals and microorganisms has been well studied. On feeding dogs with mesaconic, itaconic and citraconic acids, 64, 24 and 28 per cent respectively are recovered in urinary excretion, the remainder presumably being metabolized (Emmerich, 1939). Emmerich (1939) also

4

found that mesaconate could be detected in the urine of dogs fed with methylsuccinate. Much of the information on the degradation of these acids in the animals and microorganisms can be summarized as follows. Three main pathways of degradation are discernible, viz., (1) The succinate-pathway discovered by Brightman and Martin (1961) in a *Pseudomonas* isolate, (2) The simple hydration-cleavage system of Barker (1956), which was first found in a glutamate-grown *Clostridium tetanomorphum* and (3) The activated pathway discovered by Adler *et al.* (1957) in liver mitochondria and independently by Cooper and Kornberg (1962) in *Pseudomonas* B₂ aba. There are microorganisms in which minor variations of the above pathways are known. These pathways will now be considered in greater detail.

(1) The succinate-pathway of Brightman and Martin

On the basis of studies on an isolate, *Pseudomonas* 111, Martin and Brightman (1961) postulated that itaconate and mesaconate are converted through citramalate into succinate by a process of decarboxylation, intramolecular rearrangement and dehydrogenation. Succinate would then be oxidized via the citric acid cycle as indicated in Fig. 2(a).

The above pathway was proposed on the basis of the following observations: (i) Oxidation of both citramalate and succinic semialdehyde without lag (i.e., without induction) by cells grown on itaconate or mesaconate, (ii) The accumulation of citramalate and succinate (as observed chromatographically) on incubation of itaconate or mesaconate with cell-free extracts, and (iii) The production of succinate from citramalate, itaconate or mesaconate or succinic semialdehyde in the cell-free system. There was a large accumulation of citramalate

in the incubation mixtures containing cell-free extracts (itaconate-grown cells) and itaconate or mesaconate; this would be expected thermodynamically (since the equilibrium is in favour of the formation of hydroxyacids). The optical nature of the citramalate isomer was apparently not tested. On incubation with citramalate, itaconate or mesaconate, all the three acids together with succinic semialdehyde were found present among the products. Studies with ^{14}C -methylene-labelled itaconate or ^{14}C -methyl-labelled citramalate further indicated an intramolecular rearrangement before or after decarboxylation, leading to succinic semialdehyde. The rearrangement mechanism itself was not studied. This type of intramolecular rearrangement seems to be similar to the formation of β -methylaspartate from glutamate in *C. tetanomorphum* (Wachsman, 1956; Barker et al. 1957; Munch-Peterson & Barker, 1958; also see next page). There was no requirement of added ATP or CoA for these reactions nor could any hydroxymate derivatives be detected on incubation with hydroxylamine. These reactions evidently do not involve the formation of any activated intermediates. None of the enzymes involved in the pathway has been purified or studied further.

It was reported that a *Micrococcus* strain (Cooper et al. 1965) metabolized aconate by a mechanism [Fig. 2(b)] similar to that described by Martin and Brightman (1961). There appear to be no activated intermediates in this degradation also. Although this organism also grows on itaconate, itaconate and aconate are metabolized by different routes. Aconate delactonase and succinic semialdehyde dehydrogenase are formed only when growth occurs on aconate: itaconate degradation proceeds by a preliminary activation to itaconyl-CoA and

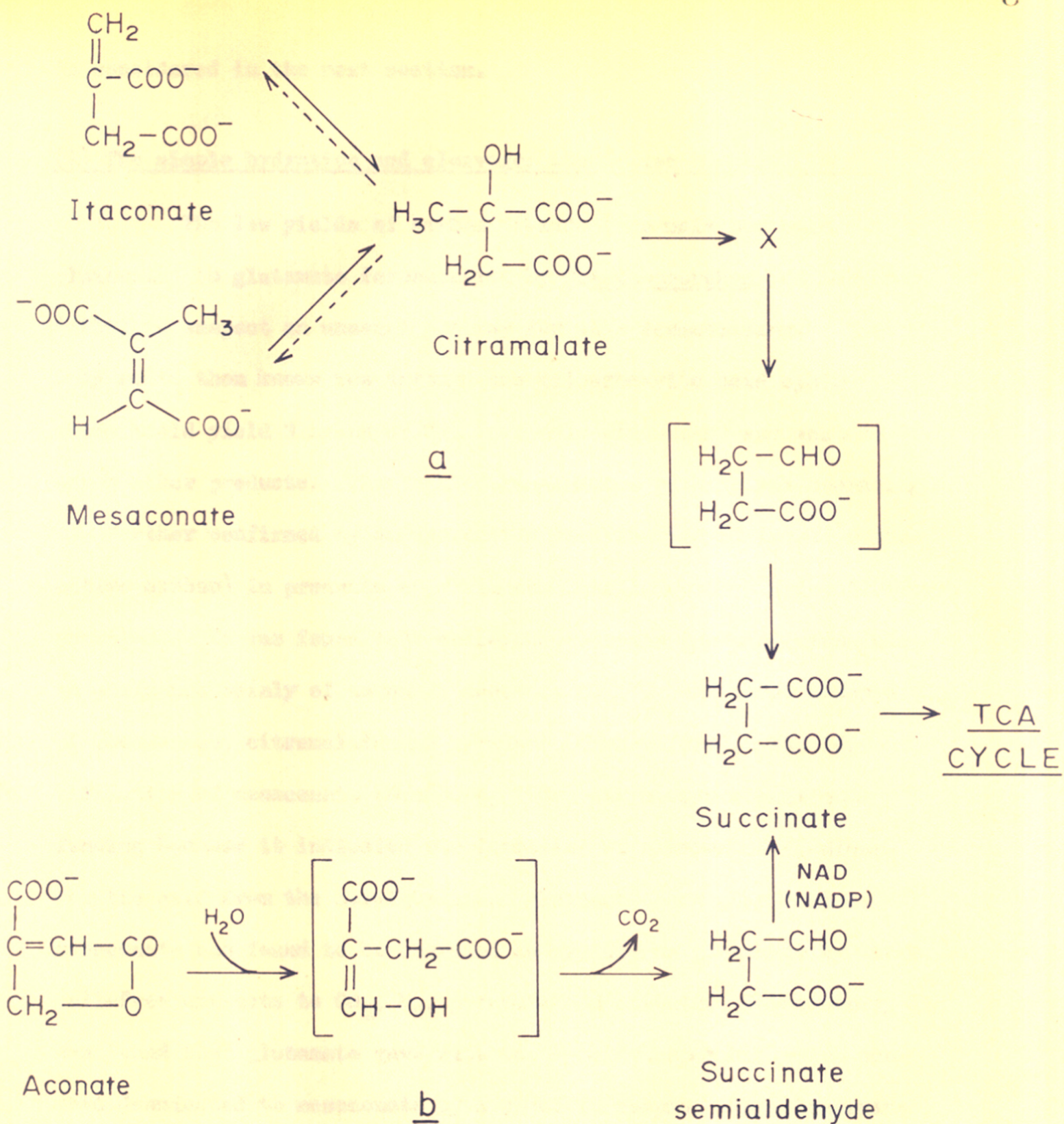


FIG. 2. POSTULATED PATHWAYS OF DEGRADATION OF :

(a) ITACONATE AND MESACONATE ACCORDING TO BRIGHTMAN and MARTIN (1961), AND

(b) ACONATE ACCORDING TO COOPER et al. (1965)

is considered in the next section.

(2) The simple hydration and cleavage: the Barker pathway (1956)

The low yields of carbon dioxide (one mole per mole glutamate) in glutamate fermentation by C.tetanomorphum led Barker (1956) to suspect an unusual pathway for this fermentation. The only route then known was through the tricarboxylic acid cycle which would yield 3 moles of CO₂ (per mole glutamate) and acetate among other products. That a hitherto-unknown pathway was operating was further confirmed by an unexpected distribution of label (radio-active carbon) in products of glutamate labelled with ¹⁴C in different positions. It was found that cell-free extracts degraded glutamate to a mixture mainly of ammonia, acetate, CO₂, H₂ and minor amounts of mesaconate, citramalate and pyruvate (Barker 1956). The identification of mesaconate (Wachsmann, 1956) was a very significant finding because it indicated the formation of a branched C₅-dicarboxylic acid from the straight-chain glutamate, and also because mesaconate was found to be degraded as rapidly as glutamate by these cell-free extracts to CO₂, H₂, pyruvate and acetate. Subsequently it was found that glutamate gave rise to β-methylaspartate which was then deaminated to mesaconate by a specific enzyme. This branching reaction is quite unusual in that it is one of the earlier examples of a reaction requiring pseudo-vitamin B₁₂ coenzyme (Barker et al. 1957). The degradation of mesaconate was shown to proceed via dextrorotatory citramalate to pyruvate and acetate. The sequence of reactions involved in this route is shown in Fig.3.

There is a great deal of information available regarding the enzymes catalysing the different reactions of this pathway. The

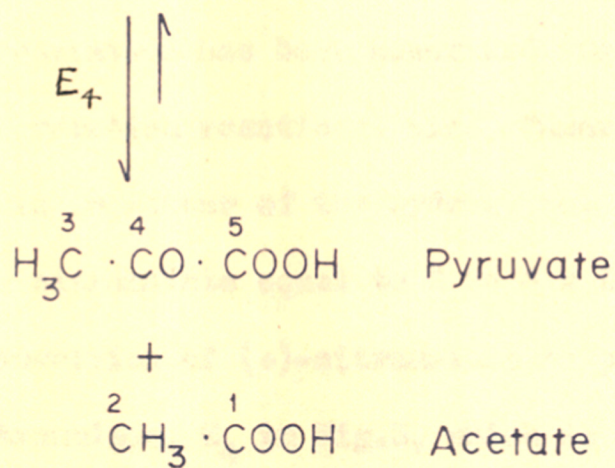
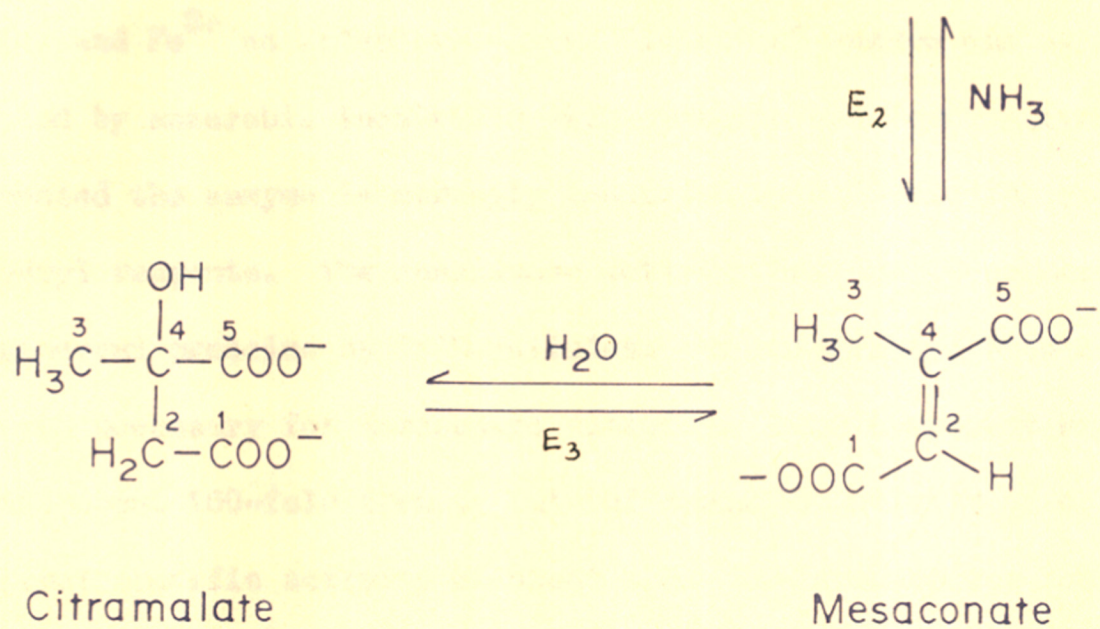
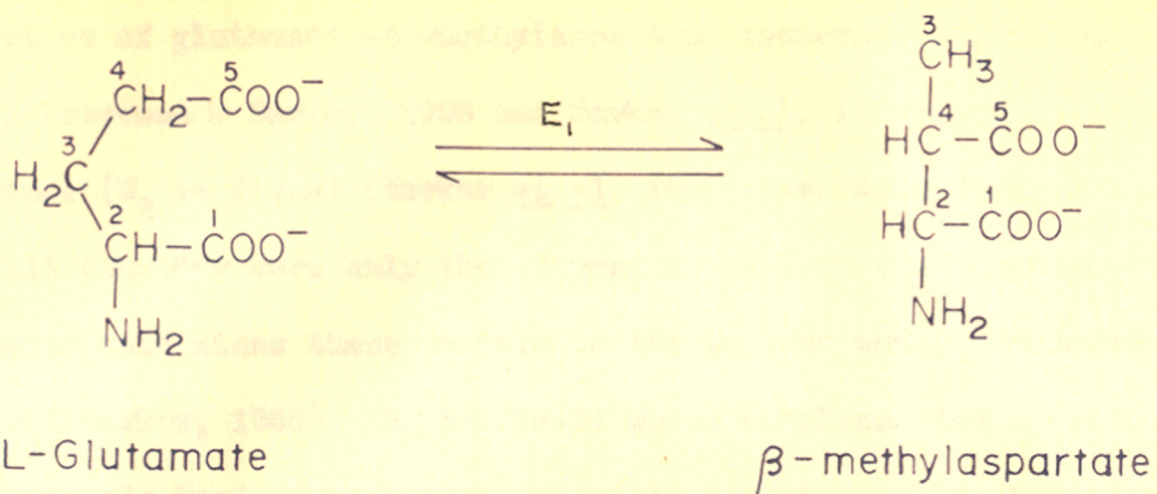


FIG. 3. PATHWAY OF GLUTAMATE FERMENTATION IN CLOSTRIDIUM TETANOMORPHUM (BARKER, 1956 and 1961)

properties of glutamate- β -methylaspartate isomerase (E_1 in Fig.3) (Munch-Peterson & Barker, 1958 and Barker et al. 1958) and β -methylaspartase (E_2 in Fig.3) (Barker et al. 1959) have been described but we shall consider here only the enzymes catalyzing the degradation of mesaconic acid since these pertain to the present work. Mesaconase (Blair & Barker, 1966) (E_3 in Fig.3) which catalyses the hydration of the double bond of mesaconate to yield (+)-citramalate requires cysteine and Fe^{2+} as cofactors. Aged "inactive" enzyme can be activated by anaerobic incubation with cysteine and ferrous iron. As expected the enzyme is strongly inhibited by Fe^{2+} -binding and sulphhydryl reagents. The mesaconase activity has been separated into two component proteins by DEAE-cellulose chromatography: both components are necessary for mesaconase activity. Each component has been purified about 100-fold from an initial specific activity of 6 to 7 to a final specific activity of about 600. The product has been characterized as (+)-citramalic acid and an enzymatic method for its preparation has been described (Barker & Blair, 1962). As in other hydration reactions, viz., fumarase and aconitase, the equilibrium is in favour of the hydroxy acid ($K \simeq 8$ and mesaconase has a K_m for citramalate equal to 2 to 4 $\times 10^{-3}M$ (Blair & Barker, 1966). The conversion of (+)-citramalate to pyruvate and acetate is mediated by citramalase, E_4 in Fig.3, which is quite specific for the dextro-rotatory isomer of citramalate. The enzyme is highly active but is unstable and loses all activity in 1 hr at 37°. It requires Mg^{2+} for activity and the equilibrium constant of the reaction is about 7 (Barker, 1961).

Mesaconase has also been shown to be present in Pseudomonas fluorescens 3081 grown on itaconate (Katsuki et al. 1961); the enzyme

has been purified 8-fold (initial specific activity 0.3 and final specific activity 2.35). Its properties are similar to those of the C.tetanomorphum enzyme in requiring ferrous ions and free enzyme sulphhydryls for activity. The pH optimum for activity is 8.2 and K_m for citramalate is $5 \times 10^{-4} M$. The equilibrium constant of the reaction is about 7. An unusual finding is the complete inhibition of the activity by cysteine and not by other sulphhydryl compounds which has not been explained.

This route of degradation does not involve activation (with ATP and CoA) of any of the intermediates.

(3) The Activated Intermediates Pathway: Lardy-Kornberg route

In contrast to the above two pathways, this route of degradation involves an initial activation of the acids to their acyl-CoA derivatives which are then metabolized. This pathway of degradation has been established from studies on liver mitochondria from rat and guinea pig (Adler et al. 1957; Wang et al. 1961) and on aerobic bacteria particularly the pseudomonads (Cooper and Kornberg, 1964).

Liver mitochondria and their soluble extracts were found to convert itaconate rapidly to pyruvate and acetyl-CoA: Mesaconate was oxidized only one-eighth and methylsuccinate one-sixth as fast as itaconate by mitochondria; citramalate is not oxidized. Soluble extracts of acetone powder acted on itaconate, citraconate or mesaconate in the presence of ATP, CoA and Mg^{2+} to form acetate, pyruvate, lactate, malate, α -ketoglutarate, glutamate and CO_2 . The major product of itaconate metabolism by soluble extracts in the presence of ATP, CoA and Mg^{2+} was found to be glutamate. C^{14} -mesaconate under

these conditions gave rise to glutamate and itaconate. ^{14}C -Citrate also yielded similar products but the conversion was much slower. Itaconyl-CoA was shown earlier (1957) to yield pyruvate and acetyl-CoA although the intermediates were not established.

The extracts fixed $^{14}\text{CO}_2$ in the presence of itaconyl-CoA, or of itaconate when supplemented with ATP, CoA and Mg^{2+} . This CO_2 fixation was found to occur after the cleavage of itaconate to pyruvate and acetyl CoA; pyruvate was first carboxylated to oxaloacetate, then with acetyl-CoA formed citrate and finally glutamate through α -ketoglutarate. Later (1961) it was established that citramalyl-CoA was the substrate of this cleavage reaction. For there was no lag in CO_2 fixation with citramalyl-CoA-whereas both itaconyl-CoA and mesaconyl-CoA showed a lag.

The different reactions have been systematically investigated. (i) Activating system: It was found that the succinate-activating enzyme (P enzyme) activates itaconate and mesaconate but not citramalate in the presence of ATP, CoA and Mg^{2+} .

(ii) Hydration: A fractionation of the mitochondrial extracts revealed that methylglutaconase catalyzes the hydration of the acyl derivatives of the unsaturated acids to citramalyl-CoA.

(iii) Cleavage: The cleavage enzyme is apparently different from that for the cleavage of β -hydroxy- β -methylglutaryl-CoA on the basis of Mg^{2+} requirement and stability. The cleavage was independent of Mg^{2+} and reversibility could not be demonstrated. The following sequence of reactions has been proposed on the basis of the above results (Fig. 4a).

Itaconate

Mesaconate

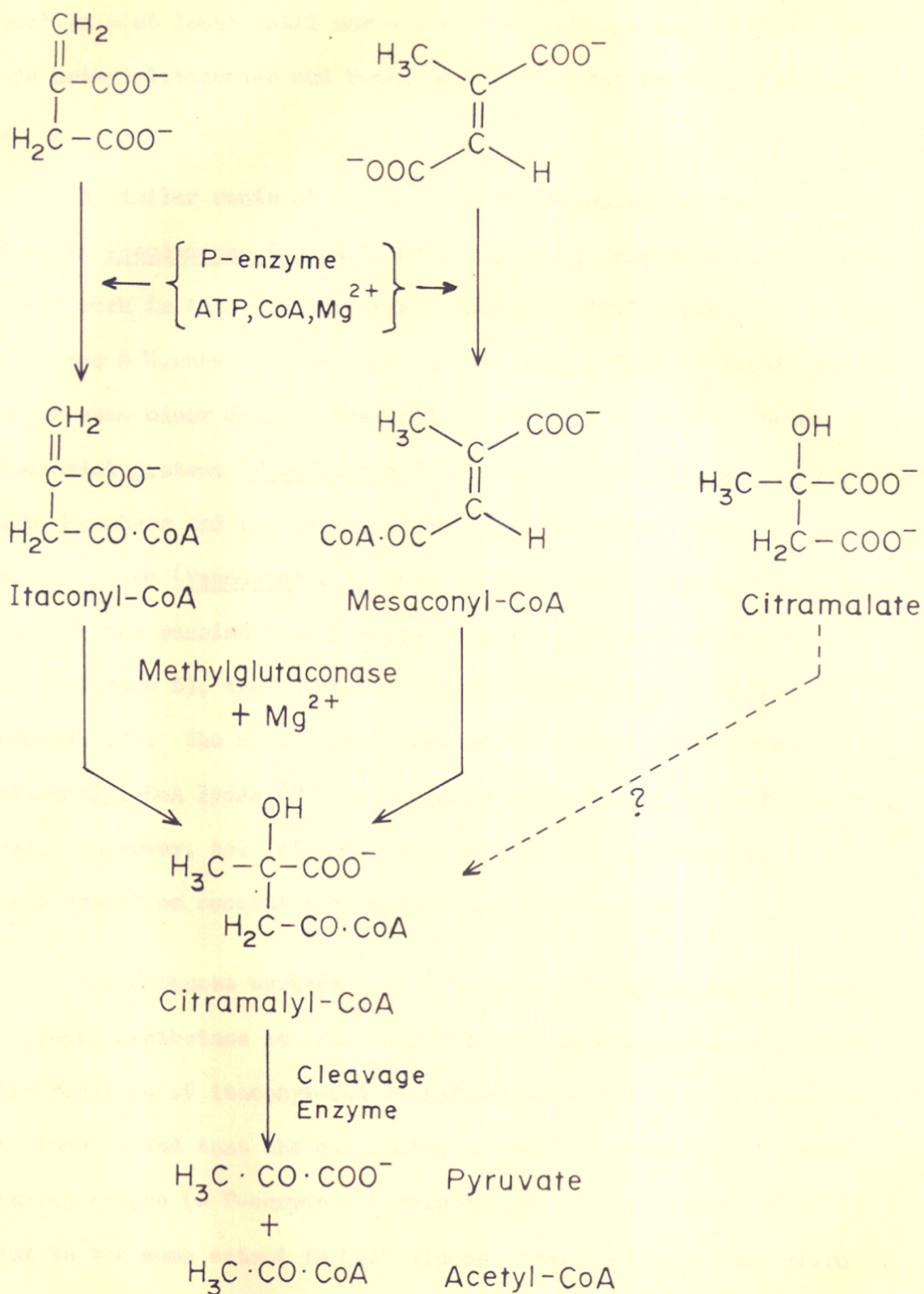


FIG. 4a. DEGRADATION OF ITACONATE, MESACONATE, AND CITRAMALATE IN THE LIVER MITOCHONDRIA OF RAT AND GUINEA PIG (WANG *et al.*, 1961)

It should be noticed that only the cleavage enzyme seems to be specific - at least until now - for this pathway: the other enzymes such as methylglutaconase and P-enzyme have broader substrate specificity.

A similar route of degradation of itaconate has been characterized in Pseudomonas B₂ aba by Kornberg et al. who have carried out extensive work in this area (Cooper & Kornberg, 1962; Grey & Kornberg, 1960; Cooper & Kornberg, 1964) and in Pseudomonas 3081 by Nagai (1963). There are some minor differences in the itaconate metabolism between the bacterial systems (Pseudomonas B₂ aba and a Micrococcus sp.) investigated by Kornberg and the liver mitochondria described above. The bacterial system (Pseudomonas B₂ aba) oxidizes free citramalate. In addition to the succinate-activating enzyme (= P-enzyme = itaconyl-CoA-synthetase = D), the bacterial systems also contain itaconyl-CoA transferase (C). The bacterial hydratase, itaconyl-CoA hydratase (A) and citramalyl-CoA lyase (B) both require Mg^{2+} , unlike the mitochondrial systems. Moreover, both of these enzymes are induced enzymes; in bacteria growth on succinate represses their formation.

The Japanese workers have also come to similar conclusions. Succinyl-CoA synthetase is identical with the itaconyl-CoA synthetase but the presence of itaconyl-CoA transferase was not tested. However, Nagai (1963) found that the citramalate CoA-transferase and itaconate activating enzyme (= P-enzyme = succinate activating enzyme) were present to the same extent in both glucose-grown and itaconate-grown cells and that only citramalyl-CoA lyase was an induced enzyme. These results with bacterial systems are schematically represented in Fig. 4(b).

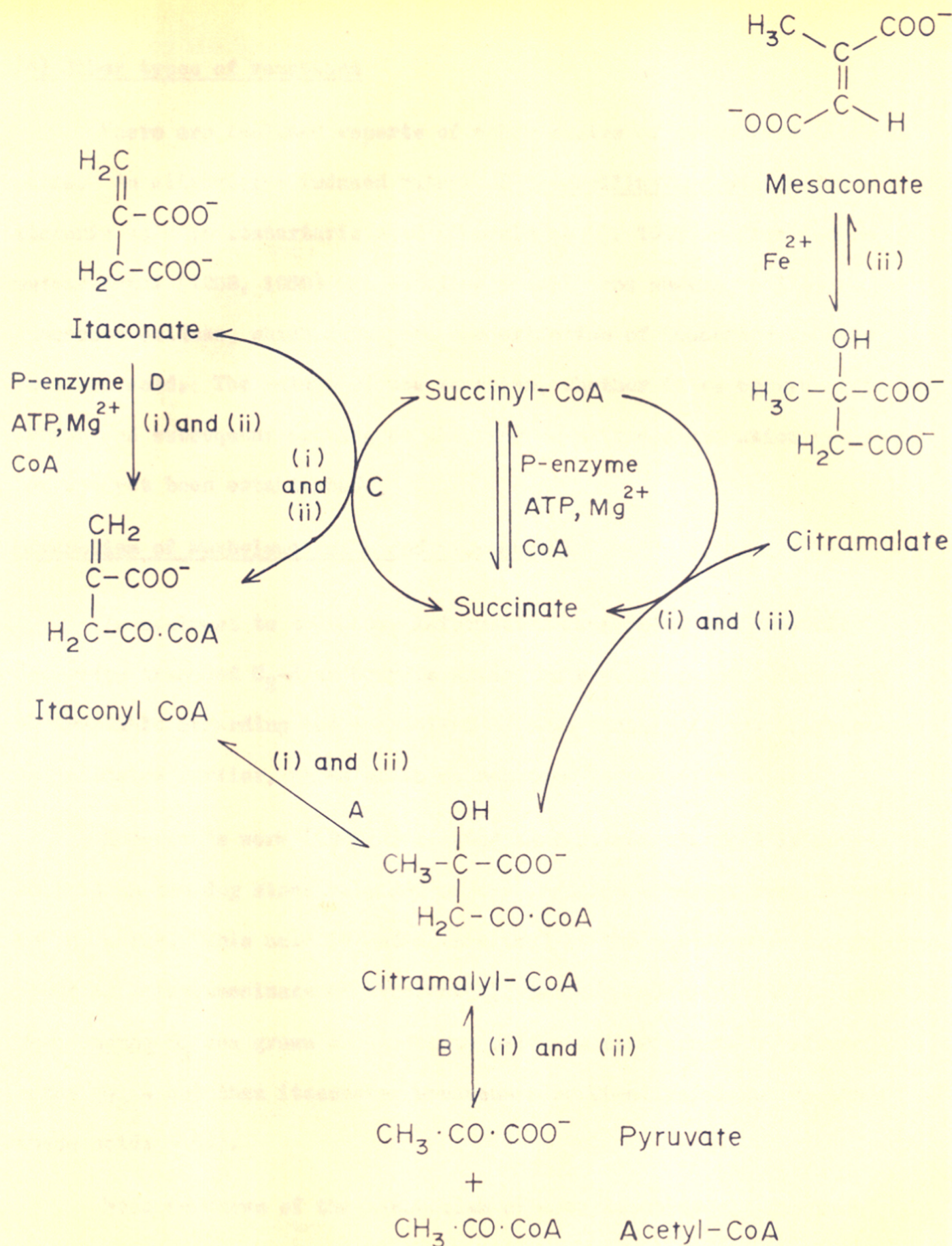


FIG. 4b. PATHWAYS OF DEGRADATION OF ITACONATE, MESACONATE AND CITRAMALATE IN PSEUDOMONADS

(i) ACCORDING TO COOPER AND KORNBERG (1962, 1964). THE ENZYMES A, B, C and D ARE DESCRIBED IN THE TEXT. (ii) ACCORDING TO NAGAI (1963)

(4) Other types of reactions

There are isolated reports of other routes of metabolism of these acids. An ultraviolet induced mutant of Aspergillus terreus oxidizes itaconic acid to itatartaric acid (Stodola et al. 1945). From such a mutant Arpai (1958, 1959) has obtained a cell-free enzyme, called itaconate oxidase, which catalyzes the oxidation of itaconate to itatartaric acid. The nature of the reaction, whether it is epoxide formation and subsequent opening of the ring or a true oxygenation reaction, has not yet been established.

Metabolism of Methylsuccinate and Citraconate

In contrast to the above information concerning the naturally occurring branched C₅-dicarboxylic acids, only preliminary information is available regarding the metabolism of methylsuccinate and citraconate. As indicated earlier, these acids do not seem to occur in nature.

Emmerich's work (1939) indicated that citraconic acid is metabolized in the dog since only 28% of the ingested material was recovered in the urine. This acid is not metabolized by the pseudomonas strain grown on methylsuccinate or itaconate (Brightman & Martin, 1961). However, Pseudomonas B₂ aba grown on itaconate oxidizes citraconate at slower rates ($Q_{O_2} = 50$) than itaconate, mesaconate or DL-citramalate (Q_{O_2} for these acids = 94).

More is known of the metabolism of methylsuccinate. Emmerich (1939) showed that dogs fed with methylsuccinate excreted mesaconic acid in the urine. Crude succinate dehydrogenase preparations isolated from horse heart (Franke & Siewert, 1944), guinea pig liver mitochondria (Adler et al. 1957) and resting cells of itaconate-grown Pseudomonas sp. (Katsuki et al. 1963; Brightman & Martin, 1961) oxidise methylsuccinate.

In a study on the stereospecificity of succinate dehydrogenase, Gawron *et al.* (1962) found that only (-)-methylsuccinate was oxidized and the product was mesaconate. In a preliminary note, Katsuki *et al.* (1963), reported that the oxygen uptake and CO_2 formed corresponded to 56 per cent degradation of the DL-methylsuccinate added. However, with cell-free extracts of itaconate-grown cells, it was found that about 96 per cent of the added DL-methylsuccinate- C^{14} disappears and that recovery of C^{14} in the products other than methylsuccinate was 83 per cent. This clearly shows that both (+)- and (-)-isomers of methylsuccinate are metabolized. The glucose-grown cells also contained a significant amount of enzymes for the metabolism of methylsuccinate except the cleavage enzyme. For, on addition of the cleavage enzyme to a cell-free extract of glucose-grown cells, nearly 35 per cent of the radioactivity of added methylsuccinate appears in acetate and CO_2 . On the basis of these results, they proposed a degradative route for DL-methylsuccinate (Fig. 5).

This scheme is based upon the label distribution in products. Neither mesaconate nor citramalate has been trapped as an intermediate nor have the enzymes catalyzing the reactions been individually characterized.

The present status of our knowledge of the mechanism of the catabolism of these acids may, therefore, be briefly summarized as follows:

1. The most reduced of the branched C_5 -dicarboxylic acids, methylsuccinic acid, is oxidized by mammalian liver mitochondria and crude succinoxidase preparations from horse heart. Apparently only (-)-methylsuccinate is oxidized by these animal preparations and the product is mesaconic acid. Itaconate-grown bacteria oxidize both (+)- and (-)-isomers of methylsuccinate and the product has been assumed, without

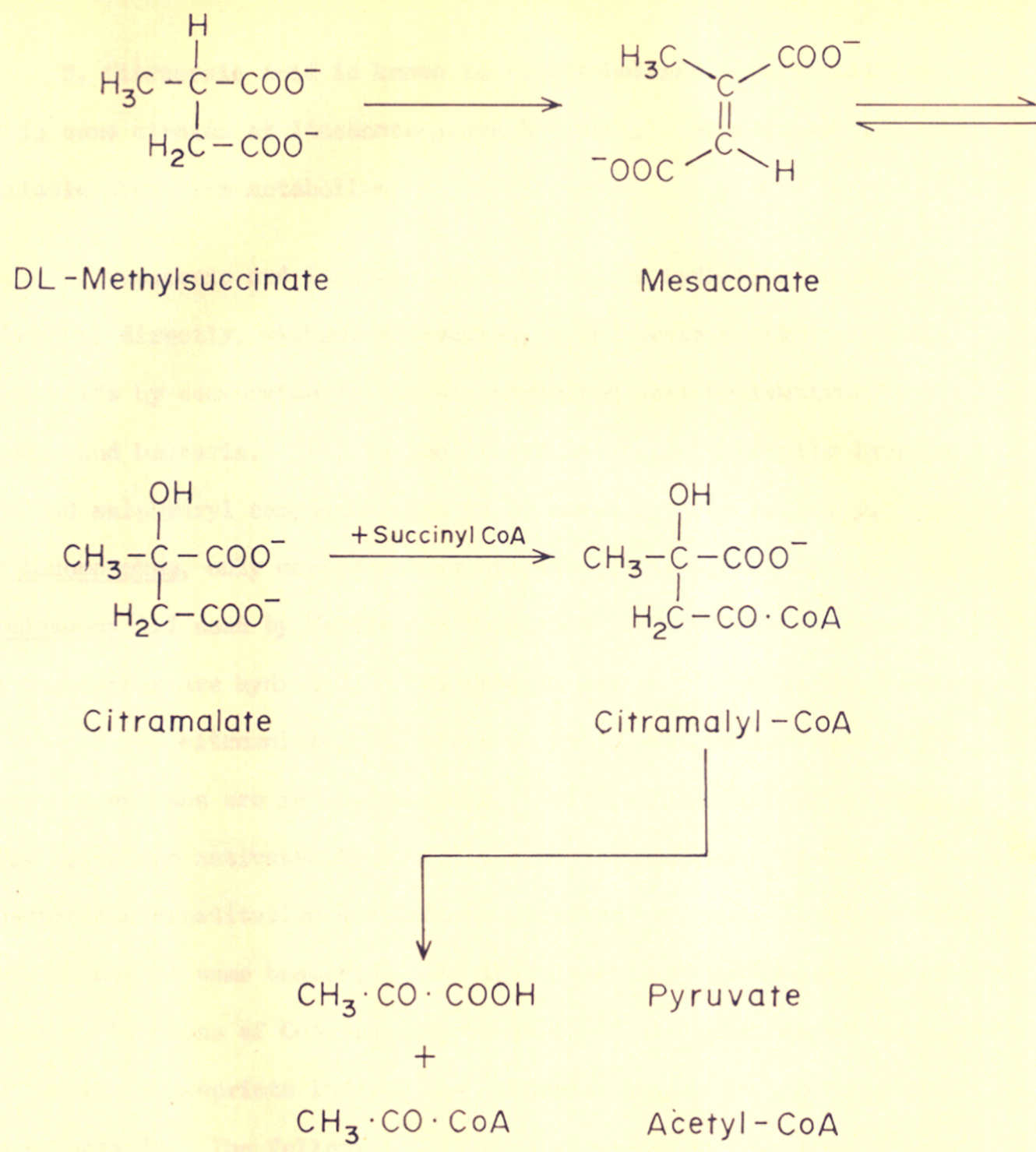


FIG.5. REACTION SEQUENCE IN THE DEGRADATION OF DL-METHYLSUCCINATE, PROPOSED BY KATSUKI et al. (1963)

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experimental evidence to be mesaconate.

2. Citraconic acid is known to be metabolized both in animals and in some strains of itaconate-grown bacteria. No further data are available about its metabolism.

3. Mesaconic and itaconic acids undergo a hydration reaction either (i) directly, without activation, by bacteria or (ii) as activated acids by conversion to the corresponding acyl derivatives by animals and bacteria. (i) In the direct hydration generally ferrous ions and sulphhydryl compounds seem to be necessary for activity. In C. tetanomorphum, only mesaconate is hydrated to (+)-citramalate. In Pseudomonas 111 used by Martin and Brightman (1961), both itaconate and mesaconate are hydrated. The product has not been characterized as (+)- or (-)-citramalate, but seems to be (+)-citramalic acid. The hydrating enzymes are induced enzymes. (ii) In the second pathway, these acids are activated by two different mechanisms. One is by the non-specific constitutive succinate-activating enzyme as in liver mitochondria and in some bacteria. And the alternative mechanism of activation is by means of CoA-transfer reaction in the presence of succinyl-CoA and the appropriate induced Co-A transferase, as in the case of other bacteria. The following well characterized enzymes seem to be generally inducible in bacteria: citramalate CoA-transferase and citramalyl-CoA lyase. In liver mitochondria, β -methylglutaconase catalyzes the hydration of the itaconyl- and mesaconyl-CoA to citramalyl-CoA.

4. The final reaction characteristic of these pathways is the cleavage reaction. Citramalyl-CoA yields acetyl-CoA and pyruvate. Free citramalate however can give rise to either acetate and pyruvate

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or a succinate-type compound and CO_2 . The cleavage enzyme shows different properties in different systems. C.tetanomorphum enzyme acts on free citramalate and requires Mg^{2+} . In pseudomonads generally and in a Micrococcus isolate the cleaving enzymes act on citramalyl-CoA apparently on the (+)-isomer only. The mechanism of the formation of succinate-type of compound is obscure.

5. The biosynthesis of the different acids may be briefly reviewed. Itaconic acid is synthesized by A.terreus. Its formation proceeds through the decarboxylation of cis-aconitic acid (Bentley & Thiessen, 1957). Some of the observations of Lal and Bhargava (1962) on the reversal of fluoride inhibition of this fermentation by pyruvate have led them to postulate a different mechanism of cis-aconitate formation, which needs to be experimentally substantiated. Citramalate is formed from glutamate in C.tetanomorphum or by photoassimilation of acetate by a reversal of the glutamate pathway in Chromatium (Losada et al. 1960) or by Rhodopseudomonas rubrum (Glover et al. 1952). Mesaconate is also formed from glutamate in C.tetanomorphum, but its origin in plants where it occurs in significant quantities is not clear.

6. Metabolism of aconate in a Micrococcus sp. has been studied and its mechanism established to be similar to the succinate pathway.

7. Itatartaric acid is formed by the oxygenation of itaconic acid in a UV-mutant of A.terreus.

8. The metabolism of the other acids, paraconic, itamalic, etc. has not been studied.

We shall now briefly (i) discuss why the present work described in this thesis was undertaken and (ii) state briefly the experiments which have been carried out and the conclusions arrived at.

PRESENT INVESTIGATIONS

The work reported here arose from the previous investigations on a cis-trans isomerase (aconitate isomerase) (Rao and Altekari, 1961) and tricarballoylate dehydrogenase (Rao and Altekari, 1962) which resembles but is distinct from succinate dehydrogenase. Other cis-trans isomerases, such as maleylacetoacetate-fumarylacetoacetate isomerase (Edward & Knox, 1955, 1956), maleylpyruvate-fumarylpyruvate isomerase (Lack, 1961) and maleate-fumarate isomerase (Otsuka, 1961) are known. Succinate dehydrogenase-type enzymes are also known: tricarballoylate dehydrogenase (Rao & Altekari, 1962) and butyryl-CoA dehydrogenase. But there are not many such systems known. This work originated with the objective of finding and characterizing such systems.

The obvious choice for the two types of reactions was the citraconate-mesaconate pair and the dehydrogenation of methylsuccinate. Citraconate was expected to isomerize to mesaconate and then undergo degradation through one of the routes mentioned earlier. (-)-Methylsuccinate is dehydrogenated to mesaconate by the animal succinate dehydrogenase system (Gawron et al. 1962): the fate of (+)-methylsuccinate is not known. It is reported that only 56 per cent of DL-methylsuccinate is oxidized by itaconate-grown cells although cell-free extracts oxidize both D- and L-isomers (Katsuki et al. 1963).

The most important part of the findings reported in the following pages are the discovery of citraconase, a new enzyme catalyzing the hydration of citraconate to laevorotatory citramalate, and studies on its purification, its properties, application and the mechanism of the hydration reaction. All of the significant results are briefly summarized below.

1. Experiments on the oxidation of the various branched C₅-dicarboxylic acids by different strains of bacteria

(isolated by the enrichment culture technique) have provided interesting results. DL-Methylsuccinate-grown cells oxidize all of these substrates (citrate, mesaconate, itaconate, (+)- and (-)-citramalate), whereas citrate-grown cells oxidize readily citrate and (+)- and (-)-citramalate.

2. Experiments on methylsuccinate oxidation have revealed that in contrast to the animal system bacterial succinate dehydrogenase system is quite distinct from the methylsuccinate dehydrogenase complex. The bacterial succinate dehydrogenase system is inert towards methylsuccinate. Preliminary data indicate that methylsuccinate dehydrogenase dehydrogenates (+)-methylsuccinate to citrate and the (-)-isomer to mesaconate. These systems are particulate and preliminary attempts to solubilize them were not successful. It is not clear whether there are two distinct dehydrogenases or whether a single non-specific enzyme attacks both isomers.

3. Citrate isomerase is a new enzyme catalyzing the hydration of citrate to (-)-citramalate. The major part of the work described here pertains to the studies on this enzyme: its characterization, purification, properties and mechanism of the hydration reaction using D₂O and its use in the preparation of (-)-citramalate. The cell-free extracts of citrate-grown cells also contain other hydratases such as maleate, aconitase, fumarate and mesaconate. The purified enzyme is free from mesaconate and maleate, and is shown to be different from the other two hydratases. Citrate isomerase requires both free sulphhydryls and Fe⁺⁺ ions for activity, as does mesaconate. Mesaconate activity in the cell-free extracts of bacteria used here is irreversibly lost by dialysis and

catalyses the formation of (+)-citramalate from mesaconate. Citramalate produced by hydration of citraconate is a 1:1 mixture of (+)- and (-)-citramalate. Citraconase activity is stable to dialysis and leads to (-)-citramalate which is not attacked by peroxidase. Citraconate is converted to citramalate from citraconate.

A study of the mechanism of enzymatic hydration of citraconate in presence of D_2O and employing the NMR spectral analysis - and comparing it with deuterated malate obtained from maleate and fumarate, has revealed that the addition of water takes place by a trans- mechanism.

4. Itaconase: It is established that itaconase of Pseudomonas 111 is distinct from mesaconase and leads to the formation of (+)-citramalate from itaconate. The cell-free extracts of Pseudomonas 111 grown on itaconate catalyzed the hydration of only itaconate and not of mesaconate. This result is in variance with that of Brightman and Martin (1961), although the bacterial strain used is presumably the same.

5. Degradation of citramalate. Attempts to elucidate the pathway of degradation of citramalate have not been successful. There is presumptive evidence that α -hydroxyglutarate is formed from citramalate. However, more intensive work must be done before any conclusions can be drawn regarding the degradation of citramalate in this bacterium.

6. Malease was first reported to be present in corn kernels (Sacks & Jensen, 1951); the product was not rigorously characterized as (+)- or (-)-malate. It was assumed to be the natural L(-)-form (Crombie, 1960) since it was oxidized in the presence of enzyme preparation from corn kernel, triphenyltetrazolium chloride and diaphorase.

Malate produced by malease of citraconate cells is a dextrorotatory compound which is not attacked by pig heart malate dehydrogenase.

The citraconate-organism however does not grow on maleate nor metabolize it beyond malate. As far as we know, this is the first report of the existence of malease in bacteria.

CHAPTER II

METHODS AND MATERIALS

METHODS AND MATERIALS

I. Bacteriological

(i) Isolation of bacteria: Five bacterial isolates were obtained from garden soil by the conventional enrichment culture technique (Stanier et al. 1963) with one of the following five acids as a sole carbon source; potassium salt of DL-methylsuccinate, mesaconate, citraconate, itaconate and maleate. The details of the isolation procedure and of the properties of the isolates are described in the chapter on "Experimental" procedure.

(ii) Maintenance of the organism: All the organisms were maintained by monthly subculture on nutrient agar slopes. After growth for 24 hr on nutrient agar slants, the cultures could be preserved for a period upto 2 years under a layer of sterile paraffin oil and storage at 5°. The cultures were routinely allowed to grow on nutrient agar slopes for 24 hr at 30° and then stored at 3 to 5°. The citraconate culture grows well and produces a fluorescent pigment on agar slopes of synthetic medium containing citraconate (composition in "Experimental"), provided "Algadan" (Danish) agar and not BDH or Difco agar was used. All of these cultures have been deposited with the National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona.

(iii) "Large Scale" growth of cells:

The medium for the growth of cells contained per litre,

Carbon source (organic acid)	5 g (pH to 7 with KOH)
NH_4NO_3	2 g
KH_2PO_4	10 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	10 mg

The phosphate solution was adjusted to pH 7, sterilized separately and added to the rest of the solution just before inoculation. Sterilization was carried out by autoclaving at 121° for 15 min.

The inoculum was built up as follows. The cells from one agar slant (not more than 15 days old) were inoculated into 125 ml of the medium in a 500 ml Erlenmeyer flask which was then incubated at 30° on a rotary shaker. After 18-20 hr growth 1.5 ml aliquots were inoculated into 125 ml medium in each of several flasks, and growth allowed to proceed as before for 12 to 15 hr (the end of the lag phase). The fermented broth was then pooled and crushed ice added to it. The cells were separated by centrifugation in a Sharples supercentrifuge and washed with 500 ml ice cold distilled water. They were used as such or stored at -15° until required.

Growth was measured by turbidity reading of cell suspensions at 660 m μ in a Klett Summerson colorimeter. The reading generally was 350 m μ at the end of 12 to 15 hr growth and corresponded to about 2 mg dry cells per ml. Dry weight of cell suspensions was determined by comparing the turbidity of such suspensions against a standard curve [of growth (dry wt.) vs. turbidity] prepared earlier. The yield of wet packed

cells was about 10 g per litre of the growth medium.

(iv) Cells for manometry: The cells for manometric experiments were grown in a mineral medium containing 0.05 M potassium phosphate buffer (pH 7) and 0.3 per cent (w/v) organic acid (neutralized with KOH). The methylsuccinate-culture was grown in a medium consisting of 0.3 per cent (w/v) methylsuccinate, 0.1 per cent (w/v) beef extract (Difco) and 0.2 per cent (w/v) peptone and the mineral salts including ammonium nitrate. The cells were harvested at three-fourths stage of the log phase by centrifugation at 2000 x g for 15 min at 5°, washed once with cold 0.05 potassium phosphate buffer and suspended in this buffer to a turbidity of 350 in the Klett at 660 m μ (approximately 2 mg dry wt.) per ml.

Lyophilized cells were prepared as follows. A thick suspension of cells (1 g wet cells in 5 ml water) was frozen at -15° and dried from the frozen state in a Stoke's Lyophilizer. These dried cells were not stored but were used immediately for experiments.

(v) Manometry: Oxidation of substrates by cells was followed by the conventional manometric techniques at 28° using air as gas phase. Each Warburg vessel contained 1 to 3 mg resting (non-proliferating) cells or 10 mg of lyophilized cells, and 150 μ moles of potassium phosphate buffer (pH 7) in the main compartment, 10 to 30 μ moles substrate in the side arm, 0.2 ml of 20 per cent potassium hydroxide in the centre well and total volume 3 ml. When chloramphenicol was added, its final concentration was 50 μ g per ml. Q_{O_2} represents the rate of oxidation: it is expressed as μ l of oxygen taken up per mg dry cells per hour.

II. ENZYMATIC

(i) Preparation of cell-free extracts: Cell-free extracts were prepared by subjecting a dense suspension of cells (5 g wet packed cells equivalent to 1 g dry cells) in 50 ml cold 0.02 M potassium phosphate buffer (pH 7) to sonic oscillation in a Raytheon Magnetostriction Sonic Oscillator (Model DF 101, 250 Watts, 10 KC) for 15 min at 5° operating at a current output of 1.1 to 1.2 A. During this period at least 90 to 95 per cent of cells were ruptured. The sonicate was centrifuged at 5° at 10,000 x g for 20 min. The supernatant containing 8 to 10 mg protein per ml was used as such or treated further as described under "Experimental" procedures.

(ii) 100,000 x g particles and "soluble" supernatant:

The cell-free extracts were centrifuged at 0° to 5° in a Spinco Model L ultracentrifuge at 100,000 x g (Rotor 40 and 39,000 r.p.m.) for 1 hr. The particles which settled down were washed once by homogenizing them in 0.05 M potassium phosphate buffer (pH 7) and recentrifuged. They were then suspended in the same buffer to a volume corresponding to that of the original cell extract.

(iii) Assay of hydratases:

(a) Citraconase (citraconate hydro-lyase or citraconate hydratase).

The procedure is fully described in the Section on the purification of citraconase [Chapter III, Section 3(iii)].

(b) Malease (Maleate hydratase, maleate hydro-lyase)

Maleate hydratase is assayed by a procedure similar to that of citraconase. Ferrous ions were not added but activation was done

with a sulphhydryl compound such as cysteine, reduced glutathione or mercaptoethanol. The reaction mixture contained in 1 ml; maleate, 5 μ moles; cysteine, 2 μ moles; Tris buffer (pH 7.5), 100 μ moles and enzyme. It was incubated at 30° for 10 min. The reaction was stopped by the addition of 1 ml of 0.66 N perchloric acid and the precipitated protein centrifuged off. The optical density of the supernatant was read at 240 m μ .

(c) Mesaconase (Mesaconate hydro-lyase, Mesaconate hydratase) was determined by the procedure of Blair and Barker (1966).

(d) Aconitase was measured by estimating the quantity of citrate formed from cis-aconitate according to Dickman and Cloutier (1951).

(e) Fumarase was assayed by the procedure of Racker (1950).

(iv) Dehydrogenases:

(a) Isocitrate dehydrogenase and malate dehydrogenase were determined according to Ochoa (1948) and Mehler et al. (1948) respectively.

(b) Succinate dehydrogenase activity was followed by the reduction of 2,6-dichlorophenolindophenol according to Bonner (1955). A modification introduced was the addition of 300 μ g phenazine methosulphate per ml of reaction mixture. This was found to enhance the rates of reduction.

Methylsuccinate dehydrogenase and tricarballic dehydrogenase activities were assayed by the above modified procedure using DL-, (+)- or (-)-methylsuccinate and tricarballic as substrates.

Lactate dehydrogenase was prepared from rabbit muscle (Kornberg, 1955).

(v) Tests for known reactions of citramalate

The detailed protocols and procedure are given in the section on "Experimental - citramalate degradation".

(a) Activation of the acids:

The activation system was the same as that described by Cooper and Kornberg (1964). It contained the citraconate-grown cell extract, Mg^{++} , succinate, ATP, CoA and freshly neutralized hydroxylamine to trap any acyl compound.

(b) Cleavage Activity:

This activity was tested under two sets of conditions: without activation and under conditions conducive to activation (i.e., in presence of ATP, CoA and succinate). The presence of α -ketoacids in the reaction mixture was tested for by means of the procedure of Friedemann and Haugen (1943) and by paper chromatography of the 2,4-dinitrophenyl hydrazones (El Hawary and Thompson, 1953).

III. CHEMICAL

Reagents and Chemicals

The following chemicals were obtained from sources mentioned: NAD, ATP, CoA, PCMB, phenazine methosulphate, FAD, FMN, and α -keto-glutaric acid from the Sigma Chemical Co., streptomycin sulphate from the Hindustan Antibiotics Ltd., Pimpri; DEAE-cellulose (Cellex-D, 0.62 m eq./g) from Bio-Rad Laboratories, sodium pyruvate from E. Merck & Co., Darmstadt; ascorbic acid prepared in this Laboratory; EDTA and o-phenanthroline from B.D.H.

Calcium phosphate gel was prepared according to the method described by Swingle and Tiselius (1951). Glass-distilled water was

used for the preparation of all solutions required for enzyme work.

Itaconic, citraconic and mesaconic acids were prepared from citric acid according to the procedure of Shriner et al. (1943). DL-Citramalic acid was prepared according to the procedure of Michael and Tissot (1892) as modified by Barker (1962). Sodium salts of (+)- and (-)-isomers of citramalic acid were prepared by a procedure developed in this Laboratory and described in a later section [Chapter III, Section 3(ii)]⁷. DL-Methylsuccinic acid was prepared by the catalytic hydrogenation of a 10 per cent (w/v) solution of itaconic acid in 95 per cent alcohol (v/v) under pressure (about 3 kg/cm²) and Pd on charcoal (0.5 g Pd per mole of itaconic acid) (Allen et al. 1939).

α - and β -hydroxyglutaric acids were prepared according to Ingold and Nicols (1922) and Pechman and Jenisch (1891) respectively. β -Methylmalic acid was prepared as described by Sherp (1946). None of these hydroxy acids except β -hydroxyglutaric acid crystallized well. They were all converted into their sodium salts and stored in the cold. All were chromatographically pure; even when 10-15 μ moles were applied to paper and chromatographed, only one large well-defined spot appeared.

The organic acids were purified by ion exchange chromatography on Dowex-1x8 (formate) columns as described by Busch et al. (1952). The p-bromophenacyl esters of the organic acids were prepared according to Kelly and Kleff (1932).

The methylesters of the acids were prepared as follows: 10 ml of a 50 per cent potassium hydroxide (w/v) solution in a boiling tube were chilled in ice bath. 25 ml of ether were added to this and cooled to 3 to 5°. Small quantities (10-20 mg) of nitrosomethylurea were

added to this tube successively after solution. The deep yellow diazomethane solution in ether was then poured out into another cold tube and dried with solid potassium hydroxide in cold. The solution was gradually added to a cold ethereal solution (5 to 10 ml) of the acid (50 mg) containing 0.5 ml absolute methanol to facilitate solution of the acid and left overnight in cold; excess of diazomethane must be present. The solution was then evaporated to dryness in a stream of air and the ester was then distilled in a microdistillation unit. The distilled methylesters were used for IR and NMR spectral analyses.

(c) Assays: Citric acid was estimated according to the procedure of Natelson et al. (1948). Samples containing 10 to 100 μ g of citric acid were used and the solutions containing the pentabromoacetone-thiourea complex were read at 430 m μ .

Protein was determined according to two procedures. In unfractionated cell-free extracts (with a high content of nucleic acids, i.e., with a ratio of O.D._{280 m μ} to O.D._{260 m μ} less than 0.7), protein was assayed with the Folin phenol reagent as described by Lowry et al. (1951). Crystalline serum albumin (Armour) served as the standard. The colour developed was measured at 500 m μ . The optical procedure of Warburg and Christian (1941) was used to determine the protein content of purified fractions.

IV. Other Procedures

Optical rotation measurements were made using the Hilger polarimeter with a 1 dm or 5 cm tube. The procedure of Krebs and Eggleston (1943) was used for estimating isocitrate and malate and that of Barker and Blair (1962) was used for citramalate. The optical rotation of the α -hydroxy acid is enhanced by molybdate in the presence of citrate and acetic acid.

Optical rotation was also measured in the absence of these additions.

Paper chromatography (descending) for the identification of organic acids was carried out according to the procedure of Lugg and Overell (1948) using Whatman number 1 filter paper. The solvent systems employed were n-butanol-formic acid-water (4:1.5:1, vol/vol) and ethyl acetate-formic acid and water (10:1:3, vol/vol). The developed and dried chromatograms were first viewed under an ultraviolet chromatolite lamp and the UV-absorbing spots marked on one side. The other side of the chromatogram was sprayed with a 0.04 per cent alcoholic solution of bromophenol blue and the yellow acid spots marked. The 2,4-dinitro-phenylhydrazones were separated and identified by ascending chromatography (El Havery & Thompson, 1953) using Whatman 3 MM paper with solvents mentioned in the section under "Experimental" procedures.

Thin layer chromatography was carried out using glass plates coated with a mixture of silica gel and plaster of Paris (as the binder) to a thickness of about 1 mm. The chromatograms were developed with the appropriate solvent systems mentioned in "Experimental" and visualized by viewing under an ultraviolet chromatolite lamp by spraying sulphuric acid and heating.

DEAE-Cellulose chromatography of proteins was performed according to the procedure of Peterson and Sober (1962). Fractions were collected using a Technicon Automatic Fraction Collector.

Measurements of pH were made with a glass and calomel electrode on the Beckman model G pH meter or the Toshniwal (Polymetron) pH meter. Precipitates of proteins obtained by addition of ammonium sulphate were centrifuged down at 5° at 10,000 x g in the International PR-2 and in

the Servall-SS1 centrifuges. The particles present in the cell-free extracts were spun down at 100,000 x g at 0°-3° in the Spinco preparative Model L ultracentrifuge. Protein homogeneity was tested in the Spinco Model E Analytical ultracentrifuge. Optical measurements were made on the Unicam SP-600 spectrophotometer for the visible range and in the Beckman DU spectrophotometer generally for the ultraviolet range. For spectrophotometric readings, 3 ml cuvettes with 1 cm light path were used. The extinction coefficients (cm^2/mole) for different compounds mentioned in the following pages were: NADH and NADPH, 6220 (340 m μ); citraconate 3000 (240 m μ); mesaconate 3850 (240 m μ); Maleate 2000 (240 m μ). The IR spectra were taken in Nujol in the Perkin-Elmer Infracord and the NMR spectra in a Varian (60 MC/s) NMR spectrometer in CCl_4 with trimethylsilane as an internal reference standard.

CHAPTER III

EXPERIMENTAL RESULTS AND DISCUSSION

SECTION 1

ISOLATION AND MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

The bacteria used in the experiments were isolated and identified according to the procedures described below. Four strains of bacteria, capable of growing on citraconate, mesaconate, itaconate and DL-methylsuccinate respectively were selected from among many isolates from enrichment on each acid. The four strains have been used in experiments described in these investigations.

(i) Isolation of the bacteria

Bacteria capable of growing on the respective acid were isolated by the conventional enrichment culture technique (Stanier, 1947). The medium employed for the isolation consisted of the following components per litre of medium.

C_5 -dicarboxylic acid		2 g (neutralized with KOH)
NH_4NO_3		1 g
K_2HPO_4		1 g
$MgSO_4 \cdot 7H_2O$		500 mg
$FeCl_3 \cdot 6H_2O$		10 mg
Agar when used		15 g

The neutralized dicarboxylic acid solution, phosphate solution and the solution containing the rest of the compounds were sterilized (121°, 15 min) separately and then mixed aseptically.

Garden soil (1 g) was suspended in 10 ml water, shaken well for 1 min and allowed to settle for 2 to 3 min. The turbid supernatant was decanted from the sediment and centrifuged at 2000 x g. The supernatant was discarded and the sediment suspended in 5 ml water. 2 ml were used for inoculating 10 ml of the medium which contained the C₅-dicarboxylic acid as the sole carbon source. The inoculated medium was then incubated on a shaker at 30° until there was visible growth (4-5 days). 10 ml of the growth were then aseptically centrifuged, the sedimented bacteria washed with sterile (0.9%) saline and suspended in 10 ml sterile saline. 0.5 ml was inoculated into 10 ml of a fresh medium of the same composition. Growth was allowed to occur as before. After growth (1-2 days), the bacteria were streaked on solid medium. Well isolated and morphologically different colonies were selected. In each enrichment 4 to 5 varieties of bacteria were obtained but only one was saved.

(ii) Morphological and biochemical characteristics

These were tested as mentioned in Table 1.

TABLE 1MORPHOLOGICAL AND BIOCHEMICAL TESTS

Test	Observations			
	Citraconate isolate	Mesaconate isolate	Itaconate isolate	Methylsuccinate isolate
1. Size	1 μ to 2 μ	1 μ to 2 μ	1 μ to 2 μ	1 μ to 2 μ
2. Gram staining (at 8 hr and 24 hr growth)	-ve	-ve	-ve	-ve
3. O ₂ requirement	Aerobic	Aerobic	Aerobic	Aerobic
4. Sporulation	-ve	-ve	-ve	-ve
5. Motility	+	+	+	+
6. Flagella	+			
7. Growth: 25°	++	++	++	++
30°	++	++	++	++
37°	++	++	++	++
45°	-	-	-	-
55°	-	-	-	-
8. Shape	Short rods			
9. Chromogenesis	Greenish diffusible fluorescent pigment			
10. Salt tolerance	5%			
11. Nitrate reduction	+			
12. Hydrolysis of starch	-			

continued

TABLE 1 (contd.)

Test	Observations			
	Citraconate isolate	Mesaconate isolate	Itaconate isolate	Methylsuccinate isolate
13. Gelatin liquefaction	-			
14. Milk	no change			
15. Agar digestion	-			
16. Citrate utilization	+	+	+	+
17. Methyl red	-	-	-	-
18. Vogor-Proskaur test (Acetyl-methyl carbimol)	-	-	-	-
19. Indole	-	-	-	-
20. Peptone water	alkaline	alkaline	alkaline	alkaline
21. Phenol	-	-	-	-
22. Yeast water + 10 per cent ethanol	no growth	no growth	no growth	no growth
23. Glucose	48 hr(acid) no gas			
Fructose	48 hr(no acid) 96 hr(acid) no gas			
Lactose	96 hr(no acid) no gas			

On the basis of the above tests and according to Bergey's Manual (1948) these strains have been identified as belonging to the genus Pseudomonas.

SECTION 2

OXIDATION OF THE BRANCHED C₅-DICARBOXYLIC ACIDS BY DIFFERENT BACTERIAL ISOLATES

The ability of the different bacterial isolates to oxidize different C₅-dicarboxylic acids was studied. Each isolate was grown in medium containing the respective C₅-dicarboxylic acid (as a sole carbon source) on which it was isolated. The procedures for growing cells for manometry, lyophilization and for preparing cell-free extracts have been described in an earlier Section.

Experiments with non-proliferating cells

The ability to oxidize the compounds varies to a significant extent with the age of the cells. In all of these experiments, cells were harvested after three-fourths completion of the log phase of growth. The results are shown in Table 2.

Chloramphenicol was added to the cell suspensions to prevent any induced enzyme formation during the experiment; however the presence of chloramphenicol does not seem to make any difference in the oxidation rates. The citraconate-grown culture oxidizes only citraconate, DL- and (-)-citramalate at high rates whereas it oxidizes methylsuccinate and mesaconate very feebly and has no action on itaconate. All of the other cultures oxidize all substrates except citraconate at similar rates. Citraconate is poorly oxidized. Besides all of them oxidize (+)-citramalate 1.5 to 2 times faster than the (-)-isomer. They also degrade readily both the isomers of methylsuccinate.

TABLE 2

OXIDATION OF C₅-DICARBOXYLATES BY RESTING CELLS OF DIFFERENT CULTURES

Substrate	CMP**	Q _{O₂} values*				
		Culture used				
		Citra- conate	Methyl- succinate	Ita- conate	Mesa- conate on Ita- conate	Mesa- conate
DL-Methylsuccinate	-	5	78	45	-	54
	+	6	79	34	-	50
Citraconate	-	90	18	6	2	5
	+	85	16	10	13	2
Mesaconate	-	7	45	30	110	39
	+	6	48	40	155	34
Itaconate	-	0	66	32	33	33
	+	0	66	38		33
DL-Citramalate	-	92	63	50	136	35
	+	-	58	50	119	30
(+) - Citramalate	-	15	80	45	-	35
	+	-	85	51	-	32
(-) - Citramalate	-	96	50	23	-	22
	+	-	45	18	-	17

* Endogenous values subtracted; they were 16, 15, 16 and 12 respectively.

** CMP: Chloramphenicol, 150 µg per flask.

Experiment with lyophilized cells

In order to find out whether permeability barriers were responsible for the poor oxidation of (i) methylsuccinate, itaconate and mesaconate by citraconate-grown culture, and (ii) of citraconate by the other cultures, an experiment with lyophilized cells was performed. The results are shown in Table 3.

The above results are very similar to those obtained with non-proliferating cells but a considerable fraction of the oxidative ability is lost by lyophilization. Itaconate and mesaconate are oxidized by all cultures except the citraconate isolate. Again all substrates are oxidized by methylsuccinate culture. Citraconate is not oxidized or only feebly oxidized by mesaconate- and itaconate-cultures. The results with the isomers of citramalate are essentially the same as those with resting cells.

The ability of succinate-grown cells to oxidize substrates was tested. The results are indicated in Table 4.

The succinate-grown cells do show some residual oxidative activity towards citramalate; perhaps the very low oxidative activities towards the other substrates are not significant. The cells however are induced, in the presence of the respective substrates, to oxidize the substrates and this induction is inhibited by the simultaneous presence of chloramphenicol (50 µg/ml).

TABLE 3

OXIDATION OF C₅-DICARBOXYLATES (BY LYOPHILIZED CELLS OF)
DIFFERENT CULTURES

Substrate	Q _{O2} values*			
	Culture used			
	Citraconate	Methyl-succinate	Itaconate	Mesaconate
DL-Methylsuccinate	2	20	20	20
Citraconate	26	5	4	8
Mesaconate	3	13	27	21
Itaconate	0	18	27	21
DL-Citramalate	31	19	20	8
(+)-Citramalate	12	13	24	19
(-)-Citramalate	40	8	12	12
Endogenous	6	12	12	14

*Endogenous values subtracted

TABLE 4

OXIDATION OF C₅-DICARBOXYLATES BY SUCCINATE GROWN
RESTING CELLS OF DIFFERENT CULTURES

		[*] Q _{O₂}			
		Cultures			
		Citraconate	Methylsuccinate	Itaconate	Mesaconate
<u>Resting cells</u>					
DL-Methylsuccinate	4	3		1	3
Citraconate	3	3		1	1
Mesaconate	0	2		0	1
Itaconate	0	0		0	0
DL-Citramalate	17	12		9	10
Succinate	173	54		34	250
Endogenous	12	27		16	14
<u>Lyophilized cells</u>					
DL-Methylsuccinate	0	1		2	1
Citraconate	1	0		0	0
Mesaconate	1	0		3	0
Itaconate	0	0		4	0
DL-Citramalate	6	0		4	6
Succinate	100	21		34	10
Endogenous	-	-		-	-

*Endogenous values subtracted

Experiments with cell-free extracts

Since both the resting and lyophilized cells seem to oxidize the respective carbon source and DL-citramalate at similar rates, it was of interest to find out whether these compounds gave rise to citramalate by a hydration reaction as in the case of the systems of Barker (1956) and of Brightman and Martin (1961). Hence the following experiments were conducted using cell-free extracts of the four bacteria. The results of the experiments and the protocols are shown in Table 5.

It is clear from the results that citramalate or an acid with very similar R_p is formed from both citraconate and mesaconate, but not from itaconate, by cell-free extracts of all locally isolated strains. Methylsuccinate under these conditions does not yield any other acid.

TABLE 5

FORMATION OF CITRAMALATE FROM THE UNSATURATED
C₅-DICARBOXYLATES AND CELL-FREE EXTRACTS

Substrate*	Cell-free extracts of cultures			
	Citraconate	Methylsuccinate	Itaconate	Mesaconate
Citraconate	+++**	+	++	+++
Mesaconate	++	+++	+++	+++
Itaconate	(-)	(-)	±	(-)

* Reaction mixture contained in 2 ml; citraconate, mesaconate or itaconate, 25 μ moles; cysteine, 20 μ moles; ferrous sulphate, 2 μ moles; and unfractionated undialyzed cell-free extract equivalent to 10 to 12 mg protein. Incubation 2 hr at 30°. Reaction was stopped by adding 2 ml of 95 per cent alcohol. The reaction mixture evaporated to dryness, taken up in 0.1 ml water and 10 μ l spotted on paper. Development with two solvents: rest of procedure as in "Methods".

** (-) no citramalate on paper; ± doubtful; + feeble positive; ++ approximately half of substrate converted into citramalate; +++, about 80 to 90% converted into citramalate.

DISCUSSION

The oxidation of individual acids by the different isolates shows some peculiar patterns. The "citrate-cells" oxidize only citrate and citramale among the substrates tested. The other strains oxidize most of the substrates at fast rates except citrate which is oxidized at very slow rates. Cooper and Kornberg (1964) found that Pseudomonas B₂ aba oxidizes itaconate, mesaconate and DL-citramale rapidly ($Q_{O_2} = 94$ for all) and citrate at half this rate. The "itaconate-mesaconate" Pseudomonads used here seem to oxidize citrate at very low rates ($Q_{O_2} = 6$) compared to the other substrates ($Q_{O_2} = 50$ to 130): the methylsuccinate strain oxidizes citrate better ($Q_{O_2} = 18$). The strains used by Brightman and Martin (1961) do not oxidize citrate at all. Hence all of these strains seem to be distinct in their oxidative behaviour. The itaconate-strain isolated here behaves very differently from Pseudomonas B₂ aba (Cooper & Kornberg, 1964) towards citrate.

It is known that DL-citramale is oxidized by the pseudomonas species used by Cooper and Kornberg (1964) and by Martin and Brightman (1961). However it is known with certainty only in the case of Pseudomonas B₂ aba that both isomers of citramale are oxidized (Cooper & Kornberg, 1964) since 93 per cent of citramale-1-C¹⁴ disappears during oxidation. Experiments described here show that all of the isolated strains oxidize both (+)- and (-)-isomers of citramale but the rate of oxidation of the two isomers are very different. The citrate-isolate oxidizes the (-)-isomer 5 to 6

times faster than the (+)-isomer. On the contrary there is an inverse relationship in the case of the other strains.

Experiments with whole cells do not indicate the pathway of metabolism of these compounds but tentative conclusions can be drawn from experiments with cell-free extracts. For example, both citraconate and mesaconate are transformed (hydrated) to citramalate (or a very similar acid) by cell-free extracts of all local isolates. We shall assume without proving here that the hydration product is citramalate. Another compound with identical R_f in systems used in chromatography here is β -methylmalic acid. However conclusive evidence is presented in the next section [3(i) and (ii)] that the product is citramalate and not β -methylmalate. This would perhaps indicate that the "activated intermediates pathway", found in liver mitochondria (Adler et al., 1957; Wang et al., 1961) and in pseudomonads (Nagai, 1963 and Cooper & Kornberg, 1961, 1964) is not operative here. Or, if operative, there is an additional route. Another plausible conclusion is that the Barker pathway also is not operative in toto since citramalate accumulates in the reaction mixture. The Brightman-Martin route does not also seem to be operative since succinate could not be detected in the chromatogram.

The hydration of free unsaturated branched C_5 -dicarboxylic acids is known to occur in C. tetanomorphum (Barker, 1956), Pseudomonas strain 111 (Brightman & Martin, 1961) and Pseudomonas 3081 (Katsuki et al., 1962). Cell-free extracts of these strains hydrate free mesaconate to citramalate. But only Pseudomonas 111 hydrates itaconate in addition. This seems to be the first report of the hydration of

itaconate but it has not been studied further. Our strains give rise to citramalate from both mesaconate and citraconate but not from itaconate.

Two findings from the above experiments need to be emphasized. 1) A new reaction, viz., the hydration of citraconate to citramalate has been discovered, and 2) the citraconate culture seems to be unique, - although Pseudomonas B₂ aba behaves somewhat similarly - in citraconate hydration to (-)-citramalate. These two findings led to further studies on the reactions of citraconate in the citraconate-culture.

SECTION 3(i)

EXPERIMENTS ON CITRACONATE CELLS AND CELL-FREE EXTRACTS

Characterization of the hydration reaction

The hydration of citraconate to citramalate, which was reported in the previous section, can occur by two alternative mechanisms. A direct hydration of citraconate to citramalate is one possibility. According to this possibility, the product of hydration of citraconate should be the optical antipode of that obtained from mesaconate, assuming that both hydrations proceed identically by a trans- or cis-addition mechanism in both cases. The other possibility is that there is first an enzymatic isomerization of citraconate to mesaconate which then is hydrated to citramalate. The product of the latter (mesaconase) reaction has been characterized as dextrorotatory citramalic acid (Barker, 1956). Even the hydration of itaconyl-CoA or mesaconyl-CoA apparently yields (+)-citramalyl-CoA, although the hydration product has not been rigorously characterized (Cooper & Kornberg, 1962; Wang et al., 1961; Nagai, 1963). A third possibility is that the product of hydration is β -methylmalic acid rather than citramalate.

In order to distinguish between these different alternatives, the following experiments were conducted using cell-free extracts of citraconate-grown citraconate culture.

(a) Experiment with fresh undialysed extracts

As already indicated, small-scale experiments (with reaction mixtures containing 5-10 mg protein of cell-free extract, 25 μ moles substrate (citraconate or mesaconate), 10 μ moles cysteine and 1 μ mole FeSO_4) showed that citramalate could be chromatographically detected with

citraconate and mesaconate. In order to ascertain the optical rotation of the product, a reaction mixture containing 50 mg cell-free extract protein, 500 μ moles substrate, 100 μ moles cysteine and 10 μ moles ferrous sulphate was incubated for 12 to 24 hr with a few drops of chloroform as a preservative. The reaction mixture was then processed as described in a later section [Section 3(ii)]. The reaction was stopped by the addition of 18N H_2SO_4 to give pH 5 followed by heating for 5 min in a boiling water bath. The supernatant was adjusted to pH 1 with 18N H_2SO_4 and continuously extracted with ether for 24 hr. The ether extracts contained only two non-volatile organic acids (as detected chromatographically) corresponding to citramalic acid and citraconic or mesaconic acid. The ether extract was evaporated to dryness, ^{and} the organic acids separated by anion exchange chromatography with Dowex-1x8 (formate 200-400 mesh).

The fractions containing the same acid (as detected by paper chromatography) were pooled together and formic acid was evaporated in vacuo. The residual acid corresponding to citramalic acid was made upto 10 ml. 9 ml were used for the preparation of p-bromophenacyl and methyl esters for identification purposes. The results of these tests are the same as described in greater detail in Section 3(ii). These tests rule out the possibility of β -methylmalate as the product of these hydrations and establish that citramalate is the product of hydration of both citraconate and mesaconate. But the more pertinent and important result of this experiment is the rotation of the products obtained from citraconate and mesaconate respectively as shown in Table 6.

The laevorotation of citramalate from citraconate was unexpected since a cis-trans isomerization and subsequent hydration to (+)-citramalate was anticipated. This would seem to establish beyond doubt that

TABLE 6

OPTICAL ROTATION* OF THE PRODUCTS OF HYDRATION OF
CITRACONATE AND MESACONATE BY CELL-FREE EXTRACTS
OF CITRACONATE CULTURE

Product from	Observed rotation
Citraconate reaction mixture	-6.7°
Mesaconate reaction mixture	+5.7°
Boiled extract + citraconate	0°
Boiled extract + mesaconate	0°

* Rotation of 1 ml aliquot (from 10 ml concentrate of the Dowex-1 eluate) made upto 10 ml in the presence of ammonium molybdate, sodium citrate and acetic acid in 2 dm tube (Krebs & Eggleston, 1943).

citraconate undergoes a direct hydration to yield laevorotatory citramalate, since hydration of mesaconate yields the dextrorotatory isomer.

It is however still not clear whether this reaction is catalyzed by mesaconase itself or by another enzyme. Mesaconase of Clostridium (Blair & Barker, 1966) or of Pseudomonas 3081 (Katsuki et al. 1962) does not seem to act on citraconate. As a preliminary step towards purification of mesaconase and its separation from other activity if any, the cell-free extracts were dialyzed at 3 to 5° overnight against cold 0.05 M potassium phosphate buffer (pH 7). The results of the hydration tests using this dialysed extract are shown in Table 7.

It is clear from these results that the two hydration reactions, viz. those of citraconate and of mesaconate are catalyzed by different enzymes. (i) Mesaconase activity can be abolished by dialysis without greatly affecting citraconase activity, and (ii) citraconase reaction yields (-)-citramalate whereas mesaconase catalyzes the formation of the dextrorotatory citramalate.

These experiments, therefore, establish that a new enzyme citraconase (citraconate hydratase or citraconate hydro-lyase) is present in citraconate-grown citraconate culture.

Before proceeding to the experiments on the purification of this enzyme, the description of a method of preparing both (+)- and (-)-isomers of citramalate using crude unfractionated sonicates of the citraconate-grown cells is given in the next Section, 3(ii). It is based on the results of the experiments described in this section. This method offers certain advantages over the presently available methods and these aspects are also discussed.

TABLE 7**EFFECT OF DIALYSIS ON HYDRATION REACTION(S)***

Enzyme extract from	Substrate	Citramalate as tested by	
		Chromatography	Rotation
Citraconate cells	Citraconate	+	-3.5°
	Mesaconate	nil	0°
<u>C. tetanomorphum</u>	Citraconate	nil	0°
	Mesaconate	+	+5°

* Experimental conditions were the same as under Table 6.

SECTION 3(ii)

ENZYMATIC PREPARATION OF (+)- & (-)-ISOMERS OF CITRAMALIC ACID

DL-Citramalic acid was first synthesized by Michael and Tissot (1892) who prepared and hydrolyzed the nitrile of acetoacetic ester. The laevorotatory isomer was isolated from apple peels by Hulme (1954). A method for the enzymatic preparation of the dextrorotatory isomer is available (Barker & Blair, 1962). There is no good procedure available for the preparation of the laevorotatory isomer. The only available method is the resolution of the DL-compound, which is tedious and wasteful (Barker, 1962).

The enzymatic procedure described here for the preparation of the two optical isomers has the advantage of (i) avoiding the wasteful resolution of the racemic mixture, and (ii) using the same unfractionated cell-free extract from the citraconate-isolate. A description of the method is given and is followed by that of the properties of the two optical isomers, the racemate and other isomeric compounds.

Preparation of (-)-citramalic acid

A freshly prepared and undialyzed cell-free extract of the citraconate-grown citraconate-isolate was used as the source of both citraconase and mesaconase. Two separate experiments were run, one for (-)-citramalate and the second for (+)-citramalate. Citraconate was the substrate for the (-)-isomer and mesaconate for (+)-isomer.

The two reaction mixtures consisted of:-

	1	2
(i) Cell-free extract (= 500 mg protein - 5 g wet packed cells)	20 ml	20 ml
(ii) Cysteine HCl (0.1 M) (neutralized just before addition and made upto 0.1 M)	10 ml	10 ml
(iii) Freshly prepared $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution (0.01 M)	10 ml	10 ml
(iv) Citraconic acid (1M soln: neutralized with KOH)	20 ml	-
(v) Mesaconic acid 1 M soln: neutralized with KOH)	-	20 ml

The total volume was adjusted to 100 ml with water. Chloroform (2 ml) was added to each of the reaction mixtures to prevent microbial contamination. The reaction mixtures were then incubated for 4 to 6 hr at 30°C. Tests were made periodically for the disappearance of the substrate and for the formation of citramalate by polarimetry. At the end of 6 hr incubation, another lot of approximately 20 mmoles of the respective substrate (citraconate or mesaconate) was added. It was observed that if all of the substrate (total quantity - 50 mmoles) was added at the commencement of incubation, the rate of hydration was a little slower. However, longer incubation in such cases seemed to yield similar quantities of citramalate. After another 6 to 8 hr incubation, a third lot of 10 mmoles of the substrate was added so that the total quantity added was 50 mmoles. The mixtures were then incubated for a further period of 48 hr. The pH was then adjusted to 4 to 5 with acetic acid and the solution heated for 10 min in a boiling water bath. The precipitated protein was centrifuged off and the clear supernatant adjusted to pH 1 with 18N sulphuric acid. This acidified solution was

extracted with ether in a continuous liquid-liquid extractor for 3 days. 25 ml of water were added at the commencement to the flask containing ether and was replaced daily. The aqueous solutions were meanwhile preserved in the cold and after complete extraction, the aqueous solutions were combined and concentrated in vacuo to a small volume (≈ 10 ml).

Isolation of (-)-citramalic acid

Barker's procedure (1962) of extracting out the residual mesaconate with ether cannot be adopted for the isolation of citraconate since the partition coefficient of citraconate is not very favourable for such a procedure (Table 8).

TABLE 8
PARTITION COEFFICIENT OF CITRACONATE,
MESACONATE AND CITRAMALATE

	Partition coefficient (ether/water)
Citraconic acid	0.2
Mesaconic acid	2.0
Citramalic acid	0.03

Dowex-1x8 (formate) chromatography

A column (5 cm x 75 cm) containing 100 g of Dowex-1x8 (formate form: 200-400 mesh) was prepared and the acids loaded, as described in "Methods". After preliminary washing with 0.5 M formic acid, (-)-citramalic acid was eluted from the column with 1 M formic acid. Chromatographic

analysis of this eluate showed that citramalate was the only detectable non-volatile acid in this fraction. The bulk of the formic acid in this eluate was removed at 50°C in a current of air. The viscous oily residue was dried further by addition of a mixture of benzene:ethylacetate (1:1) followed by evaporation and the residue left overnight in a vacuum desiccator over sodium hydroxide and calcium chloride. Crystallization could not be induced by any conventional procedure. Hence the residue was taken up in absolute ethanol and neutralized with sodium hydroxide in absolute ethanol. The precipitated sodium salt was filtered off, washed with absolute ethanol and dried. The yield from 8 g citraconic acid was about 6 g of the sodium salt of (-)-citramalic acid.

Isolation of (+)-citramalic acid

The procedure followed was similar to that of Barker and Blair (1962). The procedure for (+)-citramalate preparation was identical with that of (-)-citramalate isolation upto the stage of chromatography on Dowex-1x8 (formate). Instead of the chromatographic procedure purification of (+)-citramalate was carried out as described by these authors. The combined aqueous extracts (removed daily from the boiling flask of the ether extraction set-up) was extracted four times with an equal volume of ether in a separatory funnel to remove residual mesaconic acid. The combined ether phase was then extracted twice with 20 ml water and the aqueous phase finally reextracted twice with ether as before. The aqueous extracts containing citramalate were pooled, warmed to remove residual ether and concentrated to a syrup under reduced pressure at 50°C. The last traces of moisture were removed as before by treatment with benzene-ethylacetate (1:1). Crystallization could not be induced

in this case also, unlike in Barker's procedure. Hence the acid was converted into the sodium salt as described above. The yield of the sodium salt was about 6 g from 8 g of mesaconic acid.

PROPERTIES OF THE ACIDS

Optical rotation

The specific rotations of the free acids with and without molybdate treatment are shown in Table 9. Solutions of free acids were prepared by treatment of the sodium salts with Dowex-50 (H form) and the concentration of the acid checked by titration. The rotations of the acids, observed here, are in good agreement with the values reported in literature.

Chromatography of the products

Paper chromatography of both products obtained showed only one spot on development, even when 5-10 μ moles of the acid were spotted. The R_f values of these and related acids are given in Table 10. It is clear that the acids obtained from the reaction mixtures corresponded closely to citramalic acid. In order to obtain conclusive evidence concerning the identity of the acids, their esters were prepared as described under "Methods". The methyl esters were used for the IR and NMR spectra and the p-bromophenacyl esters for melting point determinations.

TABLE 9

SPECIFIC ROTATION OF THE ACIDS ISOLATED

	Without molybdate*		With molybdate**	
	Found	Lit. §	Found	Lit. §
(-)-Citramalic acid	-24°	-30°(b)	-1260°	-1328°(c)
(+)-Citramalic acid	+24°	+23.6° (a)	+1260	+1230°(a) enzymatic
		+37°(b)		1410°(a) synthetic

* Rotation of 3.0 per cent and 4.4 per cent solutions of the (-)- and (+)-isomers were measured in a 1 dm tube.

** 2 ml of the test solution contained 20 μ moles of citramalate, 0.1 ml glacial acetic acid, 0.2 ml 1 M sodium citrate, 0.9 ml of a saturated (29 per cent) solution of ammonium molybdate and water to 2 ml (1 dm tube).

§ (a) Figures from Barker and Blair (1962)
 (b) From Hulme (1954)
 (c) From Sai and Amaha (1965)

TABLE 10

R_f VALUES OF C₅ DICARBOXYLIC ACIDS

Acid	R _f	
	Solvent A*	Solvent B*
1. Citraconic acid	0.81	0.83
2. Mesaconic acid	0.92	0.94
3. Itaconic acid	0.82	0.86
4. DL-Methylsuccinic acid	0.85	0.86
5. Lactic acid	0.74	0.74
6. DL-Citramalic acid	0.62	0.71
7. (+)-Compound (isolated from mesaconate reaction mixture)	0.62	0.71
8. (-)-Compound (isolated from citraconate reaction mixture)	0.62	0.70
9. β -Hydroxyglutaric acid	0.51	0.65
10. β -Methylmalic acid	0.62	0.70

*Solvent A: n-butanol:formic acid:water = 4:1.5:1 (vol/vol);

*Solvent B: ethylacetate:formic acid:water = 10:1:3 (vol/vol).

Melting points of p-bromophenacyl esters

Melting points of the p-bromophenacyl esters are listed in Table 11. The melting points of the ester of (-)-citramalic acid and that of the ester of the acid isolated from the citraconate reaction mixture are almost identical. The melting point of β -methylmalic ester is quite distinct. Further confirmation that the product of the citraconate reaction is (-)-citramalic acid and not β -methylmalic acid was obtained from the IR and the NMR spectra of the methylesters.

Infrared spectra

The infrared spectra of the methyl esters of the different acids are shown in Fig.6. The IR spectra are similar in the range 4000 to 1300 cm^{-1} , but that of dimethyl β -methylmalate differs from the others in the range 1300 to 700 cm^{-1} . The three esters, viz. dimethyl DL-citramalate and methyl esters of the acids from the reaction mixtures containing citraconate and mesaconate, have almost identical IR spectra. The esters of other acids - β -hydroxyglutarate, itaconate, mesaconate, citraconate - had quite different spectra and hence not shown here.

NMR spectra

The NMR spectra of the methylesters (Fig.7) of the above four acids (used for the IR spectral examination) clearly distinguish between the tertiary methyl of citramalate (α -methylmalic) and the secondary methyl of β -methylmalate. There is a doublet in the case of the latter whereas citramalate ester shows a single peak in this region (1 to 1.5 p.p.m.)

TABLE 11MELTING POINTS* OF THE p-BROMOPHENACYL DERIVATIVES

Esters of	Found	Literature
DL-Citramalic acid	136-137°	137-137.5°**
(-)-Citramalic acid		113-113.5°**
Acid from citraconate reaction mixture	111-112°	-
Acid from mesaconate reaction mixture	99-100°	-
β-Methylmalic acid	186°	-
α-Hydroxyglutaric acid	139-140°	-
β-Hydroxyglutaric acid	166°	166°**
Citraconic acid	116°	-
Mesaconic acid	163°	-
Itaconic acid	115-116°	117°***
DL-Methylsuccinic acid	121-122°	-

* All melting points are uncorrected.

** From Hulme (1954).

*** From Shriner & Fuson (1948).

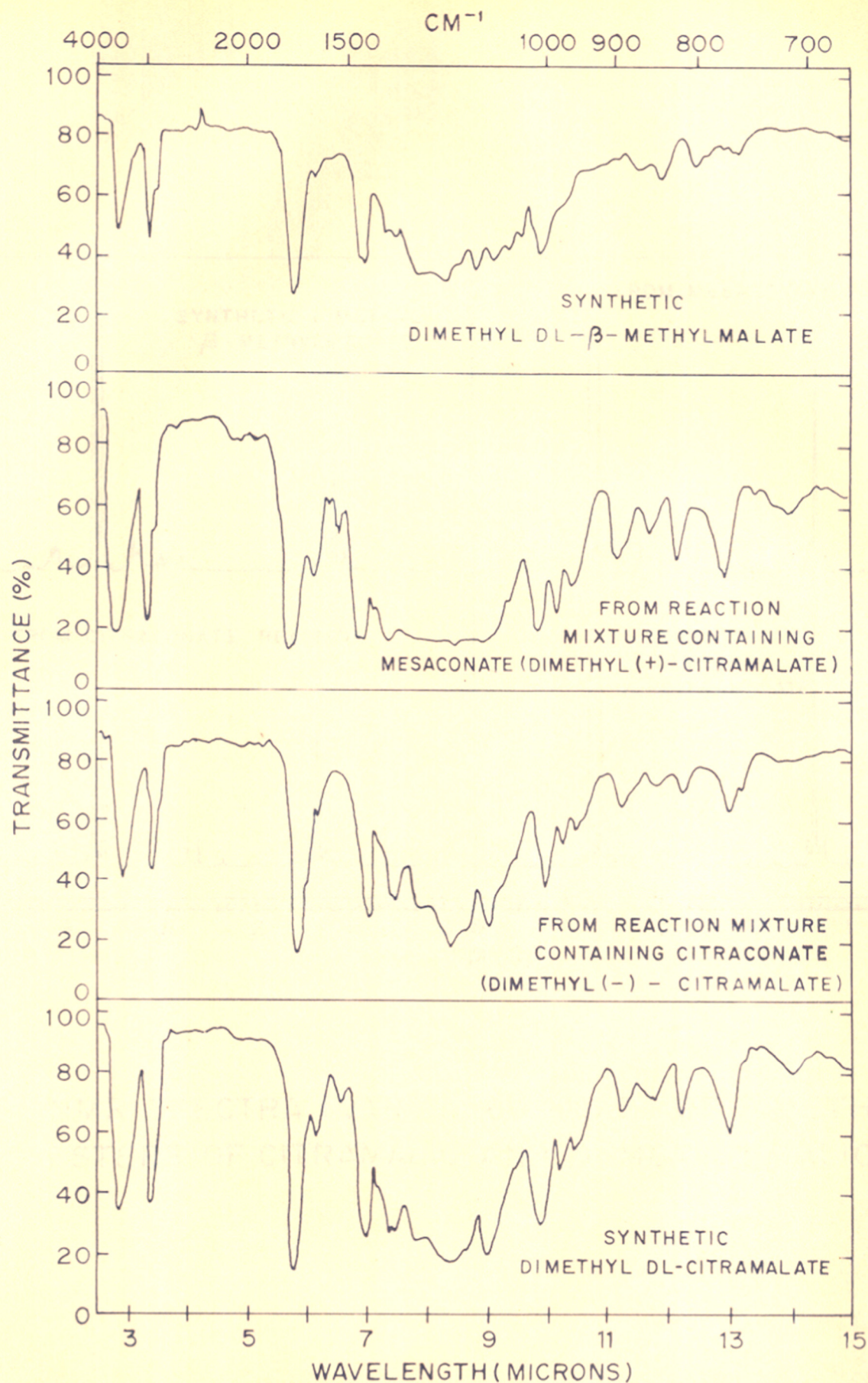


FIG. 6. IR SPECTRA OF DIMETHYL ESTERS OF CITRAMALIC AND β -METHYLMALIC ACIDS.

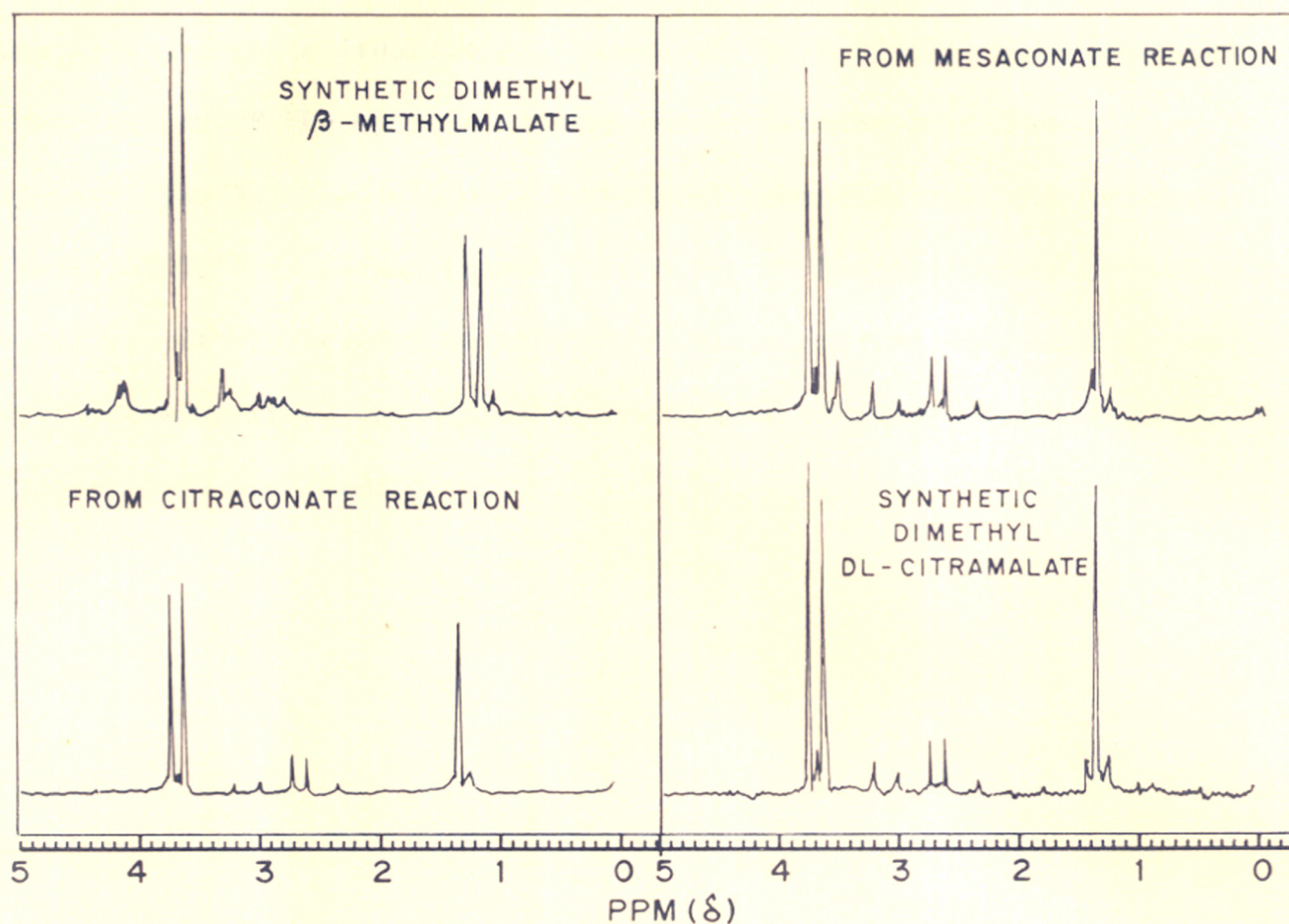


FIG. 7. NMR SPECTRA (VARIAN: 60 Mc/S.) OF DIMETHYL ESTERS OF CITRAMALIC AND β -METHYLMALIC ACIDS.

It is clear from the above data, that the product of hydration of citraconate is laevorotatory citramalate and that mesaconate yields the dextrorotatory isomer. It is also obvious that β -methylmalate is not a product since its properties - chromatographic and spectral and chemical - are quite distinct from those of the product. This procedure of preparation of the optical isomers has advantages over Barker's procedure (1962). Resolution is a wasteful procedure and Barker reports an yield of 0.76 g of each isomer from 6.4 g of DL-citramalate. This step is completely avoided in this procedure. Another advantage is the use of the same crude unfractionated cell-free extracts for the preparation of both the isomers.

SECTION 3(111)

PURIFICATION AND PROPERTIES OF CITRACONASE

The previous two sub-sections [3(i) and 3(ii)] have clearly established the fact that hydration of citraconate is catalyzed by an enzyme which is distinct from mesaconase (mesaconate hydratase or mesaconate hydro-lyase). This new enzyme has been named as citraconase (citraconate hydratase or citraconate hydro-lyase). It has been considerably purified (25-fold) and its properties studied. This section describes experiments on its purification and a study of its properties.

I. Preliminary experiments

Growth and enzyme content of the organism

Prior to studies on the purification and properties of the enzyme it is desirable to determine the growth curve of the organism in relation to citraconase content. The experimental details of preparing inoculum and media and of measuring growth and enzyme activities have been described earlier. Cells were harvested at the end of 6, 12, 18, 24 and 30 hours of growth and sonicated for 15 minutes in each case. The results of these experiments are shown graphically in Fig.8.

It can be seen from the results in Fig.8 that maximum growth of the organism occurs at about 12 hours and that maximum citraconase and malease activities are present in 12 and 15 hours of growth respectively. However for the sake of convenience the cells were routinely harvested after 15 hours of growth.

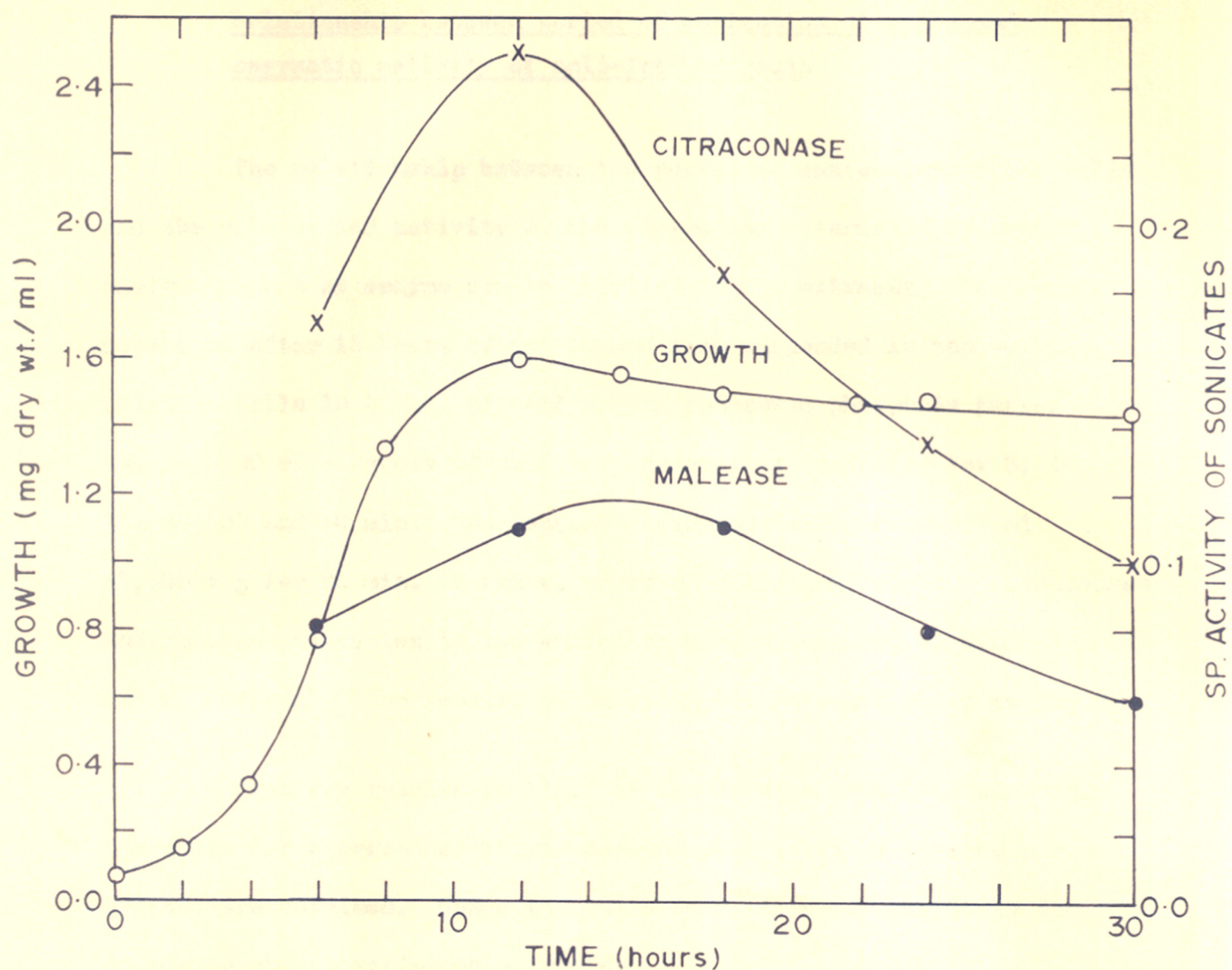


FIG. 8. VARIATION OF ENZYME (CITRACONASE AND MALEASE) CONTENT WITH GROWTH (AGE OF CULTURE).
Experimental details in the text.

Relationship between period of sonication of cells and enzymatic activity of cell-free extracts

The relationship between the period of sonication of the cells and the release and activity of the enzyme was determined so that maximum amount of enzyme can be obtained in the extracts. Cells were harvested after 15 hours of growth and were suspended in ten volumes (5 g wet cells in 50 ml) of cold 0.02 M potassium phosphate buffer (pH 7). 20 ml aliquots of cell suspension were sonicated for 5, 10, 15, 20, 30 and 60 min. The sonicates were centrifuged at 5°C at 10,000 x g for 20 min. to remove unbroken cells and debris. Citraconase and malease activities in the sonicates were determined as described under "Methods". The results of these experiments are shown in Fig.9.

From the results in Fig.9 it can be seen that by sonicating the cells for a period of 15 min maximum activities of citraconase and malease are obtained. Hence the cells were sonicated routinely for 15 min to obtain cell-free extracts.

II. Purification of citraconase

An assay procedure is essential to follow the purification stages. This has been fully described later in this section and was developed as follows:

A. Assay procedure: This was designed on the basis of the kinetic properties and activation experiments.

(1) Kinetic properties

(i) The time course study of citraconase activity was first studied. The reaction mixture contained in 1 ml, Tris-HCl buffer (pH 7.5),

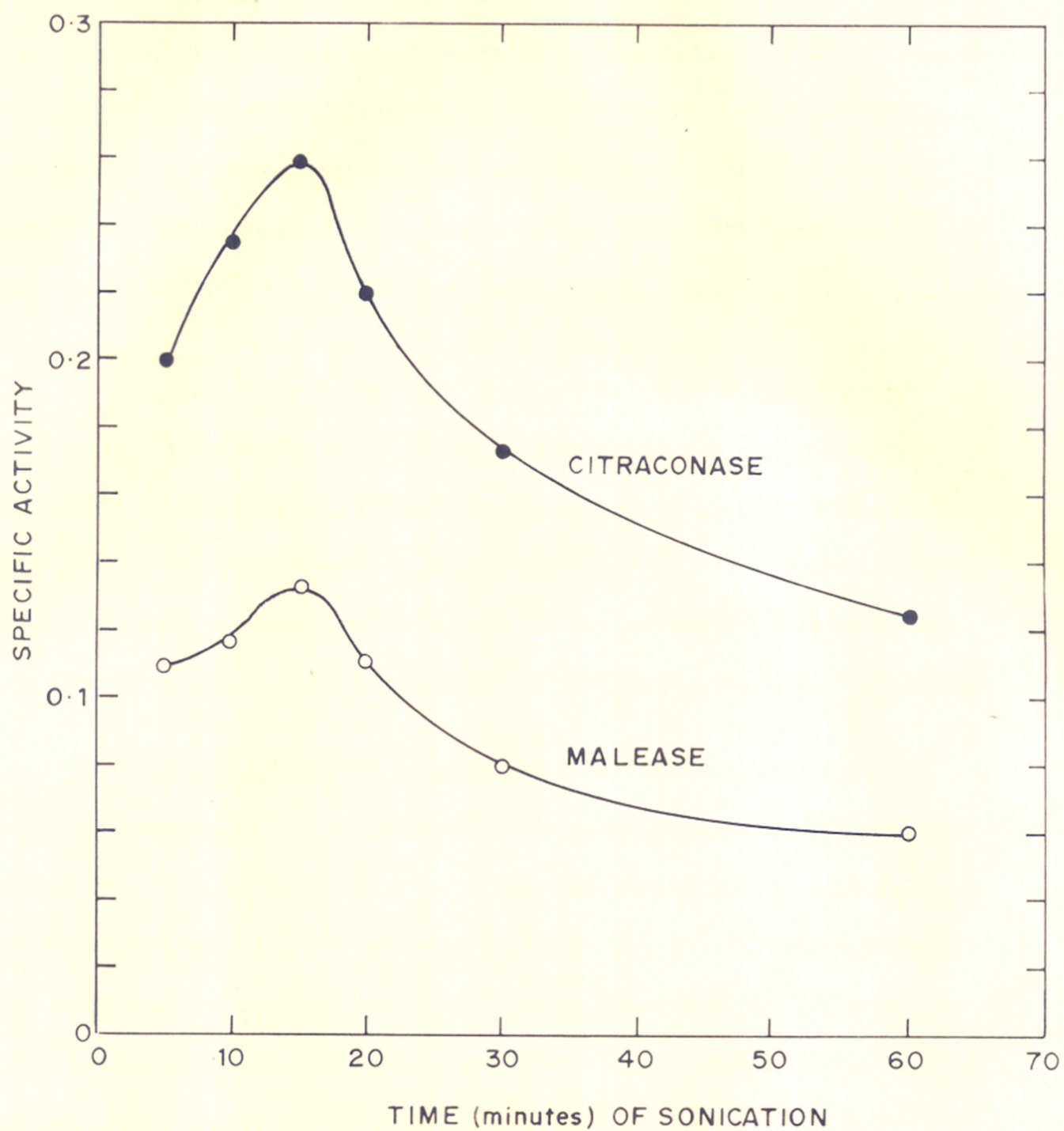


FIG. 9. EFFECT OF THE PERIOD OF SONICATION OF A CELL SUSPENSION ON THE (RELEASE & ACTIVITY OF) ENZYMES.

Experimental details in the text.

100 μ moles and potassium citraconate, 5 μ moles, 0.1 mg of enzyme protein, reduced glutathione, 10 μ moles and ferrous sulfate, 1 μ mole. The reaction was started by addition of citraconate. After different periods of incubation aliquots of the reaction mixture were treated with perchloric acid, as described later in this section and the amounts of citraconate hydrated were determined. The results of such an experiment are shown graphically in Fig.10.

It can be seen from Fig.10 that the rate of reaction is linear only during the first 10 to 12 min under the experimental conditions. During the initial linear period of 10 min, 1 μ mole of citraconate is transformed, i.e., 20 per cent of the total quantity. Hence the rate of reaction is linear if the quantity of citraconate transformed is 20 per cent (or less) of the total initial quantity.

(ii) The effect of enzyme (protein) concentration on the rate of reaction was next studied. The quantities of cell proteins varied from 0.1 to 1 mg and the incubation period was 10 min. The other conditions were the same as in (i). The relationship between the enzyme concentration and activity (as expressed by the quantity of citraconate which is transformed or has disappeared during 10 min) is shown in Fig.11.

It is clear from Fig.11 that the rate of hydration is proportional to the enzyme concentration only when the quantity of the enzyme causes a transformation of 0.1 μ mole or less in the reaction mixture under the experimental conditions.

(iii) It was also found in preliminary experiments that increasing the substrate (citraconate) concentration to above 5 mM

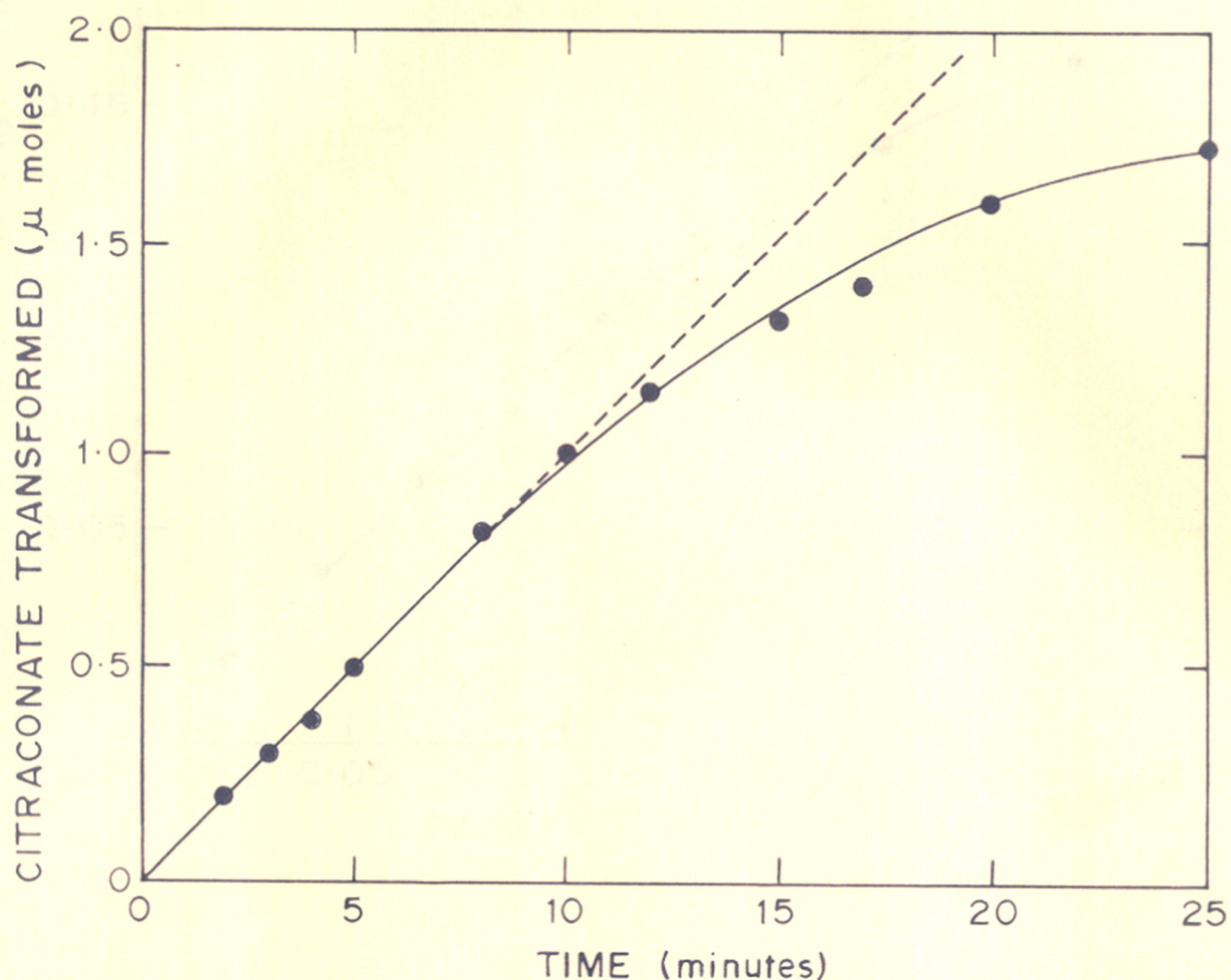


FIG. 10. EFFECT OF TIME OF REACTION ON THE ACTIVITY OF CITRACONASE.

Reaction mixture contained in 1ml: Tris-HCl buffer (pH 7.5) 100 μ moles; reduced glutathione, 10 μ moles; ferrous sulphate, 1 μ mole; potassium citraconate, 5 μ moles and 0.1 mg. of enzyme protein. The reaction was started by the addition of citraconate. Incubation period as indicated at 30°. Reaction was stopped by addition of 1 ml 0.66 N perchloric acid and amount of citraconate hydrated was estimated.

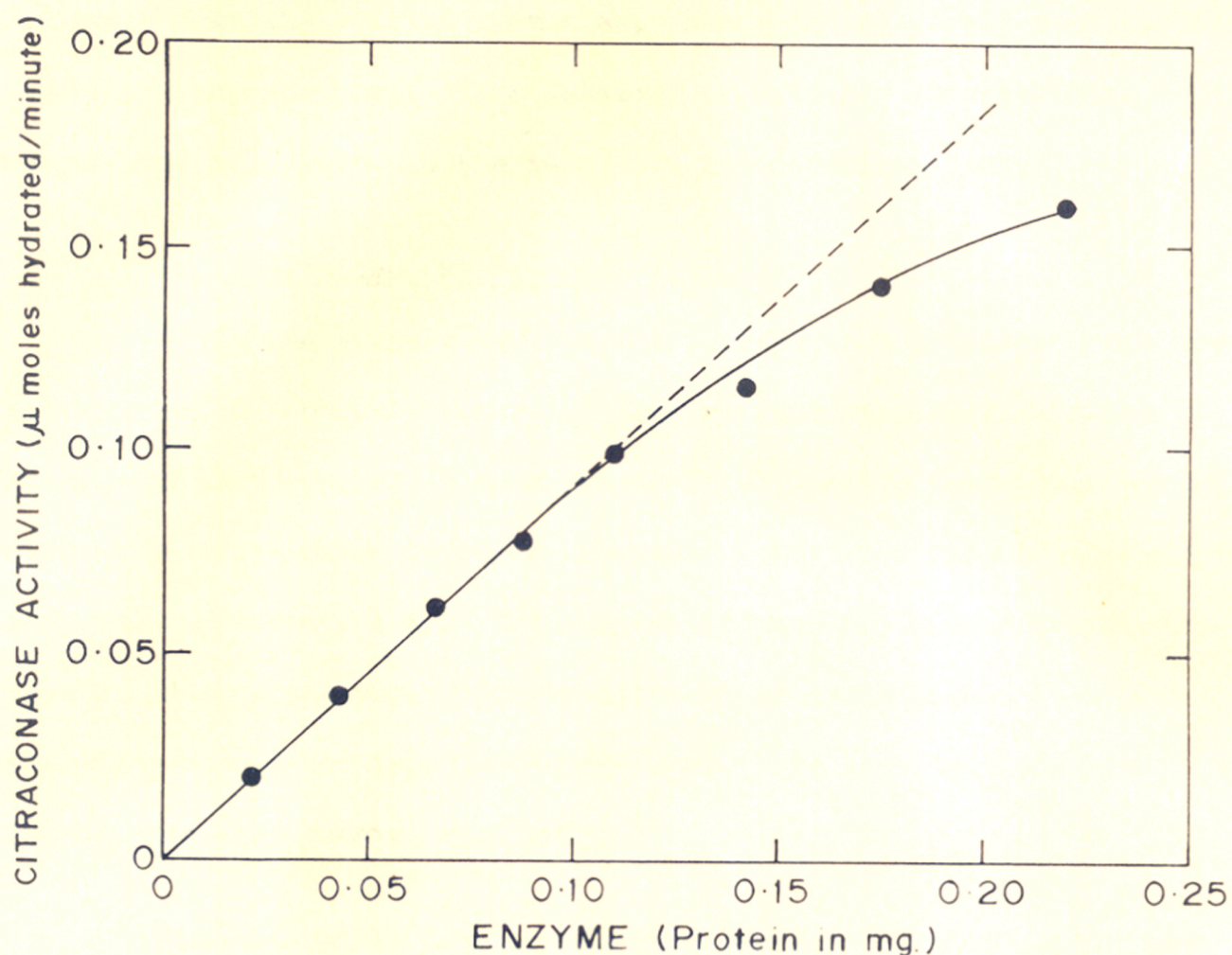


FIG. 11. EFFECT OF ENZYME CONCENTRATION ON THE ACTIVITY OF CITRACONASE.

Reaction mixture contained in 1 ml: Tris—HCl buffer (pH 7.5) 100 μ moles; reduced glutathione 10 μ moles; ferrous sulphate, 1 μ mole; potassium citraconate, 5 μ moles; protein concentration indicated as above. Incubation 10 minutes at 30°. Reaction was stopped by addition of 1 ml 0.66 N perchloric acid and amount of citraconate hydrated was estimated.

did not affect the rate of hydration. Hence the substrate concentration used routinely was 5 mM. More detailed experiments on saturation of the enzyme with substrate and the cofactors are described later.

(2) Activation experiments:

While the present work on the purification of citraconase was in progress, a report by Blair and Barker (1966) appeared in which it was stated that apparently inactive or feebly active mesaconase preparations were activated by prior anaerobic incubation with a sulphhydryl compound and ferrous ions. This activation was dependent upon anaerobiosis and upon the period and temperature of incubation. It was therefore considered important and necessary to ascertain whether citraconase also behaved similarly.

The activation experiments were carried out with partially purified preparations (specific activity = 1.0).

(i) Effect of oxygen on activation

Three reaction mixtures of identical composition were used. The reaction mixtures (1 ml) contained 0.1 M Tris-HCl (pH 7.5), 0.2 ml enzyme (2.25 mg protein), 10 mM reduced glutathione and 1 mM FeSO_4 . Thunberg tube containing the reaction mixture was evacuated for 3 min in a water pump after addition of all components while a similar tube was left exposed to air. The third one was a zero-hour control. The components were incubated for 1 hr at 30° and the enzyme activity was assayed in an aliquot (0.05 ml). The results are shown in Table 12.

TABLE 12EFFECT OF AEROBIOSIS AND ANAEROBIOSIS ON ACTIVATION

Oxygen relationship	Specific activity
1. Anaerobic (evacuated)	0.90
2. Aerobic (not evacuated)	0.33
3. Zero-hour control	0.15

It is clear that under anaerobic conditions there is a three-fold increase in activity.

(ii) Effect of temperature on activation:

This was tested at two different temperatures 0° (in ice bath) and at 30° in a thermostatic bath. The composition of the reaction mixture and other details were the same as in (i). Aliquots of reaction mixtures were removed (after 1 hr incubation) and activity determined as before. The results are shown in Table 13.

TABLE 13EFFECT OF TEMPERATURE ON ACTIVATION

Activation temperature	Specific activity
0°	0.38
30°	0.90

Anaerobic incubation at 30° for 1 hr is more efficient than at 0°.

Hence activation was done routinely at 30°.

(iii) Effect of the period of incubation on activation:

The reaction mixtures as in (i) were incubated anaerobically for different time intervals, and aliquots (0.05 ml) removed for citraconase assay. The results, shown in Fig.12 show that the specific activity increased from the initial value of 0.2 to 0.9 after one hour's anaerobic incubation. Incubation periods longer than one hour did not result in any further increase in activity.

(iv) Age of enzyme extract, activity and activation:

The activity of an extract was found to diminish with age. It could be restored by activation as shown in Table 14.

TABLE 14AGEING AND ACTIVATION

Age of extract	Specific activity	
	Before activation	After activation
1. Fresh sonicate	0.20	0.24
2. Sonicate after storage for 96 hr at -15°	0.07	0.26

On the basis of the above results, citraconase-containing extracts were routinely activated by one hour's anaerobic incubation at 30° with 100 mM Tris-HCl (pH 7.5), 10 mM reduced glutathione and 1 mM FeSO_4 . The enzyme solutions were then assayed such that aliquots for citraconase assay always contained 0.1 unit or less after activation.

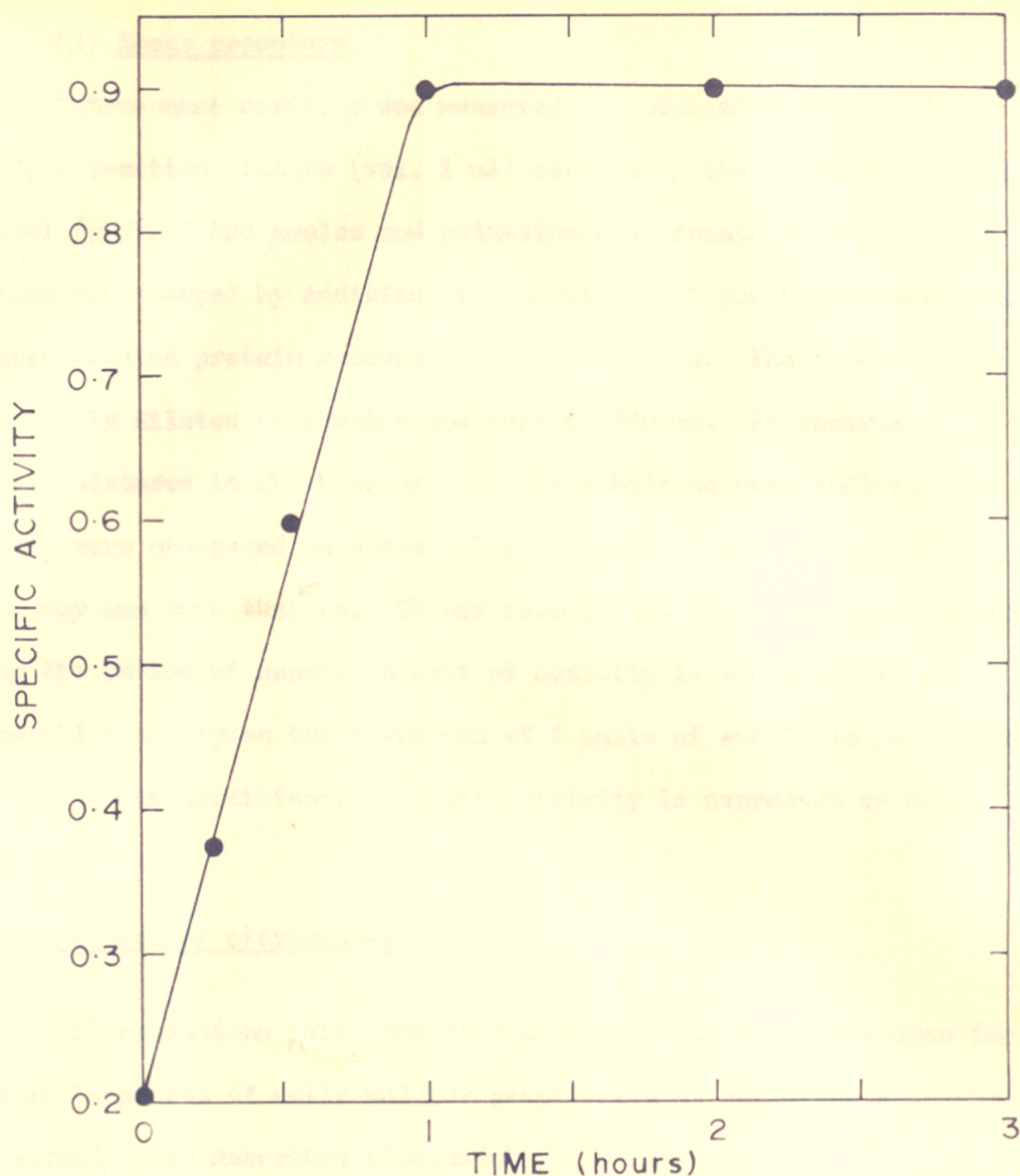


FIG. 12. EFFECT OF PERIOD OF ACTIVATION ON CITRACONASE ACTIVITY.

The reaction mixtures contained in 1 ml: Tris-HCl buffer (pH 7.5) 100 μ moles; enzyme, 0.2 ml (= 2.25 mg protein); reduced glutathione, 10 μ moles and ferrous sulphate, 1 μ mole. Thunberg tubes containing the reaction mixtures were evacuated for 3 minutes, incubated for different time intervals as indicated and aliquots (0.05 ml) removed for determination of citraconase activity as described.

(3) Assay procedure

Citraconase activity was measured by incubating for 10 min at 30°, a reaction mixture (vol. 1 ml) containing the activated enzyme, Tris-HCl (pH 7.5) 100 μ moles and potassium citraconate 5 μ moles. The reaction was stopped by addition of 1 ml of 0.66 N perchloric acid and the precipitated protein removed by centrifugation. The supernatant was suitably diluted with water and read at 240 m μ . As controls reaction mixtures in which enzyme and the substrate were individually omitted, were processed as above. The quantity of enzyme used for each assay was such that only 20 per cent of the substrate was hydrated during the period of assay. A unit of activity is the quantity of enzyme which catalyzes the hydration of 1 μ mole of substrate per minute under the above conditions. Specific activity is expressed as units per mg protein.

B. Purification of citraconase

All operations were conducted at 0 to 5°C. The procedures for large-scale growth of cells and for preparation of cell-free extracts have already been described ("Methods").

(1) Removal of nucleic acids by streptomycin sulfate

The cell-free extracts obtained by sonication contained 8 to 10 mg protein per ml or were diluted to contain 10 mg protein per ml with 0.02 M potassium phosphate buffer (pH 7.0). This concentration of protein gives uniform results in the subsequent purification procedure. Solid streptomycin sulfate (powder) was added to the cell-free extract with constant mechanical stirring. The effect of various concentrations of streptomycin sulfate was tested. The solution was stirred for 10 min

after complete addition of streptomycin sulfate and allowed to stand for 30 min. It was then centrifuged for 30 min at 12,000 x g. Citraconase activity and nucleic acids in the supernatant were tested spectrophotometrically. The results are tabulated in Table 15.

TABLE 15

STREPTOMYCIN TREATMENT, ACTIVITY AND REMOVAL OF NUCLEIC ACIDS

Streptomycin concentration in reaction mixture (per cent)	Nucleic acid		Activity recovered (per cent)
	R*	Nucleic acid present** (per cent)	
0	0.56	20	100
0.5	0.65	14.2	90
1.0	0.75	8	90
1.25	0.78	6.7	82
1.5	0.80	6.5	79
2.0	0.80	6.5	75
2.5	0.80	6.5	72

* Ratio of optical density at 280 mμ to optical density at 260 mμ.

** Nucleic acid as percentage in a mixture of protein and nucleic acid (Warburg & Christian, 1941).

Much of the nucleic acid is removed at 1 per cent streptomycin concentration with a loss of only 10 per cent of citraconase activity. Hence cell-free extracts with 1 per cent protein were routinely treated with streptomycin sulfate (1 per cent final concentration) as indicated above.

(2) Ammonium sulfate fractionation

The streptomycin sulfate-treated cell extract was next fractionated with ammonium sulfate. The quantity of ammonium sulfate (X) required for changing the degree of saturation from the initial saturation of S_1 to a final saturation of S_2 was calculated from the following equation (Jagannathan *et al.*, 1956):

$$X = \frac{50 (S_2 - S_1)}{1 - 0.28 S_2}$$

where X = g of ammonium sulfate to be added to 100 ml of a solution of saturation of S_1 to increase it to a saturation of S_2 . S_1 and S_2 are expressed as fractions of saturation at 0°C.

Powdered ammonium sulfate was added gradually with constant stirring to the streptomycin sulfate-treated cell extract to give 0.4 saturation. 22.52 g were added to every 100 ml during the course of 15 to 20 min. A pH of about 7 was maintained during this operation by periodic drop-wise addition of 2N NH_4OH . The solution was stirred for a further period of 10 min, allowed to stand 30 min and then centrifuged for 30 min at 12,000 x g. The precipitate was dissolved in 20 ml 0.02 M potassium phosphate buffer (pH 7). Powdered ammonium sulfate (18.65 g) was added with stirring during 15 min to the supernatant solution (100 ml), the solution stirred for a further period of 10 min, allowed to stand 30 min and then centrifuged for 30 min at 12,000 x g. The precipitate was dissolved in 0.02 M potassium phosphate buffer (pH 7) (16.5 ml; 165 units: SA = 0.4). The supernatant was then treated as before with ammonium sulfate (13.5 g) to 0.9 saturation and centrifuged. The supernatant was discarded and the precipitate dissolved in 10 ml of 0.02 M potassium phosphate buffer (pH 7). Each

of these fractions was then analysed for enzyme activity and protein. The results are shown in Table 16.

TABLE 16

AMMONIUM SULFATE FRACTIONATION OF STREPTOMYCIN-TREATED EXTRACTS

	Ammonium sulfate saturation			
	Initial	0 to 40	40 to 70	70 to 90
Activity recovered (per cent)	100	15	70 to 75	5
Specific activity	0.25	0.17	0.4	-

There is approximately a 1.6-fold purification by the above fractionation procedure.

(3) Calcium phosphate (gel) treatment

Experiments were next conducted to ascertain whether further purification could be achieved by calcium phosphate gel treatment. Different ratios of gel to protein (wt/wt) were tested keeping the buffer concentration constant. 10 mg protein were treated with different quantities of a suspension of gel in water (containing 70 mg dry wt. per ml), so that the final volume was 2 ml and the pH 6.5. The mixture was manually shaken gently for 5 min, allowed to stand for 15 min and then centrifuged. The supernatant solution was tested

for citraconase activity. The results are shown in Table 17.

TABLE 17

CALCIUM PHOSPHATE GEL TREATMENT OF CITRACONASE EXTRACTS

	Initial	Enzyme in gel supernatant			
		*R=2	R=5	R=7	R=10
Activity (units)	5.0	4.9	4.9	4.65	4.4
Protein (mg)	12.5	10.2	8.8	8.4	8.0
Sp. activity	0.4	0.48	0.55	0.55	0.55

*R = gel to protein (wt/wt) ratio

It is clear that the gel does not adsorb the enzyme under the conditions tested. However some inert proteins are removed by this procedure and the specific activity increases by 40 to 50 per cent (1.4-fold).

Since the enzyme solutions contained considerable amounts of ammonium sulfate, it was thought possible that this might be the reason for negative adsorption. Hence the enzyme solution was dialyzed against 0.01 M potassium phosphate buffer (pH 7) and then the above adsorption experiment was repeated. The results were essentially the same.

Hence a large batch of the ammonium sulfate fraction (0.4 to 0.7 saturation; 33 ml, 160 units, specific activity = 0.4) was

accordingly treated with calcium phosphate gel (gel:protein = 5:1, wt/wt), as described above. The gel supernatant contained practically 100 per cent of the initial activity with a specific activity of 0.55. This gel-supernatant was then refractionated with ammonium sulfate.

4) Refractionation with ammonium sulfate

The gel supernatant (33 ml) was next refractionated into 0 to 0.4, 0.4 to 0.5, 0.5 to 0.7 and 0.7 to 0.9 saturation fractions at pH 7 by successive additions of solid powdered ammonium sulfate. The procedure has already been described earlier. 50 to 55 per cent of the original activity was present in the 0.5 to 0.7 saturation fraction (16 ml, 130 units; specific activity = 0.9). The distribution of activities is shown in Table 18.

TABLE 18

ACTIVITIES IN DIFFERENT FRACTIONS AFTER REFRACTIONATION WITH AMMONIUM SULFATE

	Gel eluate	Activity in different fractions			
		0 to 0.4	0.4 to 0.5	0.5 to 0.7	0.7 to 0.9
Activity recovery (per cent)	100	0	8	80	2 to 3
Specific activity	0.55	0	0.12	0.9	0.08

Most of the activity is recovered in the 0.5 to 0.7 saturation fraction, which was next subjected to chromatography on DEAE-cellulose.

5) Chromatography on DEAE-cellulose

The 0.5 to 0.7 ammonium-sulfate saturation fraction (16 ml) was dialyzed for 8 to 10 hr with stirring against 200 volumes of 0.01 M potassium phosphate buffer pH 7 with three changes of the buffer.

The DEAE-cellulose column was set up as follows (Peterson & Sober, 1962). 10 g of DEAE-cellulose (Cellex-D, 0.62 meq/g, Bio-RAD Labs) were suspended in 500 ml of 1N NaOH at room temperature (25°C) and stirred 10 min. After filtration on a sintered glass filter, the cellulose was suspended thoroughly in 500 ml 1N HCl and filtered quickly again on a sintered glass filter: the time of contact with HCl was kept minimal. It was washed thoroughly with copious amounts of distilled water. This procedure - suspension in 1N NaOH and then in 1N HCl and washing - was repeated once.

The cellulose preparation was then suspended in about 100 ml of 0.5 M potassium phosphate buffer (pH 7), stirred for 5 min and filtered through a Buchner funnel. The cake was again suspended in a fresh batch of the same buffer and filtered and this procedure was repeated thrice. The exchanger was then washed five times in the same manner, each time with 100 ml of 0.01 M potassium phosphate buffer (pH 7). It was then suspended in 75 ml of 0.01 M phosphate buffer (pH 7) and evacuated for 30 min in a Buchner flask to remove all the entrapped air bubbles. The thick slurry was poured down a column (2 cm x 40 cm) fitted with a wad of glasswool and stopcock at the bottom. The exchanger was allowed to settle down under gravity

for 30 min and then a slight pressure was applied by means of a rubber-bulb ("hand-pump"). An exchanger column of 23 cm was obtained and was washed with 200 ml of 0.01 M potassium phosphate buffer (pH 7) added by means of a separatory funnel at the top. The rest of the operations were conducted at 0 to 5°C.

The dialysed enzyme fraction (16 ml) was divided into three equal lots and each lot was used for DEAE-cellulose chromatography. The enzyme solution (5 ml containing about 50 mg protein) was then applied to the top of the column under slight pressure (0.15 kg/cm^2). The column was then washed twice with 2 ml of 0.01 M potassium phosphate buffer (pH 7). The enzyme was then eluted from the column by gradient elution. The mixing chamber contained 250 ml of 0.01 M potassium phosphate (pH 7) and the separating funnel 0.5 M potassium phosphate (pH 7) buffers. The buffer in the mixing chamber was continuously stirred with a magnetic stirrer. The rate of addition of the strong buffer and the rate of elution were the same. The volume of each fraction was 4 ml which was collected in about 10 min. The fractions were collected in a Technicon Automatic Fraction Collector in the cold. The effluent fractions were assayed for citraconase (and other contaminating activities) and for protein. The results are shown in Fig.13. The fractions (27 to 35) containing enzyme with high specific activity (3 to 3.3) were pooled together and the enzyme was precipitated by the addition of powdered ammonium sulfate to 0.9 saturation. The precipitate was spun down at $12,000 \times g$ for 30 min and dissolved in 1 ml of 0.05 M potassium phosphate buffer, pH 7. The solution contained 10 mg protein and 30 units of citraconase activity.

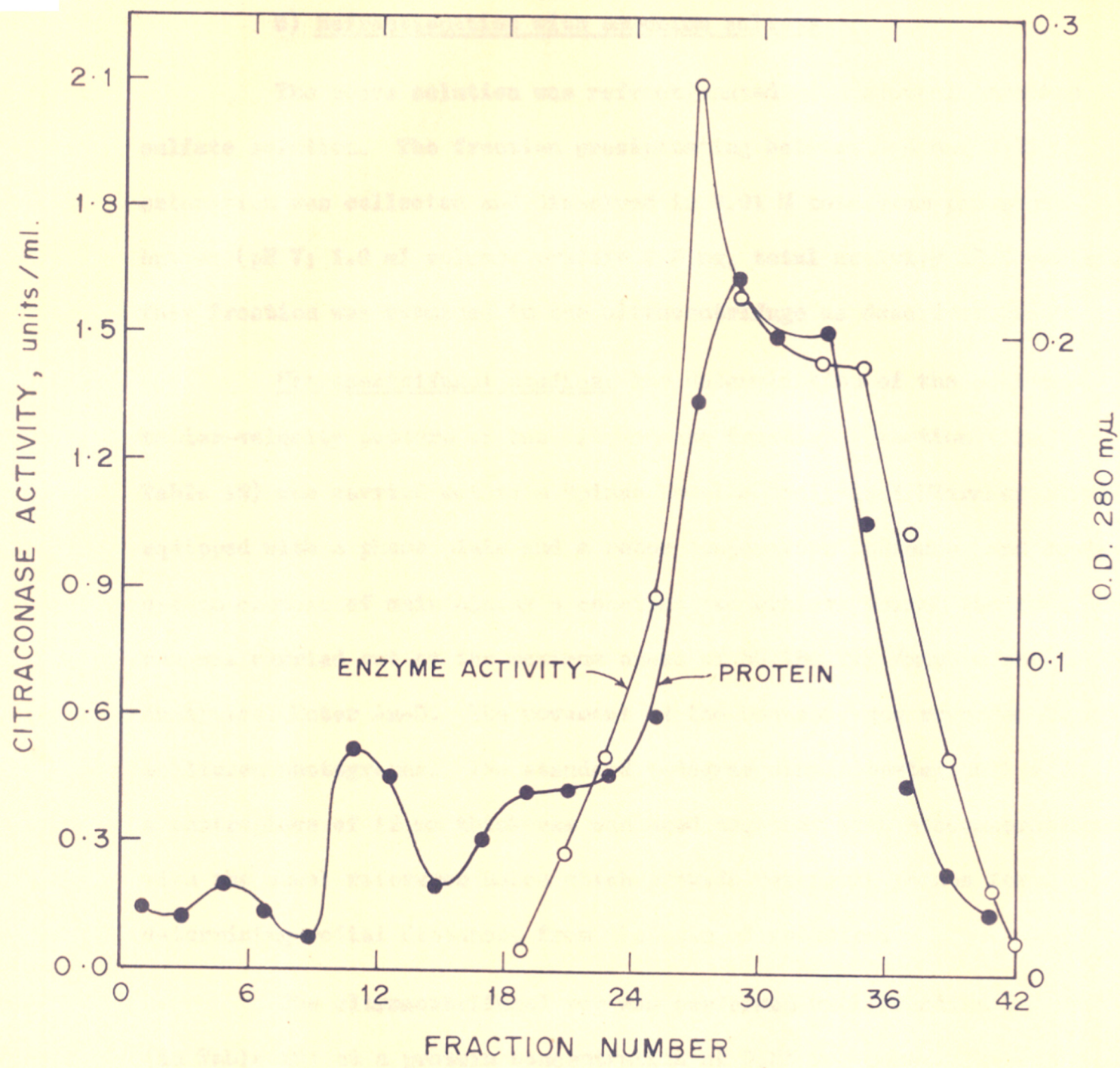


FIG. 13. CHROMATOGRAPHY ON DEAE-CELLULOSE.

O.D. 280 mμ was measured in an aliquot (0.3 ml.) of the fractions. Citraconase activity was determined as under "Methods" in aliquots (0.1 ml.) of the fractions.

6) Refractionation with ammonium sulfate

The above solution was refractionated with neutral ammonium sulfate solution. The fraction precipitating between 0.5 and 0.65 saturation was collected and dissolved in 0.01 M potassium phosphate buffer (pH 7; 1.0 ml volume, protein 3.5 mg, total activity 17.5 units). This fraction was examined in the ultracentrifuge as described below.

Ultracentrifugal studies: The determination of the sedimentation-velocity pattern of the citraconase fraction (Fraction 7 in Table 19) was carried out in a Spinco Model E Analytical Ultracentrifuge equipped with a phase plate and a rotor temperature indicator and control system capable of maintaining a constant temperature during the run. The run was carried out at the maximum speed of 59,780 rev./min in the Analytical Rotor An-D. The movement of the boundary was recorded by schlieren photographs. The standard 4-degree single-sector cell with a centrepiece of 12 mm thickness was used together with a counterbalance with the usual reference holes which provide reference points for determining radial distances from the axis of rotation.

The ultracentrifugal run was performed with Fraction 7 (in Table 19) at a protein concentration of 0.35 per cent. The run was carried out in a pre-cooled cell and rotor and at a temperature of 4.6°. Photographs were taken at intervals from the time the rotor reached two-thirds full speed.

The ultracentrifuge pattern (Fig. 14) indicates that the enzyme is not homogeneous and contains at least two other components. The uncorrected sedimentation coefficients of the three main

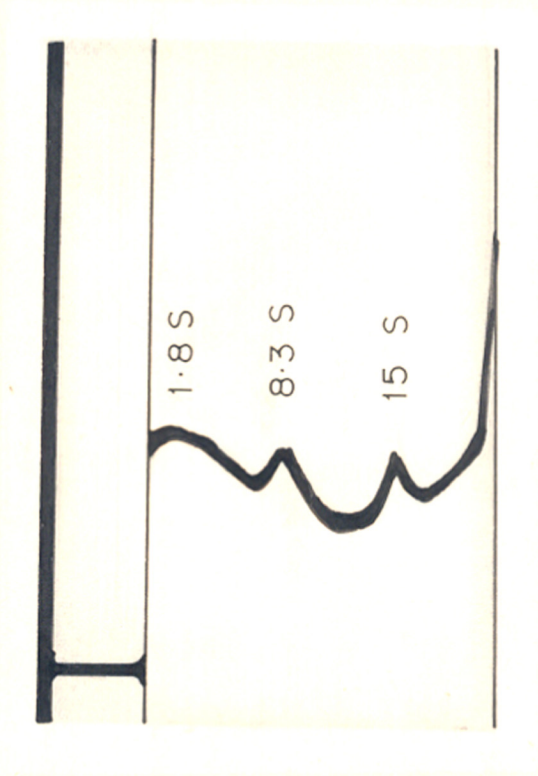


FIGURE 14. AVERAGE WAVELENGTHS OF THE FIRST THREE PEAKS OF THE SPECTRUM OF THE SUN (1961-1962)

The first peak is at 1.8 S, the second at 8.3 S, and the third at 15 S. The first peak is the most prominent, and the second and third peaks are much smaller. The wavelength of the first peak is 1.8 S, the second is 8.3 S, and the third is 15 S.

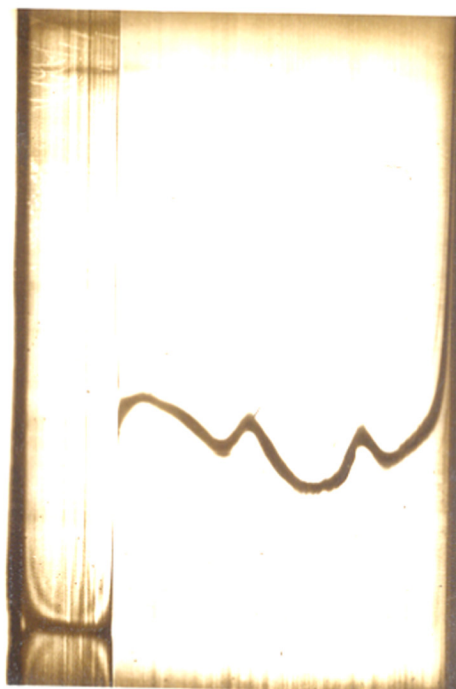


FIGURE 14. SEDIMENTATION-VELOCITY PATTERN OF FRACTION 7
(Table 19)

Sedimentation proceeds from left to right. 0.35 per cent total protein in potassium phosphate buffer, 0.05 M, pH 7. Centrifugation at 59,780 rev./min for 54 min. Rotor temperature 4.6°, diaphragm angle 45°.

peaks were calculated according to the procedure described by Schachman (1957) and they are $S_{20,w}$ 1.8, $S_{20,w}$ 8.3 and $S_{20,w}$ 15.

The results of the purification experiments are shown in Table 19.

TABLE 19
PURIFICATION OF CITRACONASE*

Fraction	Volume (ml)	Total activity (a) units	Protein (b) mg	Specific activity a/b	Recovery (per cent)
1. Cell-free extract (CFE)	120	235	1200	0.2	100
2. CFE after strepto- mycin treatment	120	210	780	0.25	88
3. Ammonium sulfate (0.4 to 0.7)	16.5	165	413	0.40	70
4. Calcium phosphate treated-eluate	33	160	290	0.55	68
5. Ammonium sulfate (0.5 to 0.7)	16	130	140	0.93	55
6. DEAE-cellulose	3	90	30	3	38
7. Ammonium sulfate fractionation (0.5 to 0.65)	3	52	10.5	4.6 to 5	22

* From 15 g of wet packed cells

The above procedures have yielded a citraconase preparation free from mesaconase and malease and resulted in a 25-fold purification. This preparation still contains at least two other components.

The fraction with a specific activity 4.6 has been used in the studies on the properties of the enzyme described in the following pages.

Procedures which were not useful in purification

In addition to those described in Table 19 other conventional procedures were also tested. These could not be used for the purpose of purification of the enzyme. Either they irreversibly inactivated the enzyme or did not enhance the specific activity of the product. These are briefly described below.

(1) Heat treatment

The cell-free extract (Fraction 1, Table 19) was rapidly heated to 50°, then kept in a thermostat at 50° for 5 min and immediately cooled in an ice bath. The precipitate was centrifuged off and the supernatant tested for citraconase activity. Both the supernatant and the precipitate were inactive. Even when the heat treatment was conducted in presence of 10^{-2} M citraconate, the enzyme activity was irreversibly lost.

(2) Acid treatment

Adjustment of pH of the sonicate (Fraction 1, Table 19) to 5 in the cold by a preliminary rough adjustment with N acetic acid and subsequent addition of acetate buffer (pH 5) to 0.05 M resulted in a significant decrease of both total (20 per cent of original) and specific (0.1 from the original 0.2) activities.

(3) Acetone fractionation

Both fractions 1 and 3 (Table 19) were used for acetone fractionation. Cold acetone (-15°C) was added to the enzyme solution in a dry ice alcohol bath at -5° and fractions were removed at acetone concentrations of 40, 50, 60, 70 and 80 per cent (v/v) and the final

temperature was not allowed to rise above 3° to 5°C. At each stage, the precipitate was collected by centrifugation and was dissolved in cold 0.02 M potassium phosphate buffer, pH 7, and dialyzed against 100 volumes of the same buffer for 2 hr. The maximum activity was present (25 to 30 per cent of the original activity) in the 60 to 70 per cent acetone fraction, but its specific activity was only a third of that of the starting material. The total recovery of enzyme was about 35 per cent. Thus there was a marked decrease in total and specific activities of citraconase.

(4) Ethanol fractionation

The procedure was similar to acetone fractionation: only fraction 3 (Table 19) was tested. Cold (-15°) alcohol (95 per cent) was used. This method also was not successful. The enzyme precipitated between 50 and 70 per cent concentration. Its specific activity was 0.3 and the yield 45 per cent.

(5) Hydroxylapatite treatment

Fraction 5 (Table 19) was dialysed against 0.01 M potassium phosphate buffer pH 7 for 8 hr with stirring. The dialysed solution was treated with hydroxylapatite prepared according to Tiselius *et al.* (1956). The enzyme was adsorbed by the adsorbent. Elution of the adsorbed enzyme was tried with potassium phosphate buffer, pH 7 of different concentrations (0.05, 0.1, 0.2 and 0.5 M) as well as by 0.3 M K_2HPO_4 solution, 0.2 M Tris-HCl buffer, pH 7.5 and 0.2 M succinate buffer pH 6. The enzyme could not be eluted by any of the solutions mentioned above. When the adsorbent along with the adsorbed enzyme was tested for citraconase activity it was found that there was complete loss of enzyme activity.

(6) Keilin-Hartree (1938) gel treatment

The fraction 7 (Table 19) was treated with calcium phosphate gel (gel:protein = 5:1) prepared according to Keilin and Hartree (1938). The gel did not adsorb the enzyme and there was no further increase in the specific activity of citraconase at this stage.

(7) Alumina C_γ treatment

Fraction 5 (Table 19) was treated with alumina C_γ (gel:protein = 5:1). The gel supernatant was tested for citraconase activity. The gel did not adsorb the enzyme under the conditions tested and there was no purification of the enzyme.

III. PROPERTIES OF CITRACONASE

The properties of citraconase were studied using the partially purified enzyme preparation (Fraction 7, Table 19) of specific activity 4.6.

a) Effect of pH on enzyme activity

The effect of pH on enzyme activity was studied at pH values ranging from 5.5 to 10. The following buffers were used: acetate buffer for pH 5.5, phosphate buffer for pH 6.2, 6.8 and 7, Tris-HCl buffer for pH 7.5, 7.8, 8.2 and 8.7 and glycine-NaOH buffer for pH 10. The assay system contained 100 μ moles of buffer, 5 μ moles of potassium citraconate and 0.05 ml of anaerobically activated citraconase in a total volume of 1 ml. The pH activity curve for citraconase is shown in Fig. 15.

The optimum pH for activity is 7.5. The activity falls off markedly on both sides of the optimum pH; there is only 43 per cent and

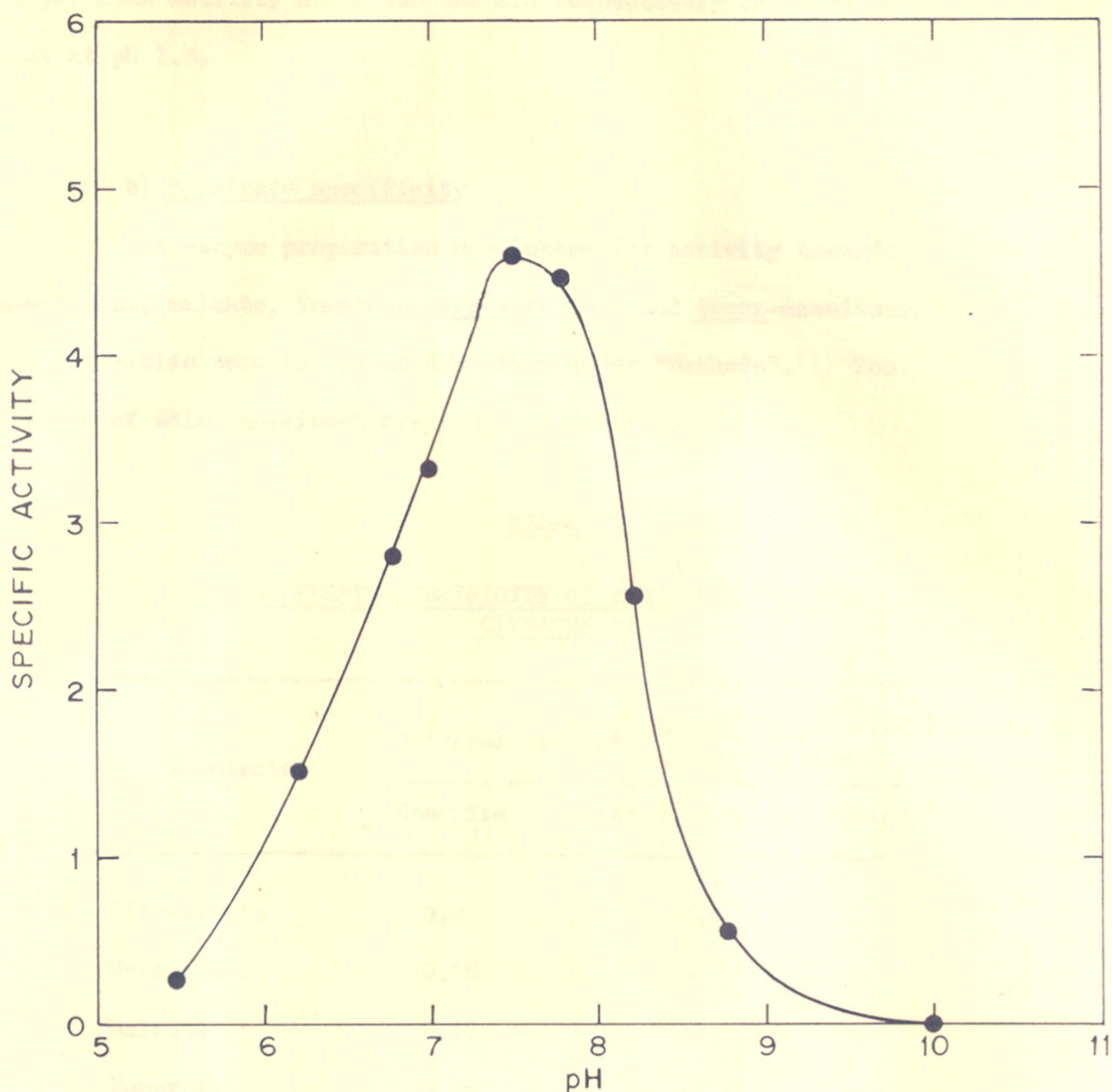


FIG. 15. EFFECT OF pH ON THE ACTIVITY OF CITRACONASE.

The assay system contained 0.1 M of acetate (5.5), phosphate (6.2; 6.8 and 7.0), Tris-HCl (7.5; 7.8; 8.2 and 8.7) and glycine - NaOH (pH 10.0) buffers. 0.05 ml of the activated enzyme was added to the buffer and 5 μ moles K-citraconate added. Volume 1 ml, incubation 10 minutes at 30°. Details of assay are given in the text.

29 per cent activity at pH 6.5 and 8.5 respectively compared to that at pH 7.5.

b) Substrate specificity

The enzyme preparation was tested for activity towards mesaconate, maleate, fumarate, cis-aconitate and trans-aconitate. The activities were tested as described under "Methods". The results of this experiment are shown in Table 20.

TABLE 20

SUBSTRATE SPECIFICITY OF PARTIALLY PURIFIED CITRACONASE

Substrate	Original extract		Partially purified preparation	
	Specific activity	Ratio*	Specific activity	Ratio*
Citraconate	0.2	1.0	4.6	1
Mesaconate	0.18	1.1	-	∞
Maleate	0.10	2.0	-	∞
Fumarate	0.12	1.7	-	∞
<u>cis</u> -Aconitate	0.10	2.0	0.005	920
<u>trans</u> -Aconitate	0	-	0	-

*Ratio of citraconase activity to the other activity. The other activities thus tested were mesaconase, malease, fumarase, aconitase and aconitate isomerase.

From the results (ratios) it is clear that the other activities which were present in the original extract are absent in the partially purified citraconase.

Other enzymatic activities

The enzyme preparation was tested for the presence of the following enzymes: lactate dehydrogenase, NADH oxidase, L-malate dehydrogenase, NADP-isocitrate dehydrogenase and citrase. Lactate dehydrogenase, NADH oxidase, malate dehydrogenase and citrase were not detectable. Isocitrate dehydrogenase (specific activity = 0.01) was present in low concentration.

c) Effect of substrate concentration on enzyme activity

The saturation of the enzyme by the substrate was investigated in the range of 0.5 to 10 mM. The rate of citraconate hydration at different citraconate concentrations is shown in Fig. 16. The maximal velocity is attained at 5 mM citraconate. Concentrations such as 20 mM are not inhibitory but very high concentrations (0.1 to 1 M) are inhibitory. The K_m at pH 7.5 and 30°C is 1.1 to 1.2×10^{-3} M.

d) Cofactors concentration and enzyme activity

The effect of cofactors - glutathione and ferrous ions concentration on the activity of citraconase was studied. The activity of the enzyme was determined at glutathione concentrations ranging from 1 to 40 mM and at a constant concentration of ferrous ions at 1 mM. In the same manner the activity of the enzyme was determined at ferrous ion concentrations ranging from 0.1 to 1.5 mM with the glutathione concentration at 10 mM. All of these refer to concentrations of the respective cofactor in the activation mixture and not the final incubation mixture. The preincubation of the enzyme

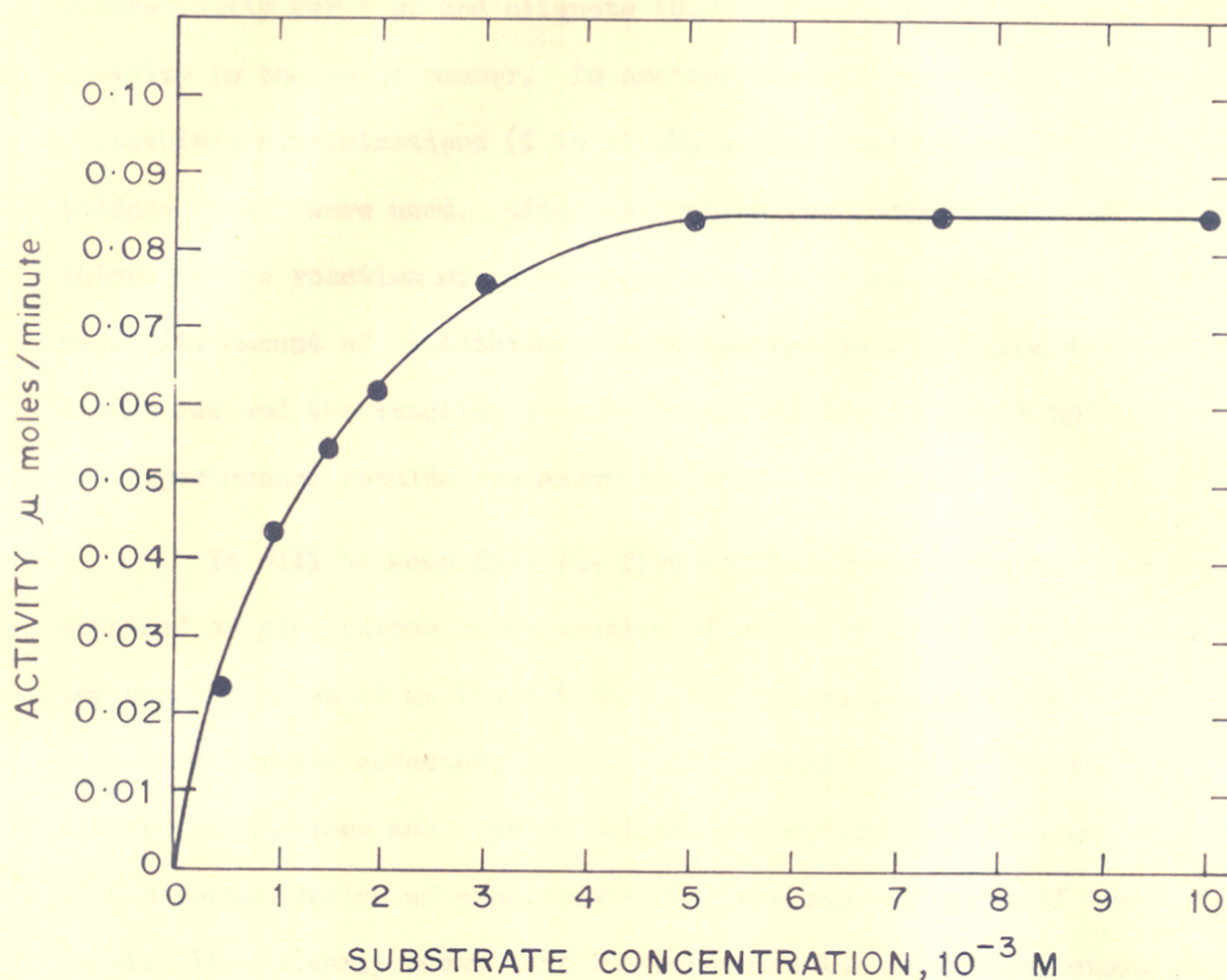


FIG. 16. EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF CITRACONASE.

The reaction mixtures contained different concentrations of citraconate as indicated and 0.1M Tris-HCl buffer (pH 7.5) in a total volume 1ml. The activated enzyme is added to each reaction mixture and incubated 10 minutes at 30°. Other details of assay as in text.

with the sulphydryl compound and ferrous ions was carried out anaerobically for 1 hr and aliquots (0.05 ml) were tested for enzyme activity in the usual manner. In another activation mixture, different glutathione concentrations (1 to 10 mM) and a constant amount of ferrous sulfate (1 mM) were used. After activation the concentration of glutathione in the reaction mixture was made up to 10 mM by addition of the requisite amount of glutathione. Citraconate was added immediately afterwards and the reaction mixture incubated for 10 min at 30°C. The experimental results are shown in Figs. 17 and 18.

It will be seen from the figures that maximum activity is obtained at glutathione concentration of at least 10 mM and ferrous ion concentration of at least 1 mM in the activation mixture. The K_m values for the cofactors are $3.1 \times 10^{-3} M$ and $1.2 \times 10^{-4} M$ for reduced glutathione and ferrous sulfate respectively. The results of the second series of experiments were the same as those of the first. This clearly shows that the concentration of the cofactors in the activation mixture is critical and not their concentration in the final reaction mixture.

e) Effect of reducing and sulphydryl compounds

The effect of some reducing and sulphydryl compounds on citraconase activity was studied. Reduced glutathione, cysteine, mercaptoethanol, thioglycollic acid and ascorbic acid were used. The compounds (10 mM) were incubated with the enzyme preparation with and without the addition of ferrous iron (1 mM) anaerobically for 1 hr. Aliquots of the activation mixture were tested for enzyme activity and the results are given in Table 21.

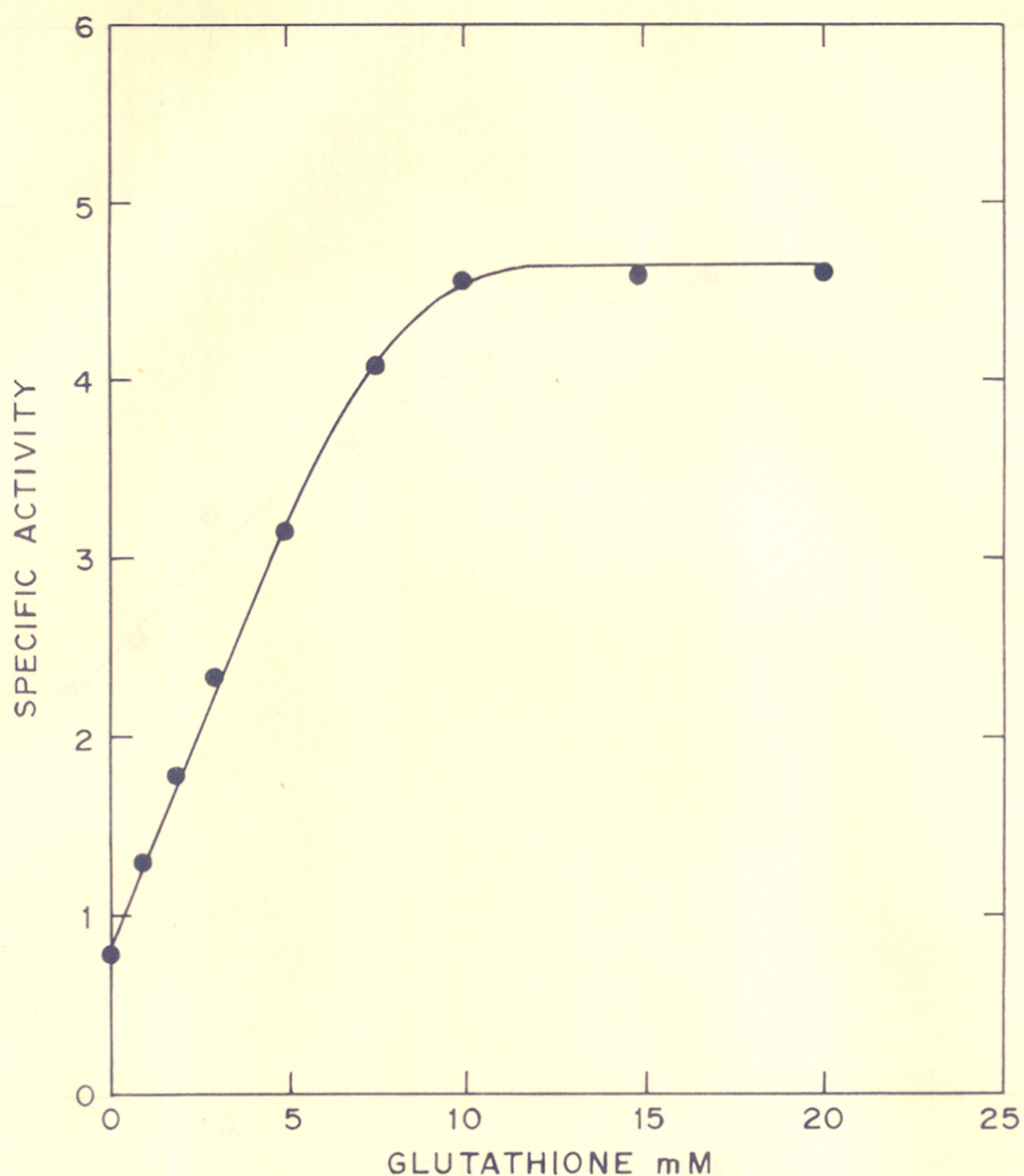


FIG. 17. EFFECT OF GLUTATHIONE CONCENTRATION ON THE ACTIVITY OF CITRACONASE.

The activation mixture contained different concentrations of glutathione as indicated and 1 mM ferrous ions. After anaerobic incubation for 1 hr. 0.05 aliquot of the activation mixture used for citraconase activity. Other details of assay as in text.

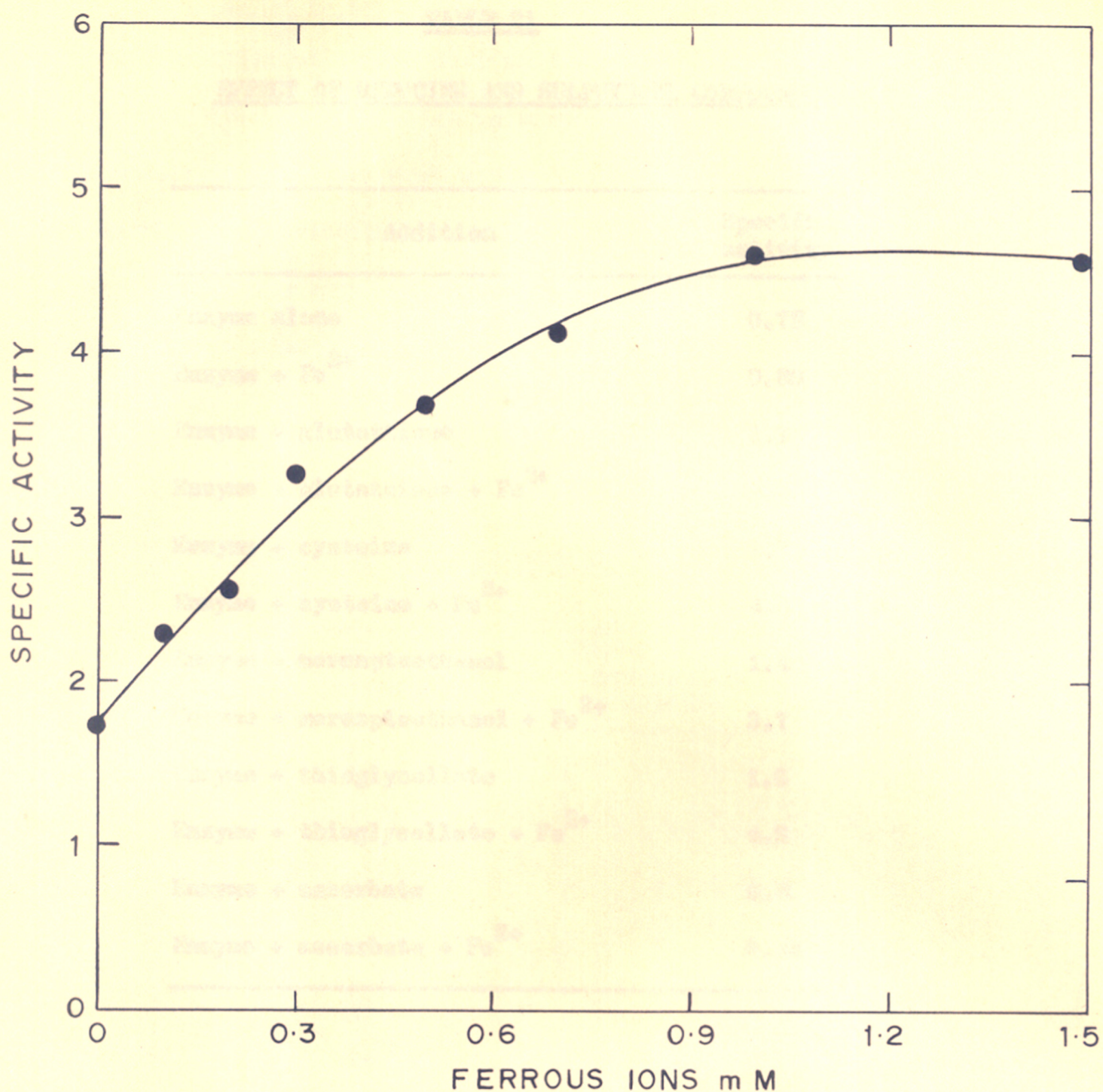


FIG. 18. EFFECT OF FERROUS ION CONCENTRATION ON THE ACTIVITY OF CITRACONASE.

The activation mixture contained 10 m M glutathione and different concentrations of ferrous ion as indicated. After anaerobic incubation for 1 hr. 0.05 ml aliquot of the activation mixture used for citraconase activity. Other details of assay as in text.

TABLE 21

EFFECT OF REDUCING AND SULPHYDRYL COMPOUNDS

Addition	Specific activity
Enzyme alone	0.78
Enzyme + Fe^{2+}	0.80
Enzyme + glutathione	1.7
Enzyme + glutathione + Fe^{2+}	4.6
Enzyme + cysteine	1.7
Enzyme + cysteine + Fe^{2+}	4.6
Enzyme + mercaptoethanol	1.4
Enzyme + mercaptoethanol + Fe^{2+}	3.7
Enzyme + thioglycollate	1.6
Enzyme + thioglycollate + Fe^{2+}	4.3
Enzyme + ascorbate	0.8
Enzyme + ascorbate + Fe^{2+}	0.85

It may be seen from the results in Table 21 that all of the sulphydryl compounds tested increase the activity of citraconase when the enzyme is preincubated with them along with ferrous ions. The extent of activation with all the sulphydryl compounds is more or less the same except for mercaptoethanol, which is only about 80 per cent as effective as other compounds. Ascorbate does not activate citraconase.

f) Effect of metal ions on enzyme activity

Metallic chlorides were used in most cases except for zinc, copper and ferrous iron which were added as sulphates. The salts of the metals to be tested were added to the activation mixture at 1 mM along with 10 mM glutathione. They were incubated anaerobically for 1 hr and aliquots from the reaction mixtures were tested for enzyme activity in the usual manner. The results of this experiment are shown in Table 22.

TABLE 22

EFFECT OF METAL IONS ON THE ACTIVITY OF CITRACONASE

Metallic ions added	Concentration in activation mixture	Specific activity
Nil		1.6
Fe ²⁺	1 x 10 ⁻³ M	4.6
Mn ²⁺	"	1.6
Mg ²⁺	"	1.6
Zn ²⁺	"	1.6
Ca ²⁺	"	1.65
Cu ²⁺	"	0.32
Hg ²⁺	"	0.31
Na ⁺	"	1.6
NH ₄ ⁺	"	1.55
Molybdate	"	1.6

It is seen from the results that among the metallic ions tested ferrous ions alone activate citraconase in conjunction with glutathione. Copper and mercury inhibit the enzyme activity under these conditions to the extent of about 80 per cent even though an excess of glutathione is present.

g) Effect of some metal-complexing agents and inhibitors
on citraconase

The enzyme was preincubated anaerobically with glutathione or ferrous ions or with both for 1 hr and aliquots (0.05 ml) were taken for activity determinations. The compound to be tested was added to the reaction mixture for assay, containing 100 μ moles of Tris-buffer, pH 7.5 and 17.5 μ g of enzyme protein and incubated for 15 min at 30°. The reaction was initiated by adding 5 μ moles of substrate. The final volume of the reaction mixture was 1 ml. Appropriate blanks in which the enzyme and the substrate were individually omitted were run. The results of this experiment are presented in Table 23.

It may be seen from the results in Table 23 that pyrophosphate and the iron-complexing agents, phenanthroline, 8-hydroxyquinoline and α, α' -bipyridyl have an inhibitory effect on the enzyme. Iodoacetate and p-hydroxymercuribenzoate which combine with free sulphydryl groups also inhibit the activity. These results suggest that free-sulphydryl groups and ferrous ions are essential for enzyme activity.

TABLE 23

EFFECT OF METAL COMPLEXING AGENTS AND INHIBITORS ON CITRACONASE ACTIVITY

Compound(s) tested	Concentration in the reaction mixture (mM)	Specific activity
1. Nil (control)		0.75
2. p-Hydroxymercuribenzoate	0.1	0.12
p-Hydroxymercuribenzoate	1.0	0.10
3. O-Phenanthroline	1.0	0.04
O-Phenanthroline	2.0	0.03
4. Iodoacetate	1.0	0.12
5. Glutathione	0.5	1.7
6. Fe ²⁺	0.05	0.8
7. Glutathione + Fe ²⁺	0.5 0.05	4.6
8. Fe + p-Hydroxymercuribenzoate	0.05 1.0	0.2
9. Glutathione + Iodoacetate	0.5 1.0	0.15
10. Glutathione + O-Phenanthroline	0.5 1.0	1.1
11. Glutathione + EDTA	0.5 1.0	1.3
12. Glutathione + EDTA + Fe ²⁺	0.5 1.0 0.05	1.5
13. Glutathione + O-Phenanthroline + Fe ²⁺	0.5 1.0 0.05	1.5
14. Glutathione + α, α' -Bipyridyl	0.5 1.0	1.2
15. Glutathione + 8-Hydroxyquinoline	0.5 1.0	1.2
16. Glutathione + Pyrophosphate	0.5 1.0	1.1

DISCUSSION

It can be seen from the results of the experiments on the purification of citraconase that a partial purification of about 25-fold from an initial specific activity of 0.2 to a final specific activity of 4.6 to 5.0 has been achieved using the various steps described. The final citraconase preparation obtained by the purification procedure described is still not homogeneous and contains at least two other components as revealed by the ultracentrifugal pattern. Application of some of the conventional procedures usually employed for purification of enzymes such as preferential heat denaturation, solvent fractionation and treatment with adsorbents other than calcium phosphate gel has resulted either in an irreversible inactivation of the enzyme or in getting a preparation the specific activity of which is not enhanced.

Among the hydratases of branched C_5 -dicarboxylates, mesaconase of C.tetanomorphum (Blair & Barker, 1966) has been the best purified (about 100-fold purification with an initial specific activity of 6 to a final specific activity of about 600). Mesaconase which is similar to citraconase has been purified only about 8-fold (from an initial specific activity of 0.3 to a final specific activity of 2.35) from P.fluorescens (Katsuki et al. 1962). Purification of itaconase which also catalyses the hydration of free itaconate to citramalate without any activation (Brightman & Martin, 1961) has not been reported so far.

Citraconase requires a sulphydryl compound and ferrous ions for activity and it resembles mesaconase of C.tetanomorphum (Blair & Barker, 1966) and aconitase from animal, microbial and plant tissues (Dickman & Cloutier, 1950; Morrison, 1954; Rahatekar & Rao, 1963) in this property. Mesaconase of P.fluorescens also requires ferrous ions for activity (Katsuki et al. 1962) but it is completely inhibited by cysteine and not by other sulphydryl compounds - a finding which has not been explained. The results of the experiments on the activation of citraconase by sulphydryl compound and ferrous ions show that anaerobic incubation of the enzyme with the activators for 1 hr at 30° is necessary for the maximal activity of the enzyme. In this respect citraconase is similar to mesaconase system of C.tetanomorphum (Blair & Barker, 1966) and to bacterial L-tartrate dehydratase (Hurlbert & Jacoby, 1964). On the other hand aconitase appears to be more readily activated by these cofactors. It is also seen that by using the procedure described above for activation the diminution in activity of aged enzyme solutions can be restored.

Experiments on the effect of reducing and sulphydryl compounds on citraconase activity indicate that in combination with ferrous ions sulphydryl compounds such as glutathione, cysteine, mercaptoethanol and thioglycollate activate the enzyme. Ascorbate does not activate citraconase. It is also seen that among the metallic ions tested for activation of citraconase, only ferrous ions activate the enzyme in conjunction with glutathione. The activation of mesaconase system of C.tetanomorphum can also be carried out by cysteine, mercaptoethanol or glutathione as sulphydryl compound.

Bacterial L-tartrate dehydratase is also activated either by cysteine or glutathione. Unpublished experiments (of Wang & Barker and reported by Blair & Barker, 1966) have shown that other divalent cations cannot replace ferrous ions in the activation process of mesaconase. Similarly none of the other metals tested except ferrous ions was effective in the activation of bacterial L-tartrate dehydratase (Hurlbert & Jacoby, 1964). Thus citraconase is similar to both mesaconase of C.tetanomorphum and bacterial L-tartrate dehydratase in this respect also.

The optimum pH for citraconase activity is 7.5. The activity falls off markedly on both sides of the optimum pH. The optimum pH for mesaconases of C.tetanomorphum and P.fluorescens is reported to be 8.2. There is only 57 per cent activity of citraconase at this pH. Thus there seems to be some dissimilarity among these enzymes.

The K_m for citraconate at pH 7.5 and 30° is 1.1 to 1.2×10^{-3} M. This value cannot be strictly compared with the values reported for mesaconases of C.tetanomorphum and P.fluorescens as the latter have been determined for the reverse reaction. Attempts to show a reversal of the citraconase reaction were not successful.

The partially purified citraconase does not act on other unsaturated di- and tri-carboxylic acids such as mesaconate, maleate, fumarate, cis-aconitate and trans-aconitate though the activity towards these substrates was present in appreciable amounts in the crude cell-free extract. The purified enzyme preparation does not have other activities such as lactate dehydrogenase, NADH oxidase, malate dehydrogenase and citrase while it shows low isocitrate dehydrogenase activity. The two other components detected in the ultracentrifuge

therefore do not represent any of the enzyme mentioned here.

The results of the experiments on the effect of metal-complexing agents and inhibitors on citraconase show that pyrophosphate and iron-complexing agents such as o-phenanthroline, 8-hydroxyquinoline and α, α' -bipyridyl have an inhibitory effect on the enzyme. Similar results have been reported for mesaconase of C.tetanomorphum. Iodoacetate and p-hydroxymercuribenzoate which combine with sulphhydryl groups also inhibit citraconase activity. Mesaconase of P.fluorescens is also inhibited by iodoacetate (Katsuki et al. 1962). The results suggest that free-sulphhydryl groups and ferrous ions are necessary for citraconase activity. Thus citraconase is similar to other hydratases such as aconitase from animal and microbial sources and mesaconase from microorganisms.

SECTION 3(iv)STEREOCHEMISTRY OF THE ENZYMATIC HYDRATION
OF CITRACONATE (AND MESACONATE)

Since citraconase-catalyzed hydration is a new reaction, it was of interest to study the stereochemistry of this reaction. This has been made a little easier in view of the work of von der M $\ddot{u}$ hl et al. (1965). These workers prepared deutero-(+)-citramalate from mesaconic acid in presence of deuterium oxide and mesaconase from C.tetanomorphum and also synthesized chemically DL-threocitramalate. An examination of the NMR spectra of two compounds showed that the addition of water (or (deuterium oxide) occurs by a trans-mechanism. Stereospecificity of other hydration reactions (formation of malate from fumarate) [(Fisher et al. 1955; England and Colowick, 1955, 1956; Farrar et al. 1957) and of the formation of citrate from cis-aconitate (England and Colowick, 1957)] have also been extensively studied and will be discussed later.

Using a similar approach (i.e., NMR examination of the methyl esters of the deuterated acids), it is found here that the mode of addition of water to citraconate and mesaconate, and to fumarate and maleate in the presence of the pseudomonad enzymes all occurs by a trans-mechanism.

1. EXPERIMENTS ON ENZYMATIC HYDRATION OF CITRACONATE AND MESACONATE

Deuterated (+)- and (-)-citramalates were prepared from citraconate and mesaconate by the procedure which has been described before, but with the following modifications.

Cells (10 g instead of 5 g) of citraconate-culture grown on citraconate as the sole carbon source and kept frozen, were thawed and suspended in 50 ml of 0.02 M potassium phosphate buffer, pH 7.0 prepared in deuterium oxide (heavy water with isotopic purity more than 99.4 per cent made available by the Atomic Energy Establishment, Trombay). The substrates for the reaction, potassium citraconate and potassium mesaconate, were prepared by dissolving solid citraconic acid and mesaconic acid in deuterium oxide and neutralising the solutions with potassium hydroxide solution prepared in deuterium oxide. The reaction mixture contained in a final volume of 50 ml the following components: potassium citraconate or potassium mesaconate, 20 mmoles; cell-free extract containing about 900 mg protein, 27 ml; cysteine (neutralised just before use with potassium hydroxide solution in deuterium oxide), 500 μ moles and ferrous sulphate, 50 μ moles. After the incubation period 5 μ l aliquots of both the reaction mixtures were chromatographed using the solvent system - butanol:formic acid:water (4:1.5:1). The developed chromatograms indicated that in the case of citraconate reaction, citraconate had completely disappeared and was converted to citramalate. In the case of the mesaconate reaction most of the mesaconate had been converted to citramalate and chromatographic examination showed only a faint spot for unconverted mesaconate.

(+)- and (-)-Citramalic acids from the above reaction mixtures were obtained according to the procedure described in Section 3(ii). They were then converted into methyl esters (as described under "Methods"). The pure, distilled dimethyl esters were taken up in carbon tetrachloride and subjected to IR and NMR spectroscopy. For comparison dimethyl ester of DL-citramalic acid was also prepared as described above. The NMR spectra of DL- as well as the optically active citramalic acids are shown in Fig. 19.

2. EXPERIMENTS ON ENZYMATIC HYDRATION OF MALEATE AND FUMARATE

The cell-free extract of the pseudomonad isolate also brings about the hydration of maleate and fumarate with the formation of (+)- and (-)-malic acids. Malease is distinct from citraconase and is covered later in Section 3(vi). The mode of addition of water to the two unsaturated acids catalysed by the enzyme (as well as the absolute configuration of the malic acids formed) has been investigated in the same manner as in the case of the enzymatic hydration of citraconate and mesaconate. The experimental set-up was the same as that described for experiments with citraconate and mesaconate. The cell-free extract of the organism and the substrates (potassium maleate and potassium fumarate) were prepared as described previously for the preparation of deuterio-citramalate. At the end of a 48-hr incubation period, aliquots of the reaction mixture were chromatographed using butanol-formic acid-water solvent system. It was found that the reaction mixtures contained malic as well as unreacted maleic or fumaric acid. The deuterio-malic acids from both the reaction mixtures were isolated by ion-exchange

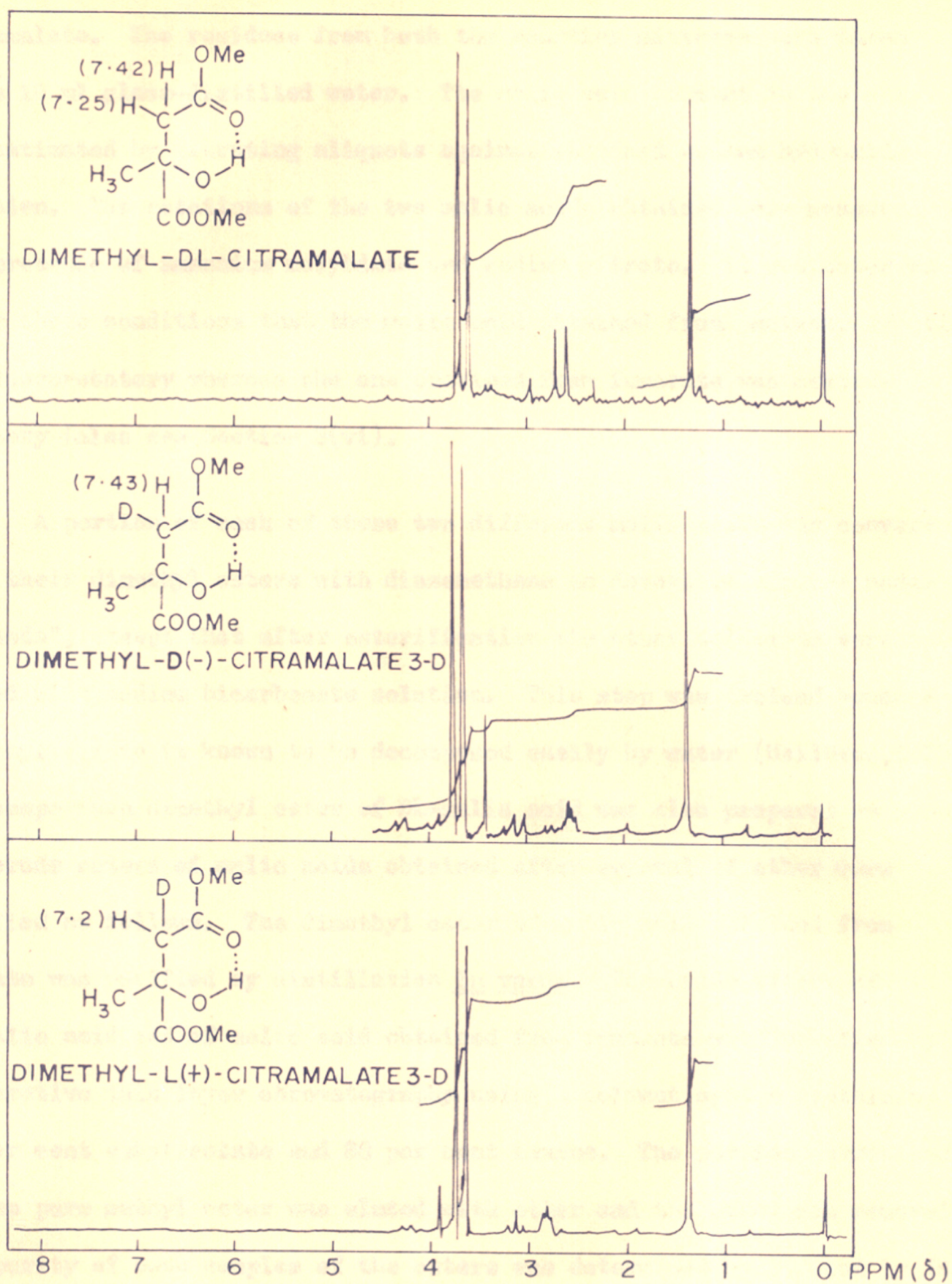


FIG. 19. NMR SPECTRA OF DIMETHYL ESTERS OF DL-CITRAMALATE, D(-)-CITRAMALATE-3D AND L(+)-CITRAMALATE-3D.

column chromatography using the same procedure as described for deuterio-citramalate. The residues from both the reaction mixtures were taken up in 10 ml glass-distilled water. The malic acid content in the solution was estimated by titrating aliquots against standard sodium hydroxide solution. The rotations of the two malic acids obtained were measured in the presence of ammonium molybdate and sodium citrate. It was observed under these conditions that the malic acid obtained from maleate reaction was laevorotatory whereas the one obtained from fumarate was dextro-rotatory (also see Section 3(vi)).

A portion of each of these two different malic acids was converted into their dimethyl esters with diazomethane as described earlier under "Methods", except that after esterification the ether solutions were not washed with sodium bicarbonate solution. This step was avoided because dimethyl malate is known to be decomposed easily by water (Heilbron, 1953). For comparison dimethyl ester of DL-malic acid was also prepared as above. The crude esters of malic acids obtained after removal of ether were purified as follows. The dimethyl ester of malic acid obtained from maleate was purified by distillation in vacuo. The crude esters of DL-malic acid and of malic acid obtained from fumarate were purified by preparative thin layer chromatography using a solvent system containing 12 per cent ethylacetate and 88 per cent hexane. The portion corresponding to the pure methyl ester was eluted with ether and the ether was removed. The purity of both samples of the esters was determined by TLC and by IR and they were found to be pure by these criteria. The NMR spectra of the dimethyl esters of the three malic acids in carbon tetrachloride solution were taken as before and are given in Fig. 20.

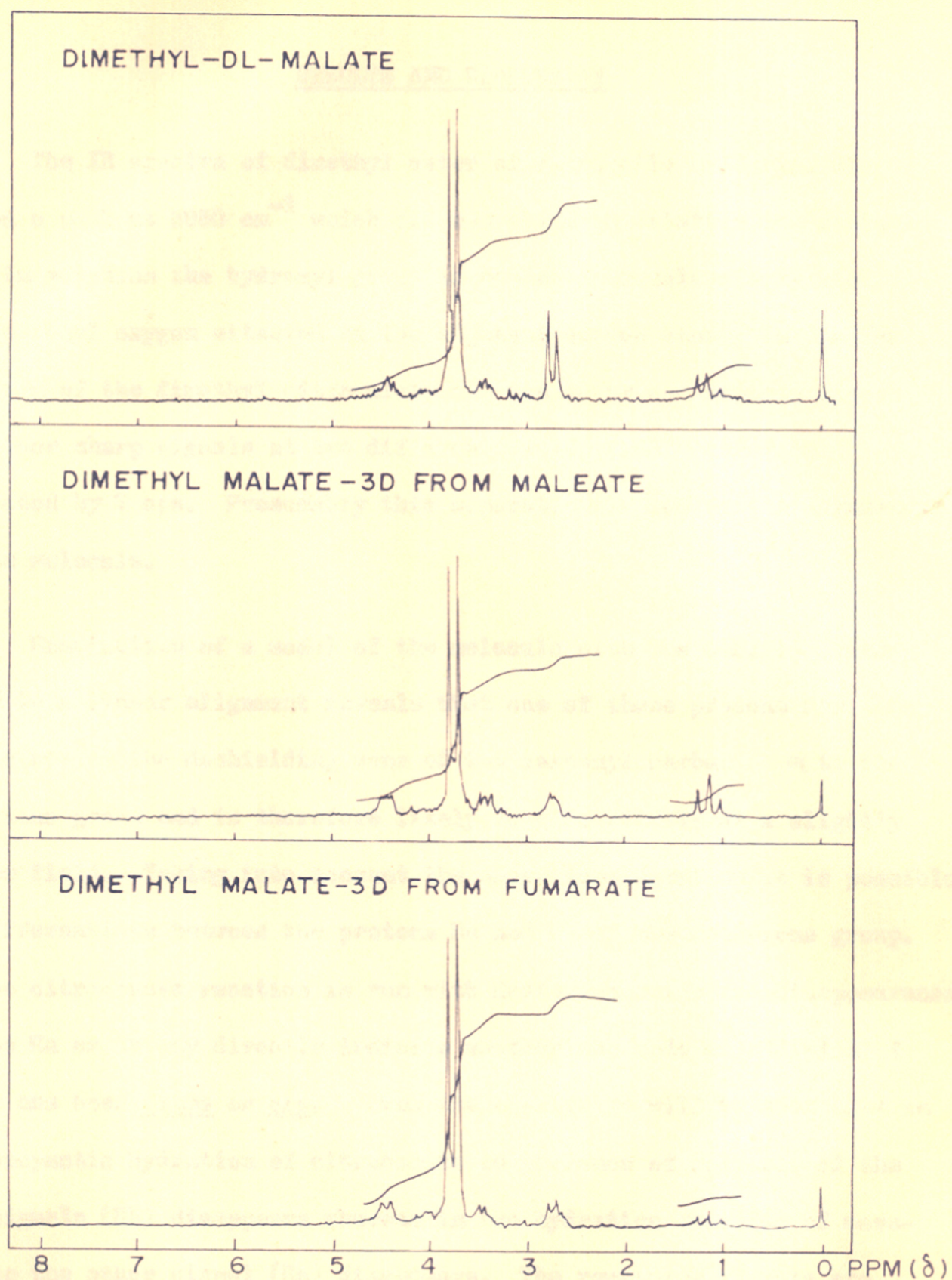


FIG. 20. NMR SPECTRA OF DIMETHYL ESTERS OF DL-MALIC ACID, AND DEUDERATED MALIC ACIDS FROM MALEATE AND FUMARATE REACTIONS

RESULTS AND DISCUSSION

The IR spectra of dimethyl ester of citramalic acid Fig. 21 showed a peak at 3050 cm^{-1} which did not shift on dilution indicating that in solution the hydroxyl group is bonded intramolecularly with the carbonyl oxygen situated on the adjacent carbon atom. In the NMR spectrum of the dimethyl citramalate the two methylene protons appear as rather sharp signals at two different fields 7.42τ and 7.25τ separated by 7 cps. Presumably this separation is due to the asymmetry of the molecule.

Examination of a model of the molecule with the O-H...O bond fixed in a linear alignment reveals that one of these protons may come very close to the deshielding zone of the carbonyl carbon, α to the methylene group and is therefore likely to give signals at a slightly higher field. Taking into account the above consideration it is possible to differentiate between the protons Ha and Hb of the methylene group. If the citraconase reaction is run with deuterium oxide the disappearance of the Ha or Hb may directly indicate whether the mode of addition of water has been trans or cis. From the spectra it will be seen that in the enzymatic hydration of citraconate in presence of D_2O , one of the two signals (Hb) disappears whereas in the hydration reaction of mesaconate the other signal (Ha) disappears. The remaining signals are split into three peaks since the introduction of deuterium on the same carbon converts the system into an AB_2 type spin-coupled one. The same phenomenon also takes place in the hydration reaction of maleate and fumarate by the enzymatic action. Since the stereochemistry of deuterio-malic acid produced by the enzyme fumarase in D_2O is well known,

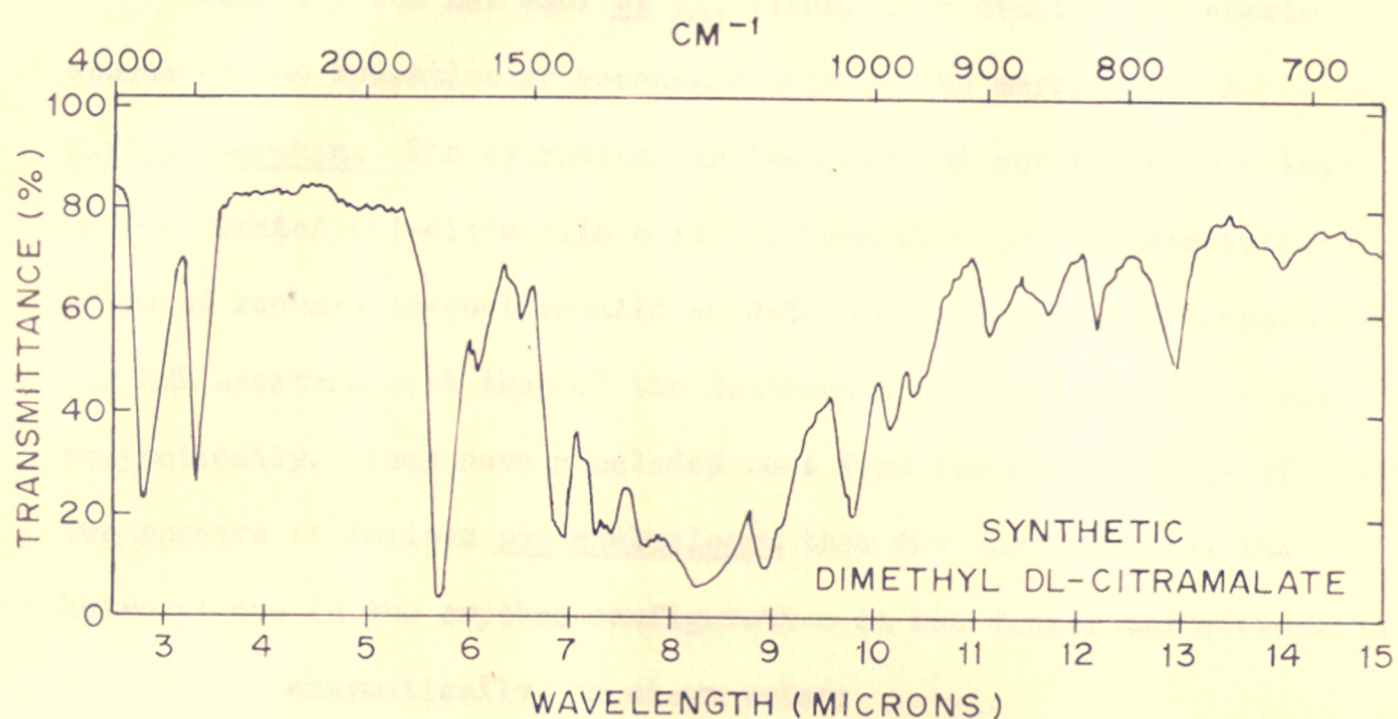
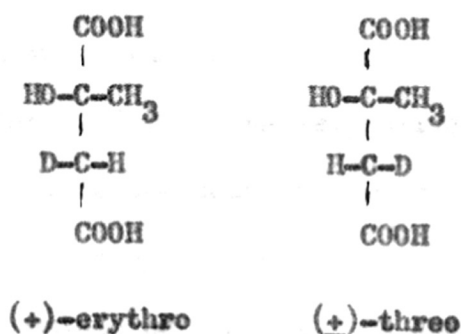


FIG. 21. IR SPECTRUM OF DIMETHYL ESTER OF DL-CITRAMALIC ACID.

the present work constitutes direct proof that the modes of addition of water in both the systems, viz. citraconate and mesaconate, maleate and fumarate are by a trans mechanism.

Recently von der Muhl et al. (1965) have studied the steric course of the hydration of mesaconic acid by the mesaconase of C.tetanomorphum. The hydration has been carried out in heavy water and deuterated (+)-citramalic acid has been obtained. These authors prepared racemic threocitramalic acid-3D synthetically and compared its NMR spectrum with that of the deuterated citramalic acid formed enzymatically. They have concluded that from the non-identity of the two spectra it follows per exclusionem that the deuterium and the hydroxyl are in the erythro-configuration in the deuterated citramalic acid formed enzymatically, as shown below:



The enzymatic addition of water to mesaconic acid is found to follow in a formal sense through a trans mechanism.

There are two contradictory interpretations of the results of the hydration of fumarate. One group which includes Farrar et al. (1957) and England and Colowick (1955, 1956) holds that the reaction occurs by a cis-addition. The other group (Gawron & Fondy, 1959 and Anet, 1960) have more convincing evidence that the reaction occurs by a trans-addition. Farrar et al. (1957) came to the erroneous

conclusion that the addition of water to fumarate is a cis addition when catalysed by fumarase. Their argument was that in the monodeutero-L-malate formed when the reaction was carried out in D_2O , the protons on carbon atoms 2 and 3 are gauche to one another, i.e., the addition of water is in cis manner. If the addition is trans, then the protons should be trans. These particular relations hold only if the carboxyl groups are trans and staggered in the most stable conformation in the final product, L-malic acid. The configuration of the mono deutero-L-malate formed in this case was assumed by observing the dipolar broadening of the proton magnetic resonance absorption in solid $DOOC.CH(D).CH(OD).COOD$. Krasna (1958) also found that fumarase of Proteus vulgaris catalysed the formation of the same monodeutero-L-malic acid as described by Farrar et al. (1957) and assigned a gauche configuration for protons on carbons 2 and 3 in the NMR absorption spectrum of the monodeutero-L-malic acid obtained. Englard (1958) investigated the mechanism of the enzymatic amination and hydration of fumarate with aspartase and fumarase respectively present in Bacterium cadaveris. He made the same assumptions as those of Farrar et al. (1957) and used again the mammalian fumarase reaction product, monodeuterated malate as a model. He concluded that bacterial fumarase also catalyses a stereospecific cis addition of water across the carbon-carbon double bond of fumarate. Later experiments by other investigators (Gawron & Fondy, 1959; Anet, 1960) revealed however, that the results in these instances mentioned above must have been interpreted according to a wrong assumption that the two carboxyls of malic acid are obligatorily trans and parallel. Anet (1960) reported an unambiguous synthesis of threeo-3-deutero-DL-malic acid and has shown that the NMR spectrum of this compound is different from that

of the monodeutero-L-malic acid obtained by Farrar et al. (1957). He has shown that the deuterated compound obtained by the latter authors is erythro and not threo and that the addition of water to fumaric acid is through a trans mechanism and not cis as had been deduced previously by Farrar et al. (1957). Gawron and Fondy (1959) have also prepared the racemic 3-monodeutero-DL-malic acid by a stereospecific synthesis by trans lithium aluminium deuteride opening of the oxide ring of 3,4-epoxy-2,5-dimethoxy tetrahydrofuran. They compared the stereochemical configuration of this compound with that of the product of the fumarase reaction. And they observed that the fumarase-catalysed hydration of fumaric acid proceeds via a trans addition and that the trans nature of this addition is in keeping with the trans nature of the cis-aconitase system. According to Farrar et al. (1957) stereochemical assignments for monodeutero-L-malic acid formed by fumarase reaction were based on NMR conclusions and on the assumption that the carboxyl group of malic acid in the solid state are in a trans relationship. Doubts of the correctness of this assumption have been raised by these authors themselves (Farrar et al.) in a personal communication quoted in Gawron and Fondy's paper (1959).

In the current work the methyl esters in which the hydrogen bonding between the tertiary hydroxyl and the β -carbonyl is a stronger force than the weaker-bonded repulsions of the carbomethoxy groups. The steric factors in these methyl esters necessitate that the carbomethoxy groups are fixed gauche to one another and in this disposition only one of the protons of the methylene group (Ha or Hb) may come very close to the deshielding zone of the bonded carbonyl.

Examination of the NMR spectrum of the free malic and citramalic acid in deuterium oxide revealed that the same two methylene protons appear at distinct signals. It is therefore highly probable that even in solution these molecules retain their hydrogen-bonded gauche conformations. Perhaps the energy available from hydrogen bonding may more than compensate that required to overcome the ionic repulsions of the carboxyl groups in the gauche conformation. This is not an unusual behaviour when one considers the helical structures of proteins and nucleic acids. These structures are maintained in solution by hydrogen bonding even against some amount of ionic and other repulsion.

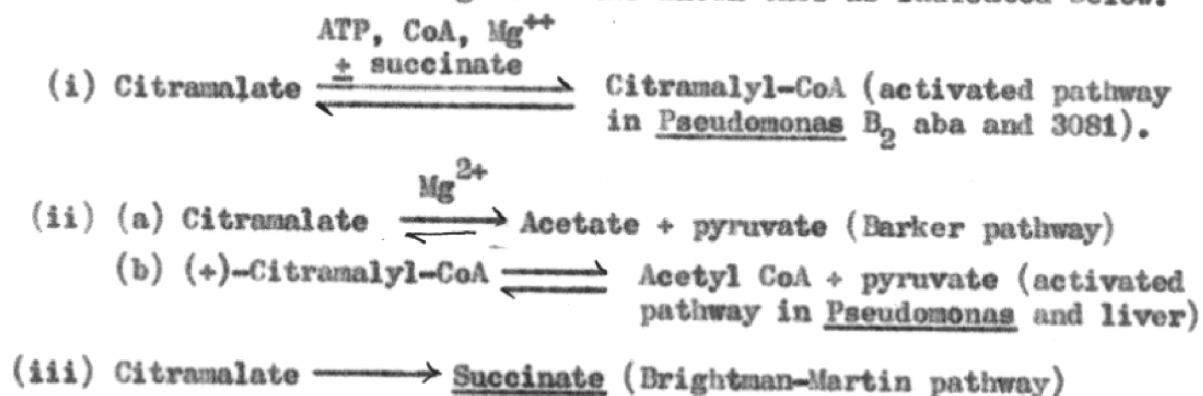
It is, therefore, logical to conclude that in the solid state the carboxyl groups of malate and citramalate are gauche to one another and not trans as supposed by Farrar *et al.* (1957) and that all of these hydration reactions involve trans addition of the elements of water. Citraconase-catalyzed hydration of citraconate also follows this general pattern of trans-addition.

SECTION 3(v)

EXPERIMENTS ON THE DEGRADATION OF (-)-CITRAMALATE

Having established that laevorotatory citramalate is the product of enzymatic hydration of citraconate, attempts were next made to elucidate the mode of breakdown of laevorotatory citramalate. These have not been very conclusive.

It has already been demonstrated in Chapter III (Section 2) that citraconate cells oxidize (-)-citramalate rapidly; in fact (-)-citramalate is oxidized 3 to 6 times faster than the (+)-isomer. The following experiments have been performed with cell-free extracts of citraconate-grown cells of the citraconate culture. The specific objectives were to find out (1) whether (-)-citramalate is activated to (-)-citramalyl-CoA i.e., to an "active" acyl compound, (2) whether pyruvate and acetate or acetyl CoA could be detected in reaction mixtures containing cell-free extract, citraconate, Fe^{2+} , GSH, ATP, CoA and Mg^{++} , or (3) whether reaction products contained any succinate. These reactions would be analogous to the known ones as indicated below.



ACTIVATION OF CITRAMALATE

This was ascertained by testing the hydroxamate formation from (-)- and (+)-isomers of citramalate in a reaction mixture similar to that of Cooper and Kornberg (1964). The procedure is similar to that of Jones *et al.* (1953) for acetate-activation. The results are given in Table 24. There is slightly more of hydroxamate formed from (-)-citramalate in presence of succinate. These are rather low values. They could be taken to indicate that citramalate alone is not activated, but that it is activated by interaction with succinyl-CoA mediated by a CoA-transferase. It is not clear from the experiments whether the feeble activation observed is due to the combined action of an activating system and a deacylase. This aspect of the work was not pursued further.

TABLE 24

HYDROXAMATE FORMATION FROM CITRAMALATE, ATP, CoA

Reaction Mixture*	Substrates	OD _{540 mμ}
1	(+)-Citramalate	515
2	(+)-Citramalate + succinate	570
3	(-)-Citramalate	520
4	(-)-Citramalate + succinate	605
5	With succinate	525
6	Without succinate	510

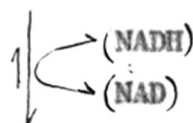
* The complete reaction mixture contained Tris-HCl buffer (pH 7), 100 μmoles; citramalate [(+)- or (-)-isomer as indicated], 50 μmoles when added; CoA, 0.1 ml containing 30 units; ATP, 10 μmoles; reduced GSH, 10 μmoles; hydroxylamine hydrochloride (freshly neutralized), 200 μmoles; enzyme extract, 1 ml; water to a final volume of 2 ml. After 2 hr incubation (30°) the reaction was stopped by the addition of 1 ml each of 3N HCl, FeCl₃ (5 per cent in 0.1 N HCl) and 12 per cent TCA solution. The solutions were centrifuged and the optical density of the supernatants was read at 540 mμ against a blank (containing 2 ml water, 3 ml of a mixture containing TCA, HCl and FeCl₃ solutions).

KETO-ACID FORMATION FROM (-)-CITRAMALATE

Pyruvate is known to be a product of cleavage of citramalate either without activation (Barker, 1956) or after activation (Cooper & Kornberg, 1964; Adler *et al.* 1957). Hence citramalate cleavage, or specifically ketoacid formation from citramalate was studied under different experimental conditions.

Preliminary tests

In order to design a suitable assay procedure for citramalate cleavage resulting in a possible formation of ketoacid, certain preliminary requirements were tested. The formation of pyruvate could be tested by coupling it with lactate dehydrogenase either endogenously present or exogenously added. Hence an oxidation of added NADH and the formation of pyruvate dependent on added citramalate would indicate the presence of a cleavage system. This would be presumably correct provided NADH oxidase and NAD-lactate dehydrogenase were absent in the cell extracts. Formation of lactate under these conditions would indicate the presence of NAD-lactate dehydrogenase.



Lactate

NAD-lactate dehydrogenase activity was tested with sodium lactate at pH 10 and could not be detected in the cell-free extracts under the experimental conditions. This lack of activity could however be compensated by addition of NAD-lactate dehydrogenase.

(b) NADH-oxidase activity of the cell extracts was tested at pH 7.4. The cell extracts do exhibit this activity (0.017 μ mole NADH

oxidized per min per mg protein). About 70 to 75 per cent of this activity was present in the 100,000 x g particles and the rest in the supernatant.

(c) Effect of cell-free extracts on pyruvate

Pyruvate if at all formed by the cleavage of citramalate should not be dissimilated further in presence of the cell extracts. The effect of cell-free extracts on pyruvate was then tested both with (50 μ moles per ml) and without arsenite: the reaction mixture contained CoA (30 units), ATP (10 μ moles) and Mg^{++} (10 μ moles). 80 and 86 per cent of pyruvate disappeared in the presence and absence of arsenite respectively.

Subsequently attempts were made to trap pyruvate or other carbonyl compounds by means of semicarbazide. In the presence of semicarbazide (15 mM), pyruvate is not metabolized by cell extracts.

(d) Test for pyruvate formation

Whether pyruvate was present in the reaction mixture was tested spectrophotometrically as follows. The reaction was conducted at pH 7 as described for the activation experiments (Table 24); semicarbazide (15 mM) was also present in the reaction mixture. After incubation for 2 hr (with (-)-citramalate), the reaction was stopped by acidification to pH 3 with 6N HCl and the solution centrifuged. An aliquot of the supernatant was adjusted to pH 7.4 by the addition of N KOH and tested for the presence of pyruvate by the addition of NADH and muscle lactate dehydrogenase. There was no oxidation of the added NADH. However on addition of pyruvate to the reaction mixture there was a rapid oxidation of NADH, indicating that the reaction mixture contained neither pyruvate nor any substance in the reaction mixture which had inhibited the oxidation of NADH. Besides, when pyruvate is used in place of (-)-citramalate in the reaction mixture

in the above protocol, it is reduced in the presence of NADH and lactate dehydrogenase showing that the reduction occurs even when semicarbazide is present. The citramalate reaction mixtures did not contain any lactate at the end of the experiment when tested according to the procedure of Barker and Summerson (1941).

Keto acid formation from citramalate

Semicarbazide was always present in the reaction mixtures. In some experimental protocols, ATP, CoA, Mg^{++} and succinate were also added to the reaction mixture. For it is known that in certain cleavage systems, citramalate is activated by succinyl-CoA and that the activation experiments have indicated activation of citramalate, although feeble.

The results of such an experiment are shown in Table 25. Keto acid formation was measured according to Friedemann and Haugen (1943).

The results show that a small quantity of a keto acid is formed from all of the three substrates but citraconate and (-)-citramalate are better substrates. Apparently succinate enhances ketoacid formation. Although the amount of keto acid formed was quite small, attempts were made to identify the keto acid(s) formed.

Attempts at identification of the keto acid

In attempts to identify the keto acid(s) formed, laevorotatory citramalate was incubated as above with cell extracts for 2 hr at 30° in the presence of semicarbazide. Then 1 ml of 0.1 per cent 2,4-dinitrophenylhydrazine was added to 6 ml of solution to convert the semicarbazide into 2,4-dinitrophenylhydrazone which was successively extracted into ethyl acetate, 10 per cent sodium carbonate, and ethyl acetate after acidification of the sodium carbonate extract. An aliquot of the sodium carbonate extract

TABLE 25

KETO ACID FORMATION FROM (+)- & (-)-CITRAMALATE

Reaction mixture*	OD ₄₃₅	
	without succinate	with succinate
1. No citramalate	0.095 (0.2)	0.103 (0.26)
2. With (-)-Citramalate	0.135 (0.34)	0.181 (0.45)
3. With (+)-Citramalate	0.100 (0.25)	0.105 (0.26)
4. With citraconate	0.130 (0.33)	0.180 (0.45)
5. With (-)-Citramalate but no enzyme	0.015 (0)	-
6. Pyruvate**	1.0 (1.0)	-

* Reaction mixture contained (μ moles in 2 ml); Tris HCl buffer (pH 7.4), 200; succinate, 2 when added; (-)-citramalate, (+)-citramalate or citraconate, 40; CoA, 30 units; ATP, 10; $MgCl_2$, 10; GSH (reduced) 10; semicarbazide hydrochloride (neutralized), 30; cell extract, 1 ml.

** Contained buffer, and 2.5 μ moles pyruvate.

Incubation 2 hr at 30°. Figures in brackets indicate μ moles of ketoacid.

was used for determining the ratios of optical densities at 420 and at 520 m μ . The ethyl acetate extract of the 2,4-dinitrophenylhydrazone was used for chromatography.

(a) Ratio of optical densities at 420 m μ and 520 m μ

An aliquot of the sodium carbonate extract was treated with 1.5 N NaOH according to the procedure of Friedmann and Haugen (1943). The results are shown in Table 26.

TABLE 26

RATIOS OF OPTICAL DENSITIES AT 420 m μ AND 520 m μ
OF THE 2,4-DINITROPHENYLHYDRAZONES

Reaction mixture	Ratio (420 m μ /520 m μ)
Experimental	1.8 to 1.9
Pyruvate standard	1.28 to 1.33*
α -Ketoglutarate standard	1.9*

*Figures from Friedemann and Haugen (1943) and from standards in the above experiment.

It is clear from the above results that the ratio of the 2,4-DNPH from the experimental reaction mixture corresponds to that of α -ketoglutarate rather than to that of pyruvate.

(b) Chromatography

The ethylacetate extract containing the hydrazone was evaporated to dryness in vacuo at 40°. The dry residue was dissolved in 0.1 ml of 0.1 N sodium hydroxide solution. About 0.5 ml of 0.1 M potassium phosphate

buffer (pH 7.2) was added to the solution and the solution was spotted on Whatman 3 MM paper along with standards. The chromatogram was developed using the solvent system of El Hawary and Thompson (1953). The results are shown in Table 27.

TABLE 27

R_F VALUES OF THE 2,4-DNEH's

<u>Solvent system*</u>	
Experimental	0.13 & a weak spot at 0.36
α -Ketoglutarate	0.15
Oxaloacetate	0.17
Pyruvate	0.47

* Contained butanol:ethanol:0.5N NH₄OH (70:10:20) system of El Hawary and Thompson (1953) for paper chromatography.

These results again confirm that it is unlikely that pyruvate is a product, and indicate that α -ketoglutarate may be a product in this reaction.

If the above result is a genuine one, then the cell-free extracts should be able to form α -hydroxyglutarate from citramalate. Oxidation-reduction is not expected to occur under the experimental conditions since there is no addition of the cofactors (NAD, NADH, FAD, FADH₂, FMN-FMNH₂) needed for this purpose. It is, however, likely that the cell extracts contain small but significant amounts of these cofactors and hence α -hydroxyglutarate may be formed. The possibility of formation of α -hydroxyglutarate from citramalate and its subsequent oxidation to α -ketoglutarate was tested by experiments with α -hydroxyglutarate.

EXPERIMENTS WITH α -HYDROXYGLUTARATE

α -Hydroxyglutarate and citramalate were incubated separately with cell extracts in presence of semicarbazide. The experimental procedure was the same as in Table 25. The total ketoacids were determined after 5 min and 20 min incubations with 2,4-DNPH. The results are shown in Table 28.

TABLE 28

KETOACIDS FROM α -HYDROXYGLUTARATE AND CITRAMALATE*

Substrate	Period of incuba- tion	OD ₄₃₅		OD (420 m μ) / OD (520 m μ)
		-succinate	+succinate	
(-)-Citramalate	5'	-	0.240	1.8-1.9
	20'	0.13	0.255	
α -Hydroxygluta- rate	5'	-	0.187	1.8-1.9
	20'	0.152	0.225	
None	20'	0.049	0.055	1.12

* Initial incubation period (with semicarbazide but without 2,4-DNPH) was 2 hr at 30°.

** Incubation period with 2,4-DNPH (after the reaction) for the formation of the hydrazones.

It may be seen from Table 28 that there is keto acid formation from both (-)-citramalate and α -hydroxyglutarate and that ratio of the OD at 420 m μ to OD at 520 m μ corresponds to that for α -ketoglutarate. The addition of both NAD and NADP to the reaction mixtures enhanced the formation of ketoacids by 15 to 20 per cent.

An attempt was made next to identify the ketoacid in the citramalate and α -hydroxyglutarate reaction mixtures. The 2,4-DNPH was successively extracted with ethyl acetate, 10 per cent Na_2CO_3 and again ethylacetate after acidification of the carbonate extract. The ethyl acetate extract layer was then concentrated and chromatographed. The results are shown in Table 29.

TABLE 29

R_f VALUES OF THE 2,4-DNPH FROM CITRAMALATE
AND α -HYDROXYGLUTARATE REACTIONS

		R_f^*		
		A	B	C
Substrates	(-)-Citramalate	0.13	0.05	0.66
	α -Hydroxyglutarate	0.14	0.05	0.67
Standards	Oxaloacetate	0.17	0.1	0.87
	α -Ketoglutarate	0.15	0.05	0.67
	Pyruvate	0.47	0.34	0.58 & 0.41
	Glyoxylate	-	0.24	0.57

Solvent system * A = n-Butanol:Ethanol:0.5 N NH_4OH
of El Hawary & Thompson (1953)
B = n-Butanol saturated with 3 per cent
(v/v) ammonia (Cavallini *et al.* 1949)
C = 0.05 N NaOH (Lewis & Weinhouse, 1957)

It is clear that the ketoacid seems to be α -ketoglutarate as indicated by the R_f values of the 2,4-DNPHs in different solvents and by the ratio of optical densities at 420 to 520 m μ . However it must be stressed that only the isolation in quantity and identification by other

of the citraconase reaction and there is no evidence for the amination of citraconate. The second one is possible, but there is really no clear evidence for it.

However, the formation of the straight chain compound α -keto-glutarate from (-)-citramalate is a very interesting reaction - if it is not an artifact. More evidence is necessary both concerning the identity of the product itself (i.e., α -ketoglutarate) and regarding the existence of the other reactions postulated above.

SECTION 3(vi)MALEASE ACTIVITY OF CITRACONATE CELLS

The enzyme malease catalyzes the hydration of maleic acid to malic acid. It was first found in corn kernels by Sacks and Jensen (1951): under the experimental conditions used by these authors fumaric acid was not formed from maleate. The optical nature of malate formed from maleate by the action of malease present in corn kernels has not been characterized. Because the enzyme preparation from the corn kernels reduced triphenyl-tetrazolium chloride in the presence of diaphorase and maleate, it was presumed that the product formed was (-)-malic acid (Crombie, 1960).

The citraconate cells used in these studies do not oxidize maleate but do contain malease and hence convert maleate to malate. The following experiments were conducted in order to ascertain (i) the optical nature of the product of malease action and (ii) whether citraconase and malease are identical.

Optical activity of the product

The reaction was conducted under the same conditions as for citraconase except that Fe^{++} was not added to the reaction mixture. The products were isolated as described under "Stereochemistry of Citraconate" [Section 3 (iv)]. The results of the measurement of optical rotation of malic acid from fumarate and maleate in the presence of cell-free extracts of citraconate cells are shown in Table 30.

It has also been observed that the malate formed from maleate is not attacked by pig heart malate dehydrogenase whereas malate formed from fumarate is readily dehydrogenated by this enzyme.

TABLE 30

SPECIFIC ROTATION VALUES OF MALIC ACIDS

Product	With molybdate and citrate*	
	Found	Literature**
1. Malate obtained from maleate	-1420°	
2. Malate obtained from fumarate	+1460°	
3. Standard L(-)-malate Na salt	+1400°	+1400° to +1470°
4. Standard D(+)-malate Na salt	-1424°	

* 4 ml of the test solution contained 13 mg of malate, 0.2 ml glacial acetic acid, 0.4 ml 1M sodium citrate, 1.8 ml of a saturated (29 per cent) solution of ammonium molybdate and water to 4 ml. Rotation was measured in a 5 cm tube.

** Figures from Krebs and Eggleston (1943).

The purity of the standard acids - both (+)- and (-)-isomers, was also checked by determination of specific rotations with and without molybdate addition as described by McKenzie and Plenderleith (1923); without any addition: L(-)-malic acid, found -3.05° (not reported in literature), L(-)-malate Na salt, found -10.1° (not reported in literature), D(+)-malic acid, found +3.1°, reported +3.07°, +3.46°; D(+)-malate Na salt, found +10°, reported +8.29°; with molybdate addition: L(-)-malic acid, found +540°, reported +568°; D(+)-malic acid, found -540°, reported -560°.

Mechanism of enzymatic hydration of maleate and fumarate

The enzymatic hydration of maleate and fumarate by the unfractionated cell-free extract of the citraconate culture has been carried out in presence of D_2O and employing NMR as in the case of citraconate and mesaconate hydration. These experiments have been fully described in a previous section (Chapter III, Section 3-iv). The experiments have revealed that the addition of elements of water takes place also by a trans mechanism as in the case of citraconate and mesaconate hydration reactions.

Fractionation of cell-free extract for malease activity

The cell-free extract of citraconate cells was fractionated through various steps as described earlier for citraconase purification and the malease activity in the various fractions was tested. The results of these experiments are given in Table 31.

Most of the malease activity is present in the 0.4 to 0.5 ammonium sulphate fraction after calcium phosphate gel treatment (step 5) although the overall purification and recovery of the activity are low. In this fraction the corresponding specific activity of citraconase is 0.12.

The variation of ratios of the two activities malease:citraconase from 2 to almost zero and the absence of malease in the 0.5 to 0.7 ammonium sulphate saturation fraction indicate that the two activities are quite distinct entities. It is surprising that growth on citraconate induces malease formation also, although the organism does not oxidize maleate and malate produced from maleate. Perhaps this is an instance of gratuitous induction. The other enzymes concerned with the degradation of (+)-malate are not formed since the organism and its cell-free extracts do not dissimilate (+)-malate under diverse conditions.

TABLE 31MALEASE ACTIVITY IN VARIOUS FRACTIONS

Fraction	Total malease activity (units) a	Protein (mg) b	Specific activity a/b	Recovery (Per cent)	Ratio of Malease Citraconase
1. Cell-free extract*	120	1200	0.1	100	0.5
2. After strep- tomyacin treatment	96	780	0.12	80	0.46
3. Ammonium sulphate (0.4-0.7)	60	413	0.145	50	0.36
4. Calcium phos- phate gel treated eluate	55	290	0.19	46	0.34
5. Ammonium sulphate 0.4-0.5	27	112	0.24	22.5	2
6. 0.5 to 0.7		140			negligible

*From 15 g of wet packed cells

SECTION 4

ITACONASE OF PSEUDOMONAS 111

The enzyme itaconase catalyses the hydration of itaconic acid to citramalic acid. Brightman and Martin (1961) showed the presence of this enzyme in the cell-free extracts of a pseudomonad isolated by them. They observed that by the action of the enzyme there was accumulation of citramalate from either free itaconate or mesaconate. This is the first report of the hydration of free itaconate to citramalate. Thus the pathway of itaconate metabolism in this pseudomonad is different from that in liver mitochondria or the other pseudomonads studied. As already mentioned the product has not been characterized as (+)- or (-)-citramalate. The experiments described here have been conducted with two objectives: First, to ascertain which optical isomer of citramalate is formed by itaconase action and second, whether itaconase activity in this organism is distinct from mesaconase.

The organism (Pseudomonas 111) used in these studies was obtained from Professor Martin and was grown in itaconate medium (Brightman & Martin, 1961) containing 0.05 per cent (w/v) 'Bacto' Casamino Acids instead of Basamin used by the above authors. The cell-free extract of the organism was prepared as described (Brightman & Martin, 1961).

Experiments with undialysed cell-free extract

The experiments of Brightman and Martin (1961) have been performed anaerobically. Two sets of experiments were carried out. In one the incubation of the substrate (itaconate) along with the undialysed cell-free extract was carried out in an atmosphere of nitrogen in Warburg manometric

flasks as described by Brightman and Martin (1961). In another the incubation was carried out with air as gas phase and without shaking. The results of the experiments and the protocols are shown in Table 32.

TABLE 32

FORMATION OF CITRAMALATE FROM ITACONATE
AND CELL-FREE EXTRACT OF PSEUDOMONAS 111

Products formed**	Condition of incubation	
	Under nitrogen	Aerobic
Citramalate	+++	+++
Succinate	++	++
Mesaconate	+	+

** Reaction mixture contained in 2 ml: sodium itaconate, 10 μ moles; potassium phosphate buffer (pH 7), 20 μ moles and undialysed extract equivalent to 20 mg protein. Incubation for 2 hr at 30°. The rest of the procedure as in Table 5.

+ means feeble positive; ++, approximately 10% of the substrate converted into succinate; +++, about 75% converted into citramalate.

It is clear from Table 32 that citramalate and succinate are formed from itaconate both under aerobic and anaerobic conditions.

Action on mesaconate

An experiment was also carried out to ascertain whether mesaconase was present in the cell-free extract. Citramalate or succinate could not be detected by chromatography. Only a very feeble spot for citramalate was discernible when the reaction mixture was supplemented with Fe^{2+} and cysteine.

Experiments with dialysed cell-free extract

Most of the itaconase activity was lost on dialysis of the cell-free extract against 0.05 M Tris buffer (pH 7.0) for 8 hr. Very little citramalate was observed chromatographically. The activity lost on dialysis could not be restored by the addition of cysteine and ferrous ions. The same result was obtained with mesaconate also.

Optical nature of citramalate formed from itaconate

The optical nature of citramalate formed from itaconate was measured in the presence of ammonium molybdate and sodium citrate (Krebs & Eggleston, 1943). The concentration of the citramalate formed was checked by titration and taken accordingly for the determination of optical rotation. The specific rotation $[\alpha]_D^{27}$ of the citramalate formed was +126°. Hence the citramalate formed from itaconate by the action of itaconase is dextrorotatory.

DISCUSSION

It may be seen that free itaconate is hydrated to citramalate in Pseudomonas 111 and that mesaconate is not acted upon. This clearly shows that itaconase activity is distinct from mesaconase. It should be mentioned that the cell-free extracts of Pseudomonas 111 used in these experiments had hardly any mesaconase activity. In contrast, Brightman and Martin (1961) reported that the cell-free extracts of this bacterium contained mesaconase activity also. This discrepancy in results is hard to explain. Itaconate gives rise to dextrorotatory citramalate. In the other systems also, apparently both itaconate and mesaconate yield dextrorotatory citramalate or its CoA derivatives (Adler et al. 1957; Wang et al. 1961; Nagai, 1963; Cooper & Kornberg, 1964).

SECTION 5

METABOLISM OF METHYLSUCCINATE

Methylsuccinate does not occur in nature, but is degraded by animals and bacteria (Emmerich, 1939; Gawron et al. 1962). Katsuki et al. (1963) reported that both (+)- and (-)-isomers disappear on incubation with cell extracts of itaconate-grown Pseudomonas 3081. Although their results indicated that citramalate was an intermediate in the degradation of DL-methylsuccinate, it is not clear whether the initial dehydrogenation is caused by the succinoxidase complex itself or by a different system. Further it is not known whether both isomers of DL-methylsuccinate give rise to mesaconate by dehydrogenation as postulated by Katsuki et al. (1963) or whether one of them, viz., the (-)-isomer yields mesaconate as is known in the animal succinoxidase system (Gawron et al. 1962) and the other isomer giving rise to citraconate. Attempts were therefore made to get the following information: (i) Is the methylsuccinate dehydrogenase complex distinct from bacterial succinoxidase? (ii) What are the products of dehydrogenation of the (+)- and (-)-isomers of methylsuccinate?

(1) Oxidations by resting cells

The results of oxidation of the two isomers of methylsuccinate by the different cultures are given in Table 33. The conditions of the experiments are as described in Table 2. The results clearly show that the cultures used oxidize isomers of methylsuccinate equally rapidly. The methylsuccinate culture seems to oxidize the (-)-isomer more rapidly than the (+)-isomer. For further experiments only the methylsuccinate culture was used.

TABLE 33

**OXIDATION OF THE ISOMERS OF METHYLSUCCINATE
BY RESTING CELLS OF DIFFERENT CULTURES**

Substrates	[*] Q _{O₂}		
	Methylsuccinate- grown methyl- succinate culture	Itaconate- grown itaconate culture	Mesaconate- grown mesaconate culture
DL-Methylsuccinate	78	45	54
(+)-Methylsuccinate	55	50	50
(-)-Methylsuccinate	80	45	60
Citraconate	18	6	5
Mesaconate	45	30	39
(+)-Citramalate	80	45	35
(-)-Citramalate	50	23	22
Endogenous	15	16	12

*Endogenous values subtracted

(2) Experiments with 100,000 x g particles of cells

The methylsuccinate culture was grown on two different media, one with DL-methylsuccinate as the sole carbon source and the other with succinate. The initial experiments indicated that the succinate and methylsuccinate dehydrogenase activity were exclusively present in the 100,000 x g particles. Hence these particles were used for the following experiments.

(a) Is methylsuccinate dehydrogenase distinct from succinate dehydrogenase complex?

The suspension of 100,000 x g particles from both succinate-grown cells and those from methylsuccinate were tested for the dehydrogenase activity in Thunberg tubes. The results of such an experiment are shown in Table 34.

TABLE 34DEHYDROGENATION* OF METHYLSUCCINATE AND SUCCINATE

100,000 x g particles from cells grown on	Time (min) for complete decolourization		
	Endogenous	DL-Methylsuccinate	Succinate
Succinate	∞	∞	25
Methylsuccinate	∞	120**	13
Tricarballoylate	∞	∞	25

*Each Thunberg tube contained 0.5 ml particles (= 20 mg dry cells = 10 mg protein), 200 μ moles potassium phosphate (pH 7.4) in the tube and 1.5 μ moles, 2,6-dichlorophenolindophenol and 20 μ moles substrate in the side arm. Total volume 4 ml, gas phase hydrogen and temperature 28°.

**1 hr lag before reduction commenced.

The results clearly indicate that methylsuccinate dehydrogenase is distinct from succinate dehydrogenase. Methylsuccinate dehydrogenase is not

present in cells grown on succinate, but is formed when cells are grown on methylsuccinate (itaconate or mesaconate). It is, therefore, an induced system.

(b) What is the product of dehydrogenation?

The experimental set-up was similar to the one described above, but larger quantities of the constituents were used. DL-Methylsuccinate and (+)- and (-)-isomers were separately incubated for 6 to 12 hr at 30° with particulate preparations of methylsuccinate-grown cells in large Thunberg tubes (total volume 20 ml, particles 4 ml, dye 10 μ moles initially and 5 μ moles added twice after each decolourization, substrate 30 μ moles and potassium phosphate buffer (pH 7.5) 1 mmole). After complete decolourization of the dye the reaction was stopped by the addition of 20 ml ethanol. The precipitated protein was centrifuged down. The supernatant solution was then evaporated to dryness in vacuo (40 to 45°C) and taken up in 0.4 ml water and chromatographed.

The results of paper chromatography indicated that DL-methylsuccinate gives products which have the same R_f values (both as acids and UV absorbing spots) as those ^{of} mesaconate and citraconate. (+)- and (-)-isomers seemed to yield products corresponding to citraconate and mesaconate.

However, when "mixed chromatography" (i.e., when citraconate or mesaconate was added to the reaction mixture and then chromatographed), the "reaction" spots and the added acids seemed to have a mobility some 10 per cent and 5 per cent different from the standards. Hence the above tentative conclusions need to be very much more rigorously confirmed.

DISCUSSION

The experiments with the bacterial systems clearly demonstrate that the bacterial succinate dehydrogenase complex is quite distinct from the animal counterpart. The former does not attack methylsuccinate at all whereas the latter dehydrogenates (-)-isomer to mesaconate. The bacterial system is an induced system, although induction is not very specific. For example even when grown on mesaconate or itaconate, the bacteria exhibit the capacity to oxidize both (+)- and (-)-isomers of methylsuccinate.

The results indicated that (+)-isomer perhaps yields citraconate whereas (-)-isomer yields mesaconate. This is in line with the results of oxidation of (-)-isomer by animal succinoxidase system. It is possible but not proved by these experiments that the (+)-isomer yields citraconate which in presence of citraconase of cell-free extracts yields (-)-citramalate.

An interesting question arises if it is assumed that the above results are genuine and not artifacts. How is (-)-citramalate oxidized in these organisms? Cells grown on itaconate, mesaconate, or DL-methylsuccinate oxidize both isomers of citramalate, as seen in the experiments described earlier and in Kornberg's experiments (Cooper & Kornberg, 1964). Is (-)-citramalate racemized and then oxidized according to the known routes, or is it metabolized by a different pathway? More data are needed before this could be settled and also to establish the tentative conclusion that (+)- and (-)-isomers of methylsuccinate yield respectively citraconate and mesaconate.

CHAPTER IV

GENERAL DISCUSSION

DISCUSSION

The most reduced of the branched C_5 -dicarboxylic acids is methylsuccinic acid. Both (+)- and (-)-isomers of methylsuccinic acid are known to be oxidized biologically. Only laevorotatory methylsuccinic acid is oxidized by mammalian liver mitochondria (Adler et al. 1957) and succinate dehydrogenase preparations (Gawron et al. 1962); the product is mesaconate. Results obtained here show that bacterial succinate dehydrogenase complex is quite distinct from the animal counterpart in that the former does not attack methylsuccinate at all. The results of Katsuki et al. (1963) and experiments described here (Chapter III, Section 5) show that both (+)- and (-)-isomers of methylsuccinate are metabolised by pseudomonads isolated here. Katsuki et al. (1963) have assumed without experimental evidence that the product from both (-)- and (+)-isomers is mesaconate. The results reported here indicate that (+)-isomer of methylsuccinate perhaps yields citraconate whereas (-)-isomer yields mesaconate. Thus it seems that degradation of the optical isomers of methylsuccinate yield geometric isomers, the branched unsaturated C_5 -dicarboxylic acids.

The general pattern is that the unsaturated acids are hydrated either directly or after activation to branched C_5 -hydroxy acids. Thus citraconic, mesaconic and itaconic acids are hydrated directly to citramalic acid by bacteria isolated here, Pseudomonas 3081 and by Pseudomonas 111. Itaconic acid is more commonly activated to itaconyl-CoA before it is hydrated to citramalyl-CoA (liver mitochondria and in certain pseudomonads). The hydration of citraconic acid to laevorotatory citramalic acid catalysed by citraconase is the most important finding in the present studies.

This hydration of citraconate is similar to mesaconase reaction of C.tetanomorphum (Barker, 1956), of P.fluorescens (Katsuki et al.1962) and itaconase of Pseudomonas 111 (Brightman & Martin, 1961). In the requirement of a sulphhydryl compound and specifically ferrous ions for activity, citraconase resembles mesaconase of C.tetanomorphum (Blair & Barker, 1966) and aconitase from animal, microbial and plant tissues (Dickman & Cloutier, 1950; Morrison, 1954; Rahatekar & Rao, 1963). The activation of citraconase by anaerobic incubation at room temperature with a sulphhydryl compound and ferrous ions is similar to that of mesaconase of C.tetanomorphum (Blair & Barker, 1966) and bacterial L-tartrate dehydratase (Hurlbert & Jacoby, 1964).

It has been shown by earlier investigators that the addition of water takes place by a trans mechanism in enzymatic hydrations such as fumarase and aconitase reactions. Using NMR spectroscopy the present studies show that the hydrations of citraconate and mesaconate also occur by a trans-mechanism and confirm that the hydration of maleate and fumarate is brought about by trans-addition.

Among these hydratases of C_5 -dicarboxylates, mesaconase has been the best purified (from an initial specific activity of 6 to a final specific activity of 500 to 600). Citraconase has been partially purified from an initial specific activity of 0.2 to a final specific activity of 4.6 to 5. Mesaconase of P.fluorescens has been purified to about 8-fold from an initial specific activity of 0.3 to 2.35. Mesaconase of C.tetanomorphum consists of two component proteins both of which are essential for activity.

Present investigations show that the product of hydration of citraconate by citraconase of the organism is laevorotatory citramalate.

Mesaconases of the citraconate-culture, of C.tetanomorphum (Barker 1956) and of P.fluorescens (Katsuki et al. 1962) also catalyse the formation of (+)-citramalate from mesaconate. The results reported here clearly show that optical isomers of citramalate are formed by the enzymatic action from the geometrical isomers - citraconate and mesaconate and that the two activities are distinct. There is another instance of the formation of optical isomers from geometrical isomers found in the formation of β -ketoadipic acid from cis, cis-muconic acid by the enzymes of P.fluorescens (Sistrom & Stanier, 1954). In this case, the lactonising enzyme present catalyses the formation of both (+)- and (-)-isomers of the lactone (γ -carboxymethyl-4- α -butenolide) from cis-cis and cis-trans isomers of muconic acid. The optical nature of the citramalate formed from itaconate had not been characterised by earlier investigators (Brightman & Martin, 1961). The present studies show that the citramalate formed from itaconate by itaconase of Pseudomonas 111 is dextrorotatory.

Citramalate or citramalyl CoA that is formed by the hydration of either mesaconate or itaconate is next cleaved to pyruvate and acetate or acetyl CoA in bacteria and animal kingdom. Preliminary results here indicate that most probably pyruvate is not a product of degradation of laevorotatory citramalate and that α -ketoglutarate may be a product. However, more evidence is necessary both concerning the identity of the product itself (i.e., α -ketoglutarate) and regarding the existence of other reactions in the degradation of laevorotatory citramalate.

In addition to the presence of citraconase and mesaconase in the citraconate organism, the enzyme malease which catalyzes the hydration of maleate to malate is also present. Malate formed from maleate is

dextrorotatory and is not attacked by pig heart malic dehydrogenase. However, malease is distinct from citraconase. It is surprising that growth on citraconate induces malease formation also, although the organism does not oxidise either maleate or (+)-malate produced from maleate. Perhaps this is another instance of gratuitous induction. It should be mentioned in passing that the rotation of optically active malate is reversed by the addition of molybdate and citrate. This effect seems to be characteristic of malate and not of other α -hydroxy acids tested.

CHAPTER V

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

(1) Four strains of bacteria capable of growing on citraconate, mesaconate, itaconate and methylsuccinate were isolated from soil by enrichment culture techniques.

(2a) The ability of the four isolates to oxidise different branched C_5 -dicarboxylic acids has been studied. It is found that the citraconate-grown culture oxidises only citraconate and (+)- and (-)-isomers of citramalate at high rates; methylsuccinate and mesaconate are oxidized very feebly and itaconate is not oxidized at all. The other cultures oxidize all substrates used except citraconate at similar rates.

(2b) In addition all of them oxidise (+)-isomer of citramalate 1.5 to 2 times faster than the (-)-isomer. On the contrary there is an inverse relationship in the case of citraconate culture in that it oxidizes the (-)-isomer 5 to 6 times faster than the (+)-isomer. Cell-free extracts of all the isolates hydrate both citraconate and mesaconate to citramalate.

(2c) Succinate-grown cells of the isolates exhibit low oxidising activities towards the substrates. The cells, however, are induced to oxidize the substrates in the presence of respective substrates and this induction is inhibited by chloramphenicol.

(3) The most important finding in the present investigations is the enzymatic hydration of citraconate. The product of hydration is laevorotatory citramalate. The new enzyme that catalyses the hydration of citraconate to laevorotatory citramalate has been termed "citraconase".

Citraconase present in the citraconate organism is quite distinct from mesaconase which is also present in the same organism.

(4a) Citraconase has been partially purified about 25-fold from an initial specific activity of 0.2 to a final specific activity of 4.6 to 5.0. The various steps with purification procedure consist of treatment with streptomycin sulphate for removal of nucleic acids, fractionation with ammonium sulphate, negative adsorption with calcium phosphate gel and chromatography on DEAE-cellulose. The above procedures have yielded a citraconase preparation which is still not homogenous and which contains at least two other components as revealed by the ultracentrifugal pattern.

(4b) Some properties of the partially purified citraconase have been studied. The enzyme requires activation by a sulphydryl compound such as reduced glutathione, cysteine, thioglycollate or mercaptoethanol and ferrous ions for maximal activity; ascorbate has no activating effect.

(4c) The activation is carried out by preincubation of the enzyme and the cofactors anaerobically at room temperature. No other metallic ion tested - manganese, magnesium, zinc, calcium, nickel, cobalt, sodium and ammonium activate citraconase in conjunction with glutathione.

(4d) The optimum pH for citraconase activity is 7.5 and the activity falls off markedly on both sides of the optimum.

(4e) The partially purified citraconase does not act on other substrates such as mesaconate, maleate, fumarate, cis-aconitate and trans-aconitate than citraconate. It does not contain lactate dehydrogenase, NADH oxidase, malate dehydrogenase and citrase. Very low isocitrate dehydrogenase activity was present in the partially purified enzyme preparation.

(4f) The K_m for citraconate at pH 7.5 and 30° is 1.1 to 1.2 x 10⁻³M. The K_m values for the cofactors are 3.1 x 10⁻³M and 1.2 x 10⁻⁴M for reduced glutathione and ferrous sulphate respectively.

(4g) Iron complexing agents such as o-phenanthroline, 8-hydroxyquinoline and α,α' -bipyridyl have an inhibitory effect on citraconase. Copper and mercury inhibit the enzyme activity under these conditions to the extent of about 90 per cent. Iodoacetate and p-chloromercuribenzoate are markedly inhibitory. Pyrophosphate is also found to be inhibitory.

(5) The stereochemistry of enzymatic hydration of citraconate, mesaconate, maleate and fumarate by the cell-free extract of citraconate organism has been investigated using NMR spectroscopy. It is found that the mode of addition of water to all the above substrates occurs by a trans-mechanism.

(6) An enzymatic procedure for the preparation of both (-)- and (+)-isomers of citramalate from citraconate and mesaconate respectively, using unfractionated cell-free extract of citraconate-grown cells has been described. The procedure has the advantage of avoiding the wasteful resolution of the racemic mixture for the isolation of optical isomers and of using the same unfractionated cell-free extract. The properties of the two isomers, the racemate and other related isomeric compounds have been described.

(7) Attempts to elucidate the mode of degradation of laevo-rotatory citramalate have been made. The results indicate that most probably α -ketoglutarate and not pyruvate may be a product of degradation. However, more intensive studies need to be done before any conclusions can be drawn.

(8) It is found that the citraconate organism also contains malease, the enzyme catalysing the hydration of maleate to malate. However it is shown that citraconase and malease activities are distinct from one another and that the malate formed from maleate is dextro-rotatory. The malate formed from maleate is not acted upon by pig heart malic dehydrogenase.

(9) Present studies show that itaconase activity of Pseudomonas 111 is distinct from mesaconase and that the citramalate formed from itaconate is dextrorotatory.

(10a) Results of the studies on the metabolism of methylsuccinate by methylsuccinate-culture clearly indicate that methylsuccinate dehydrogenase of the organism is distinct from succinate dehydrogenase complex. Methylsuccinate dehydrogenase is not present in cells grown on succinate. Thus bacterial succinate dehydrogenase is quite distinct from the animal counterpart in its specificity.

(10b) The results also indicate that citraconate and mesaconate seem to be the products of dehydrogenation of (+)- and (-)-isomers of methylsuccinate respectively, although more data are needed to establish this tentative conclusion.

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