EVIDENCE FOR THE EXISTENCE OF THE TRICARBOXYLIC ACID CYCLE AND GLYOXYLATE PATHWAY IN CERTAIN MARINE FILAMENTOUS FUNGI

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by

Devi Vembu

A Thesis Submitted to the Faculty of the
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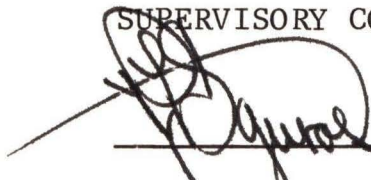Florida Atlantic University
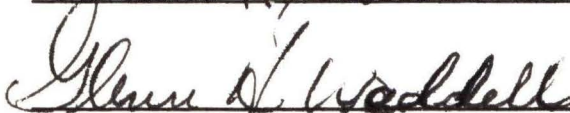Boca Raton, Florida
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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. P. L. Sguros, Department of Biological Sciences. It was submitted to the Faculty of the College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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INTRODUCTION

The role of marine fungi in biological and chemical aspects of the sea awaits complete revelation. Even the criteria upon which the definition of a marine fungus should be based remain tenuous. Johnson and Sparrow (1961) defined a marine fungus as that which is "... capable of developing to reproductive maturity even though exposed at some point in their growth to salinities of 30 $^o/_{oo}$ (parts per thousand) or more, either while continually submerged or intermittently inundated by tidal waters."

Vishniac (1956) reported that marine fungi are likely to occur in the same aerobic communities as bacteria, while Wood (1964) viewed them as strongly aerobic saprophytes able to grow generally in a pH range of 3.2-9.6.

Moore and Meyers (1959) originated the term "Thalassiomycetes," in an attempt to collect indigenous "sea fungi" into a single systematic group. However, Kohlmeyer and Kohlmeyer (1971) recommended that this concept be abandoned, since marine fungi are an ecological, not a taxonomic group. According to these authors, most of the higher marine fungi may have derived from the different groups of terrestrial fungi, while only a few genera may be

truly of marine origin. The high incidence of monotypic genera, resulting in a ratio of 1:2 between genera and species, may account for the belief that these fungi have evolved from their terrestrial ancestors without time for splitting into closely related species. This theory tends to contradict the statement of Denison and Carroll (1966) that "Marine fungi are not simply a random sample of terrestrial groups which have strayed into the sea."

An excellent historical survey of the marine fungi has been presented by Johnson and Sparrow (1961) who recorded that the first authentic report of a marine fungus occurred in 1846 when Durieu and Montagne described the Ascomycete Sphaeria posidoniae. In 1859, Westendorp described Sphaeria albopunctata (= Leptosphaeria albopunctata) and more Leptosphaeria species were reported by Ellis and Everhart in 1885 from collections of tide water phanerogam culms. From 1859 to 1889 the literature variously reported marine fungi in calcareous animal structures and in parts of marine invertebrates. The first marine Labyrinthula, L. macrocystis, was described by Cienkowski in 1867, but 65 years elapsed before Dangeard, Renn and Young, and Vishniac and Watson published additional work. Studies of Ichthyosporidium hoferi,

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a fungal pathogen of fish, by Johnstone after the turn of the century, were outstanding, even though similar diseases of salt water teleosts were known previously. Reports of fungi thought to live symbiotically with algae also are an interesting phase in the development of marine mycology. Such a relationship between the fungus Guignardia alaskana and the alga Prasiola borealis was first studied by Reed in 1902. Other workers, Sparrow, for example, succeeded in focussing attention on marine Phycomycetes with their publications on fungi which inhabit marine algae.

Contemporary marine fungal physiology probably commenced with the work of Barghoorn and Linder (1944), who furnished the first information on the nutritional requirements of a number of isolates and included observations on the effects of temperature, salinity and hydrogen ion concentration on growth. Carbon and nitrogen requirements, subsequently, were determined on selected species by Gustaffson and Fries (1956), Johnson, Ferchau and Gold (1959), Sguros and Meyers (1962), and Sguros, Simms and Rodrigues (1971). Detailed nutritional data on vitamin requirements were added by Sguros, Meyers and Reynolds (1961), Sguros, Meyers and Simms (1962) and Sguros and Simms (1963a,b, 1964). The salt requirements of marine Phycomycetes

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were reported by Vishniac (1960) while the inorganic requirements of higher forms were studied by Sguros and Simms (1963b, 1964).

Little work has been reported on specific enzyme systems in marine fungi. While Barghoorn and Linder (1944) presented the first indirect evidence on the lignolytic activity of marine fungi, Meyers and Reynolds (1959) were the first to qualitatively demonstrate cellulolytic enzymes in six different species of Deuteromycetes, using reducing sugar production as an index of hydrolysis. Jensen and Sguros (1971) studied specificity and stability characteristics of the cellulolytic complex in two species. Preliminary studies conducted by Sguros, Rodrigues and White (1971) and Rodrigues, Sguros and White (1970), showed the presence of phosphofructokinase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydratase, isocitrate dehydrogenase and malate dehydrogenase. Such data suggested the simultaneous presence of the Embden-Meyerhof, hexose monophosphate, Entner-Doudoroff and tricarboxylic acid (TCA) pathways of hexose degradation in marine fungi.

The TCA cycle was proposed first to account for the terminal stage of oxidation of carbohydrates to carbon dioxide

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and water (Krebs and Johnson, 1937). The key observation was
that citrate was formed in animal tissue from oxalacetate and
a second unknown substance. It was speculated that the
unknown might be pyruvate, acetate or a related compound.
Since this historic work, a great many details of the original
cycle have been elaborated. Foremost was the identification
of the unknown substance, namely acetyl coenzyme A, and the
isolation of **citrate** synthase.

While the TCA cycle was proposed originally as a mechanism
for the oxidation of carbohydrates, later studies expanded its
role in CO_2-fixation (Utter, 1961), energy production (Boulter
and Derbyshire, 1957) and supplying amino acids through either
transamination or reductive amination (Ehrensvard et al., 1951).
Such amino acids now are known to be used for the synthesis of
proteins, purines, pyrimidines and porphyrins. Energy production
is achieved by coupling TCA cycle dehydrogenases with an
electron transport system. The net effect of the cycle is the
complete oxidation of pyruvic acid:

$$CH_3COCOOH + 2.5O_2 \longrightarrow 3CO_2 + 2H_2O + 344 \text{ kcal}$$

Accessory to the TCA cycle is the so-called glyoxylate
by-pass first proposed by Kornberg and Krebs (1957). The

probable function of this pathway is to supplement the TCA cycle by providing C_4-dicarboxylic acids to compensate for the depletion of 2-oxoglutarate and oxalacetate caused by transamination and to avoid the loss of carbon as CO_2 at the C_6 to C_5 degradation step.

Among the many microorganisms examined for the TCA cycle, bacteria have been a major source of material, although some work has been done on yeasts (Krebs, 1952). Because of technical difficulties, filamentous fungi, in general, have been less intensively studied. Many workers have used indirect methods to assess the importance of such cyclic mechanisms in the fungal economy (Carson et al., 1951). Studies on Aspergillus niger with C^{14}-labelled glucose revealed that part of the citric acid formed from glucose arose from re-cycled C_4-dicarboxylic acids (Shu, Funk and Neish, 1954). However, respiratory studies and chemical investigations of individual reactions have tended to support the supposition that the TCA cycle is of major importance to Ashbya gossypii (Mickelson and Schular, 1953), Penicillium chrysogenum (Casida and Knight, 1954), Streptomyces coelicolor (Cochrane and Peck, 1953) and Blastocladiella emersonii (Khouw and McCurdy, 1969).

The first indications that the TCA pathway might be

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functioning in terrestrial fungi came from nutritional studies. Several yeasts utilized various intermediates known to be involved in the TCA cycle as sole carbon sources for growth (Barnett and Kornberg, 1960). However, other work showing that closely related microbial forms were unable to use such carbon sources did not prove the absence of the TCA cycle; in fact, negative results frequently were due to the permeability barrier of the intact cells (Cochrane, 1958).

The significant role played by impermeability was revealed when it was found that, in a number of species, TCA cycle intermediates could be metabolized by cell-free extracts, but not by intact cells (Barron and Ghiretti, 1953). In certain cases, the permeability barriers of fungi have been overcome by employing acidic media (Barron, Ardao and Hearon, 1950), esters of TCA cycle intermediates (Beevers, Goldschmidt and Koffler, 1952), alternate freezing and thawing of mycelia (Moses, 1955), or drying mycelia over P_2O_5 (Wessels, 1959) or deoxycholate (Kovac, 1961).

The purpose of studying the carbon metabolism of filamentous marine fungi is manifold; such studies contribute to a number of areas of significance:

(1) Biochemical description of a relatively unknown group of

microorganisms.

(2)   Biochemical comparisons to establish or deny taxonomic relationships within marine fungal taxa and between marine and terrestrial groups of fungi.

(3)   Determination of the effect of marine environmental conditions on metabolic profiles.

(4)   Establishment of the role of this group of organisms in marine food cycles and biochemistry.

(5)   Provision of information for preventing deleterious activities, e.g., wood destruction at shore installations.

(6)   Provision of information for enhancing beneficial activities, e.g., waste cellulose destruction in the marine environment.

In pursuit of all these goals, this present work concerns the focus of necessary methodology and elucidation of the terminal oxidation system employed by selected marine fungal isolates under simulated natural environmental conditions.

MATERIALS AND METHODS

Cultures. The marine fungal cultures used were the Deuteromycete, Culcitalna achraspora (FAU accession number 230) (Meyers and Moore, 1960), and the Ascomycete, Halosphaeria mediosetigera (FAU accession number 700) (Johnson, 1958). Cultures were obtained from Dr. S. P. Meyers, presently of the Department of Food Technology, University of Louisiana, Baton Rouge.

Reagents. Reagents of high commercial grade were employed for the preparation of media, buffers and analytical solutions. Enzyme preparations and chemicals were obtained from Sigma Chemical Co., Calbiochem, Nutritional Biochemicals Corp., Fisher Scientific Co. and Matheson, Coleman and Bell. Deionized water, obtained from a Barnstead Demineralizer (standard cartridge), was used throughout these studies. Artificial sea water (ASW) was prepared according to Lyman and Fleming (1940) as shown in Table 1.

Growth media. Organisms were grown routinely in a liquid semisynthetic medium, designated 1410, consisting of glucose 5.0, tris(hydroxymethyl)aminomethane (Tris) 1.21, NH_4NO_3 2.40 and yeast extract 1.0 g/liter in ASW, adjusted to pH 7.5 with 12 $\underline{N}$ HCl. The medium was dispensed and autoclaved

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TABLE 1. Composition of sea water according to Lyman and
Fleming (1940)

Salts	mg/100 ml	Ions represented	
		Cations	Anions
Sodium chloride	2,350.0	Na^+	Cl^-
Magnesium chloride	500.0	Mg^{++}	SO_4^{--}
Sodium sulfate	390.0	Ca^{++}	CO_3^{--}
Calcium chloride	110.0	K^+	Br^-
Potassium chloride	70.0	Sr^{++}	BO_3^{---}
Sodium bicarbonate	20.0		F^-
Potassium bromide	10.0		
Boric acid	3.0		
Strontium chloride	2.0		
Sodium fluoride	0.3		

for 20 min at 15 psi. The conditions of growth and maintenance of the cultures were based on the general principles and methodology devised earlier by Sguros and Meyers (1962).

Equipment and conditions. Cultures were grown in Pyrex conical flasks on a New Brunswick shaker reciprocating at 66-8 cm strokes per min (spm) at 25 C. Bellco wide-bore (1 mm minimum) volumetric pipettes were used for quantitation and transfer of standard inocula. Waring stainless steel semi-micro and 1-liter blendors were employed to homogenize mycelia. Mycelial extracts were clarified in an International HR-1 refrigerated centrifuge at 4 C. A Gilson-Lardy Warburg respirometer, Type US1-34, was used for the determination of oxidative capacity in whole cells and cell extracts (Umbreit, Burris and Stauffer, 1964) at 25 C for 2 hr at a shaking rate of 144 spm. Sonic disruption of mycelia was accomplished with a Branson S-75 Sonifier. Routinely, 120 ml of vegetative culture in a Rosett cell was immersed in an ice-salt mixture at -15 C and disintegrated at 20 kHz for 20 min. Cell-free extracts were dialyzed overnight in collodion membranes against distilled water at 4 C. A Bausch and Lomb "Spectronic 20" spectrophotometer was used for protein determinations, while a Cary 14 recording spectrophotometer was used for

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enzyme measurements with UV or visible light, depending on the type of assay. All precise weighing procedures were carried out with a Mettler semi-microbalance, Model B6.

Mycelial quantitation. Quantitative procedures for inoculum production and growth (Sguros et al., 1962) were accomplished by filtering mycelial samples on tared Whatman GF/A glass filter discs and drying in vacuo at 60 C for a minimum of 6 hr. The discs were weighed to the nearest 0.1 mg. All mycelial weights are given hereafter as dry weights.

Chemical methods. Protein was determined by the biuret method (Layne, 1957) and the presence of dehydrogenases was detected with 2,6-dichlorophenolindophenol (DPIP) (Rodrigues et al., 1970).

Microbiological methods. Cultures were started by transferring mycelial growth from a 6-day old culture, previously grown in 100 ml of medium 1410 in 500-ml flasks and stored for up to 30 days at 4 C, to 500-ml flasks containing 100 ml of fresh medium 1410. Cultures were incubated on the reciprocating shaker for 6 days (late linear growth phase) at 25 C, at which time one flask was removed from the shaker, the contents transferred to a sterile semi-microblendor and homogenized for 35 sec. Next, a 500-ml flask containing 100 ml

of fresh medium 1410 was inoculated with 4 ml of the homogenate.

This flask was shaken also for 6 days after which the growth

was filtered aseptically on Whatman #1 paper on a Millipore

support and washed four times with 50-ml portions of distilled

water. The washed mycelial pad was transferred to a sterile

semi-microblendor and homogenized in 50 ml of distilled water

for 30 sec. Three 2.0 ml samples were drawn for quantitation

and the remainder stored in the blendor overnight at 4 C.

After dry weight determinations, the stored suspension was

diluted with distilled water to bring the mycelial weight to

2.0 mg/ml (Appendix 1). The contents were homogenized for 5

sec to ensure uniform mixing. The number and size of flasks

and media volumes used were determined by the amount of

inoculum required for the experiment. The required amount of

inoculum for each flask size and volume of medium per flask

were previously specified by Sguros and Meyers (1962) as shown

in Table 2. These cultures, intended to provide standard

experimental inocula, were grown finally for 6 days to the

late linear phase on the shaker.

Experimental inocula were similarly treated for

standardization. The volume of suspensor used was always

half of the volume of culture medium to ensure dilution, rather

TABLE 2. Effect of scale-up procedures on the mycelial yield

of H. mediosetigera (700), C. achraspora (230)

and H. alopallonella (710) in medium 1410

(Sguros and Meyers, 1962)

Designations	Conditions and results				
A Flask[1] volume (ml)	125	250	500	1000	2000
B Medium volume (ml)	25	50	100	200	400
C Inoculum volume (ml)	1	2	4	8	16
D Inoculum weight (mg)	2	4	8	16	32
E Medium surface area (cm^2)	32.3	45.2	75.4	113.0	154.0
F Medium depth (cm)	0.9	1.4	1.6	2.0	2.4
G Surface-volume ratio E/B	1.29	0.91	0.75	0.57	0.39
H Surface-depth ratio E/B	33.9	32.3	47.0	56.5	64.0
Culture 700					
Total yield (mg)	54	113	225	454	957
Yield (mg/ml)	2.2	2.3	2.3	2.3	2.4
Fold increase	27	28	28	28	30
Culture 230					
Total yield (mg)	53	105	213	440	872
Yield (mg/ml)	2.1	2.1	2.1	2.2	2.2
Fold increase	27	26	26	28	27
Culture 710					
Total yield (mg)	50	101	210	492	952
Yield (mg/ml)	2.0	2.0	2.1	2.5	2.4
Fold increase	25	25	26	31	30

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[1]Erlenmeyer shake-flasks

than concentration, to the intended ratio of 2.0 mg/ml. This ratio was chosen as each species weighed approximately 2.0 mg/ml at 6 days. Extra flasks were inoculated routinely to provide a continuous source of viable inocula for future experiments.

To ensure the consistency of stock cultures, growth curves previously established by Sguros and Simms (1963a) in medium 1410 were repeated. Ten sets of triplicate flasks for each culture were inoculated with 1.0 ml standard inoculum and shaken simultaneously. Every 24 hr, one set of triplicate flasks was removed for quantitation after filtering the contents of each flask on a tared disc, washing and drying as before. Growth was expressed in terms of mg mycelium/ml of medium.

Whole cell manometry. Respirometric studies were conducted to determine the age at which each culture exhibited maximum endogenous oxidative capacity. Based on growth curves obtained previously, a sufficient number of 1-liter flasks containing 200 ml of medium 1410 were inoculated to obtain the required amount of resting mycelia. Cultures were harvested, filtered and washed every 24 hr for a period of 120 hr. In each case, the resulting mycelial pad was homogenized

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for 35 sec in a minimum amount of 10 $\underline{mM}$ phosphate buffer, pH 7.5, and the homogenate transferred to a conical flask. The blendor was rinsed with sufficient buffer to give a final mycelial concentration of 4 mg/ml. Each Warburg flask was charged with 2.5 ml of cell suspension (Sguros and Rodrigues, 1966), 0.2 ml of 40% KOH in the center-well and 0.5 ml of distilled water in the side arm. Three 2.5 ml samples of the same cell suspension were taken immediately for quantitation and results expressed as Q_{O_2} (dry weight).

Enzyme extractions. Fungal enzymes were extracted by acetone powder (Greene, 1969) and sonic disintegration (Niederpruem, 1965) techniques. The crude cell-free extracts from the acetone powder were employed both for spectrophoto-metric enzyme assays and manometric studies, while those obtained from sonically disintegrated cells were used only for manometric studies.

To prepare mycelial acetone powder, a suitable number of flasks containing 1410 broth were inoculated and, depending on the age of maximum oxidative capacity of the culture, harvested at 72 hr or 96 hr. Mycelia were vacuum-filtered on Whatman #1 paper in a Büchner funnel and washed three times with distilled water, the volume of each portion being

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one-tenth the original volume of the medium. The remaining procedure was carried out at 4 C in a cold room, using pre-cooled equipment and reagents.

The washed mycelial pad was homogenized in a semi-microblendor with 3 volumes of acetone (w/v), at -20 C for 60 sec, filtered and again blended for 30 sec with half the initial volume of acetone. The resulting suspension was re-filtered, washed three times with an equivalent volume of cold diethyl ether, and stored in a vacuum dessicator at -25 C.

The crude cell-free extracts were prepared by suspending 1 g of the powder in 10 ml of 100 mM N-tris(hydroxymethyl) methyl-2-aminomethane sulfonic acid (Tes) buffer at pH 7.5. The suspension was held in the cold for 10 min without agitation and centrifuged at 10,000 X g for 1 hr. Centrifugation speed was limited to 10,000 X g to prevent the excessive loss of respiratory particles by sedimentation with the cellular debris (Rao, Sirsi and Ramakrishnan, 1962).

The extracts were decanted and dialyzed when the NAD- and NADP-linked isocitrate dehydrogenases of both cultures and the isocitratase of H. mediosetigera were assayed.

Nondialyzed extract controls, lacking NAD or NADP or substrate, gave higher spectrophotometric readings than test systems in which all components were present. Dialysis against distilled water at 4 C overnight reversed this phenomenon to within experimental tolerance. The protein content of the dialysis residue was determined and its oxidative capacity qualitatively tested with DPIP.

The procedure for obtaining sonic extracts of mycelia was identical to that for preparing acetone powder, to the first point of cold storage. Before use, the mycelial pad was thawed and homogenized with 100 mM Tes buffer, pH 7.5, to give a final concentration of 10 mg/ml. Three 2 ml samples were taken for quantitation (Appendix 1). Extraction by sonic oscillation was accomplished by taking 120 ml of the above homogenate, subjecting it to 20 kHz for 20 min at -15 C, and centrifuging at 10,000 X g for 1 hr (Niederpruem, 1965; Rodrigues et al., 1970). The extracts were dialyzed under the same conditions as described for the acetone powder, their protein content determined, and oxidative capacity tested with DPIP.

Enzyme assays. The crude cell-free extracts obtained from acetone powder were assayed for aconitase, NAD- and

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NADP-linked isocitrate dehydrogenase, 2-oxoglutarate

dehydrogenase, succinate dehydrogenase, fumarase, malate

dehydrogenase and isocitratase. Determinations were performed

spectrophotometrically at 25 C, using a 1 cm light path. The

change in absorbance was measured routinely for at least 90

sec. The methodology is summarized in Table 3. Certain

assays required special handling and are treated separately

in the text below.

Aconitase in cell-free extracts was activated by mixing

equal volumes of thiomalate-Tris buffer and 4 $\underline{mM}$

$Fe(NH_4)_2(SO_4)_2$ solution. Ten ml thiomalate-Tris buffer was

prepared by dissolving 30 mg thiomalate in approximately 7 ml

of water, adding 0.4 ml of 1 $\underline{N}$ NaOH, 1.0 ml of 1 $\underline{M}$ Tris-HCl

buffer, pH 7.6, and diluting to volume with water. The buffer

and $Fe(NH_4)_2(SO_4)_2$ were deoxygenated by gassing with nitrogen

for 10 min in tubes which then were sealed until used. The

extracts were incubated at 37 C for 25 min and then held in

ice where activity could be maintained for 6 hr.

In the case of isocitrate dehydrogenase (NADP-specific),

Tris-HCl buffer was prepared to contain, in 1 ml, 1 mmole

ethylenediamine tetraacetate (EDTA), 0.3 mmole dithiothreitol

and 100 mmoles Tris-HCl.

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TABLE 3. Enzyme assay systems for <u>H</u>. <u>mediosetigera</u> (700) and <u>C</u>. <u>achraspora</u> (230)

| | | | | | | | | Culture | | Water[1] | Total | Wave |
Enzymes	References	Buffers	Ions	Coenzymes	Other co-factors	Color reagents	Substrates	700 cell protein (mg)	230	(ml)	volume (ml)	length (nm)
Aconitase	Fansler and Lowenstein, 1969	Tris-HCl 0.6 ml (60 umole) pH 7.6	NaCl 0.6 ml (0.3 mmole)				Cis-aconitate 0.3 ml (0.1 umole)	0.1 ml (0.79)	0.15 ml (1.14)	1.4 (1.35)	3.0	240
Isocitrate dehydrogenase (NAD)	Cook and Sanwal, 1969	Tris-acetate 2.4 ml (0.48 mmole)	MgCl$_2$ 0.1 ml (10 umole)	NAD 0.1 ml (1.5 umole)	AMP 0.04 ml (8 umole); lipoamide oxidoreductase 0.01 ml	DPIP 0.1 ml (0.53 mmole)	DL-Isocitrate 0.1 ml (2.5 umole)	1 ml (9.2)	1 ml (5.8)	-	3.85	600
Isocitrate dehydrogenase (NADP)	Cleland, Thompson and Barden, 1969	Tris-HCl 1 ml (100 mmole) pH 7.4	MnSO$_4$ 0.2 ml (20 mmole)	NADP 0.2 ml (1.5 mmole)			DL-Isocitrate 0.05 ml (80 mmole)	-	0.5 ml (3.75)	- (1.95)	3.0	340
2-Oxoglutarate dehydrogenase	Reed and Mukherjee, 1969	KH$_2$-K$_2$HPO$_4$ 0.2 ml (100 umole) pH 8.0	MgCl$_2$ 0.2 ml (20 umole)	NAD 0.2 ml (4 umole) CoA 0.04 ml TPP 0.02 ml (20 mmole)	Cysteine·HCl 0.2 ml (6 umole)		2-Oxoglutarate 0.02 ml (2 umole)	Various concentrations[2]		-	-	340
Succinate dehydrogenase	Veeger, DerVartanian and Zeylemaker, 1969	NaH$_2$-Na$_2$PHO$_4$ 1 ml (0.3 mmole) pH 7.6	KCN 0.1 ml (3 umole)		EDTA 0.1 ml (0.3 umole) bovine serum 0.1 ml 3% (w/v)	K$_3$Fe(CN)$_6$ 0.2 ml (15 umole)	Succinate 0.3 ml (40 umole)	0.45 ml (3.55)	0.45 ml (2.29)	1.1	3.35	455
Fumarase	Hill and Bradshaw, 1969	NaH$_2$-Na$_2$PHO$_4$ 0.25 ml (50 umole) pH 7.3					DL-Malate 1 ml (50 umole)	0.01 ml (0.065)	0.075 ml (0.443)	1.75	3.01 (3.075)	250
Malate dehydrogenase	Davies, 1969	KH$_2$-K$_2$HPO$_4$ 1 ml (0.6 mmole) pH 7.0		NADH 0.5 ml (0.4 umole)			Oxalacetate 0.5 ml (1.5 umole) for culture 700 and (0.02 umole) for culture 230	0.1 ml (1.3)	0.1 ml (0.59)	0.9	3.0	340
Isocitratase	Gottlieb and Ramachandran, 1960	KH$_2$-K$_2$HPO$_4$ 1 ml (200 umole) pH 6.85	MgCl$_2$ 0.4 ml (15 umole)		Phenylhydrazine·HCl 0.1 ml (10 umole) Cysteine·HCl 0.4 ml (4.5 umole)		DL-Isocitrate 0.5 ml (5 umole)	0.7 ml (1.61)	1 ml (3.4)	-	3.1 (3.4)	324

[1]Amount of water with and without parentheses pertains to cultures 230 and 700, respectively.

[2]Various concentrations of extracts were used in attempting to show the presence of 2-oxoglutarate dehydrogenase activity.

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While attempting to assay 2-oxoglutarate dehydrogenase, 2-oxoglutarate and acetyl coenzyme A (CoA) were added simultaneously to a mixture containing potassium phosphate buffer, cysteine·HCl, $MgCl_2$, NAD and TPP. The blank cuvette contained the same amount of buffer, $MgCl_2$ and NAD as was present in the test samples, with water to bring the total volume to 1.56 ml.

Succinic dehydrogenase present in the enzyme solution was diluted with 30 mM deoxygenated sodium phosphate buffer containing 0.1% bovine serum albumin to the extent that the change in extinction did not exceed 0.02-0.08 units/min.

For assaying malate dehydrogenase, oxalacetic acid was prepared in 0.6% EDTA.

Enzyme manometry. Conventional manometric techniques (Umbreit et al., 1964) were employed variously to study enzymatic reactions in (a) crude cell-free extracts obtained by sonic disintegration, (b) crude cell-free extracts prepared from acetone powder and (c) whole cells. These studies were carried out in support of spectrophotometric data on acetone powder extracts. Enzyme solutions prepared from sonic extracts were dialyzed against distilled water at 4 C overnight. The protocol for respirometric studies is summarized in Table 4.

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TABLE 4. Respirometric protocol for H. mediosetigera (700) and C. achraspora (230).

Warburg flask compartment	Reactants	Dialyzed sonic extracts				Non-dialyzed acetone powder extracts				Whole cells			
		700		230		700		230		700		230	
	Units	umole	ml	umole	ml	umole	ml	umole	ml	umole	ml	umole	ml
Main	Sodium phosphate buffer	100	1.0	100	1.0	100	1.0	100	1.0	-	-	-	-
	NAD	0.5	0.1	0.5	0.1	0.5	0.1	0.5	0.1	-	-	-	-
	NADP	0.35	0.1	0.35	0.1	0.35	0.1	0.35	0.1	-	-	-	-
	ATP	5.0	0.1	5.0	0.1	5.0	0.1	5.0	0.1	-	-	-	-
	MgSO$_4$	10.0	0.2	10.0	0.2	10.0	0.2	10.0	0.2	-	-	-	-
	Protein (mg)	(1.45)	0.5	(2.25)	0.5	(1.45)	0.5	(2.25)	0.5	-	-	-	-
	Whole cells	-	-	-	-	-	-	-	-	-	2.5	-	2.5
	Water	-	0.88	-	0.88	-	0.88	-	0.88	-	-	-	-
Center	40% KOH	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
	Phenazine methosulfate	0.98	0.02	0.98	0.02	0.98	0.02	0.98	0.02	-	-	-	-
Side	Substrate[1]	7.5	0.5	7.5	0.5	7.5	0.5	7.5[2] 2.5[3]	0.05	2.5	0.5	2.5	0.5
Total volume of flask contents			3.2		3.2		3.2		3.2		3.2		3.2

[1] Substrates used were glucose, citrate, cis-aconitate, DL-isocitrate, 2-oxoglutarate, succinate, fumarate, malate, oxalacetate and ethyl esters of fumarate, malate and oxalacetate.

[2] All substrates at 7.5 umole with the following exceptions: 22.5 umole of DL-isocitrate and 15 umole of oxalacetate in presence of acetone powder extracts (culture 230); 15 umole of malate and isocitrate in presence of sonically disintegrated cells (culture 230); 22.5 umole of oxalacetate in presence of sonically disrupted cells (culture 700).

[3] In the case of whole cells and acetone powder extracts of culture 230, 2.5 umole of esters was used. Whole cells (4 mg/ml) in 10 mmole sodium phosphate buffer.

RESULTS

Growth curve studies revealed that maximum growth for
H. mediosetigera and C. achraspora was obtained at 5 days and
8 days, respectively (Fig. 1), although harvesting both
cultures at 6 days resulted in a consistently uniform and
active mycelial inocula. Dry weight profiles showed only
slight variation from those reported by Sguros and Simms
(1963a).

Respirometric studies on whole cells showed that
respiration in the absence or presence of glucose or malate
was maximal at culture ages of 72 hr and 96 hr for H.
mediosetigera and C. achraspora, respectively (Fig. 2 and 3).
At such times, under the experimental conditions, Q_{O_2} (dry
weight) maxima, in the absence or presence of malate or
glucose, were 10.1-14.9, 15.2-19.5, and 14.5-20.5, respec-
tively, for C. achraspora, and 7.7-12.3, 11.9-15.6, and
14.6-18.0, respectively, for H. mediosetigera. This variation
was roughly in accordance with that obtained by Rodrigues
et al. (1970).

While preparing the acetone powder, it was noticed that
the precipitate changed color from brown to light gray during
the process of dehydration. Rehydration of the powder in air
resulted in its reversion to the brown color. Extraction of

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Fig. 1. Growth profiles for <u>C</u>. <u>achraspora</u> (◯) and
<u>H</u>. <u>mediosetigera</u> (△) in medium 1410 at 25 C on the
reciprocating shaker at 66-3 cm spm.

Fig. 2. Warburg studies conducted on H. mediosetigera. Each flask contained 2.5 ml of whole cells in 10 mM sodium phosphate buffer, pH 7.5, in the main compartment and 0.5 ml of 10 mM substrate in the side arm. Center-wells contained 0.2 ml of 40% KOH (w/v); the gas phase was air and temperature 25 C.

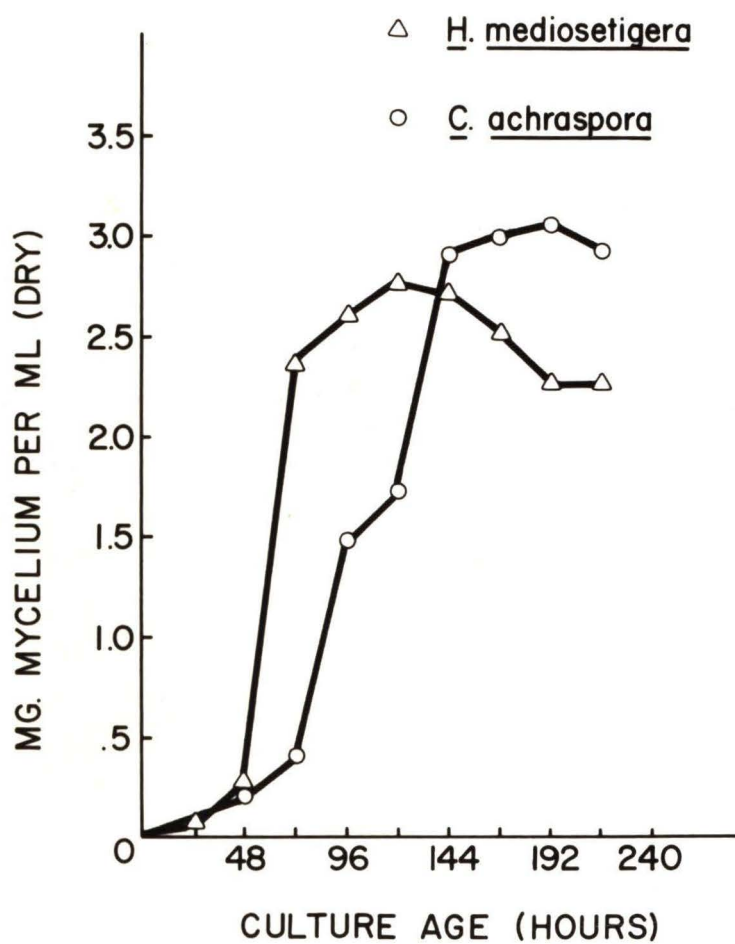H. mediosetigera

△ Endogenous
○ Malate
□ Glucose

Fig. 3. Warburg studies conducted on C. achraspora. Each flask contained 2.5 ml of whole cells in 10 mM sodium phosphate buffer, pH 7.5, in the main compartment and 0.5 ml of 10 mM substrate in the side arm. Center-wells contained 0.2 ml of 40% KOH (w/v); the gas phase was air and temperature 25 C.
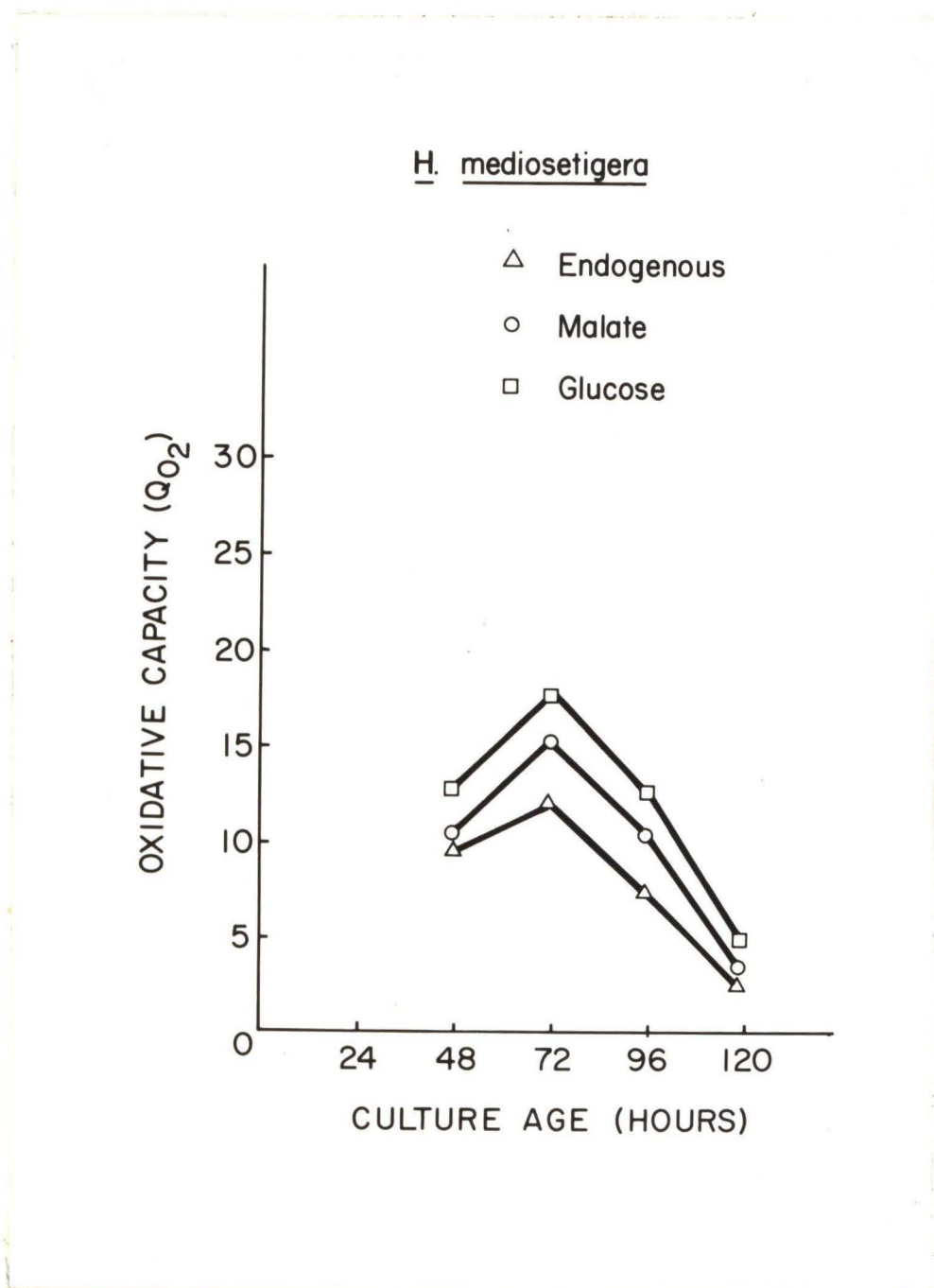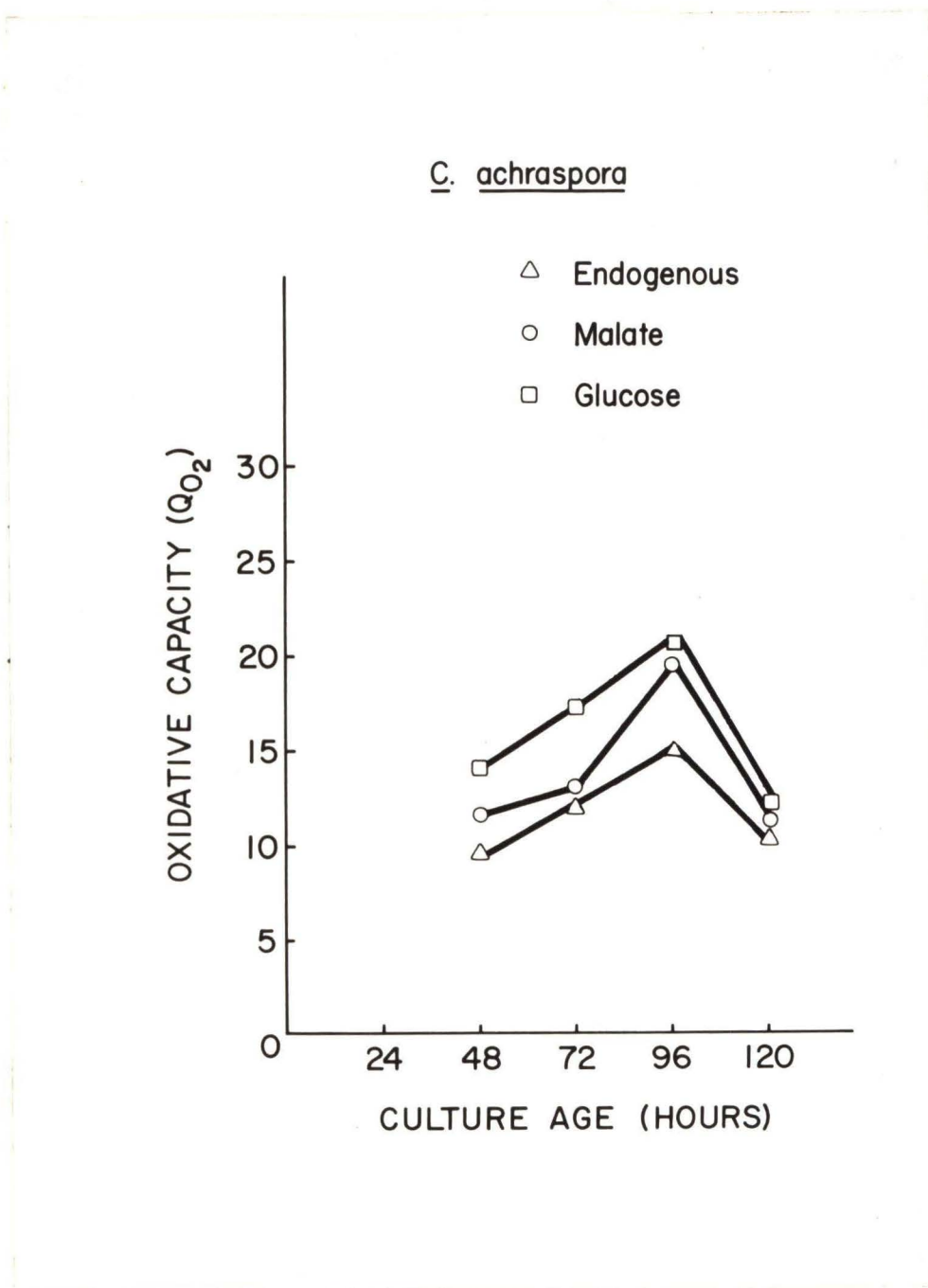

C. achraspora

△ Endogenous
○ Malate
□ Glucose

protein from dark-colored powder was quantitatively less effective.

Extracts obtained from acetone powder were clear and almost colorless, unlike those obtained from sonically disintegrated cells which were turbid and yellowish, although both reduced DPIP. When examined by the biuret method, acetone powder extracts showed 5.2-7.5 mg protein/ml, while sonically disrupted cells showed only 2.0-2.25 mg protein/ml.

During attempts to demonstrate individual TCA cycle and glyoxylate pathway enzymes, crude cell-free extracts obtained from acetone powder showed the presence of aconitase, isocitrate dehydrogenase (NAD- and NADP-specific), succinate dehydrogenase, fumarase, malate dehydrogenase and isocitratase. Repeated attempts to demonstrate the presence of 2-oxoglutarate dehydrogenase by the NAD reduction method were unsuccessful. As has been mentioned, only in the case of isocitratase (H. mediosetigera) and isocitrate dehydrogenase (both cultures), were dialyzed cell-free extracts required.

Evidence for the presence of aconitase (Fig. 4 and 5) was indicated by the decrease in extinction values at 240 nm due to the disappearance of cis-aconitate. The activity in C. achraspora showed a linear response with time (Fig. 4), whereas

Fig. 4. Aconitase of <u>C</u>. <u>achraspora</u>. The cuvette contained (in 3.0 ml) 60 umole of Tris-HCl buffer, pH 7.6, 0.3 umole of sodium chloride, 0.1 umole of cis-aconitate and 1.14 mg of protein. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

ACONITASE

<u>C</u>. <u>achraspora</u>
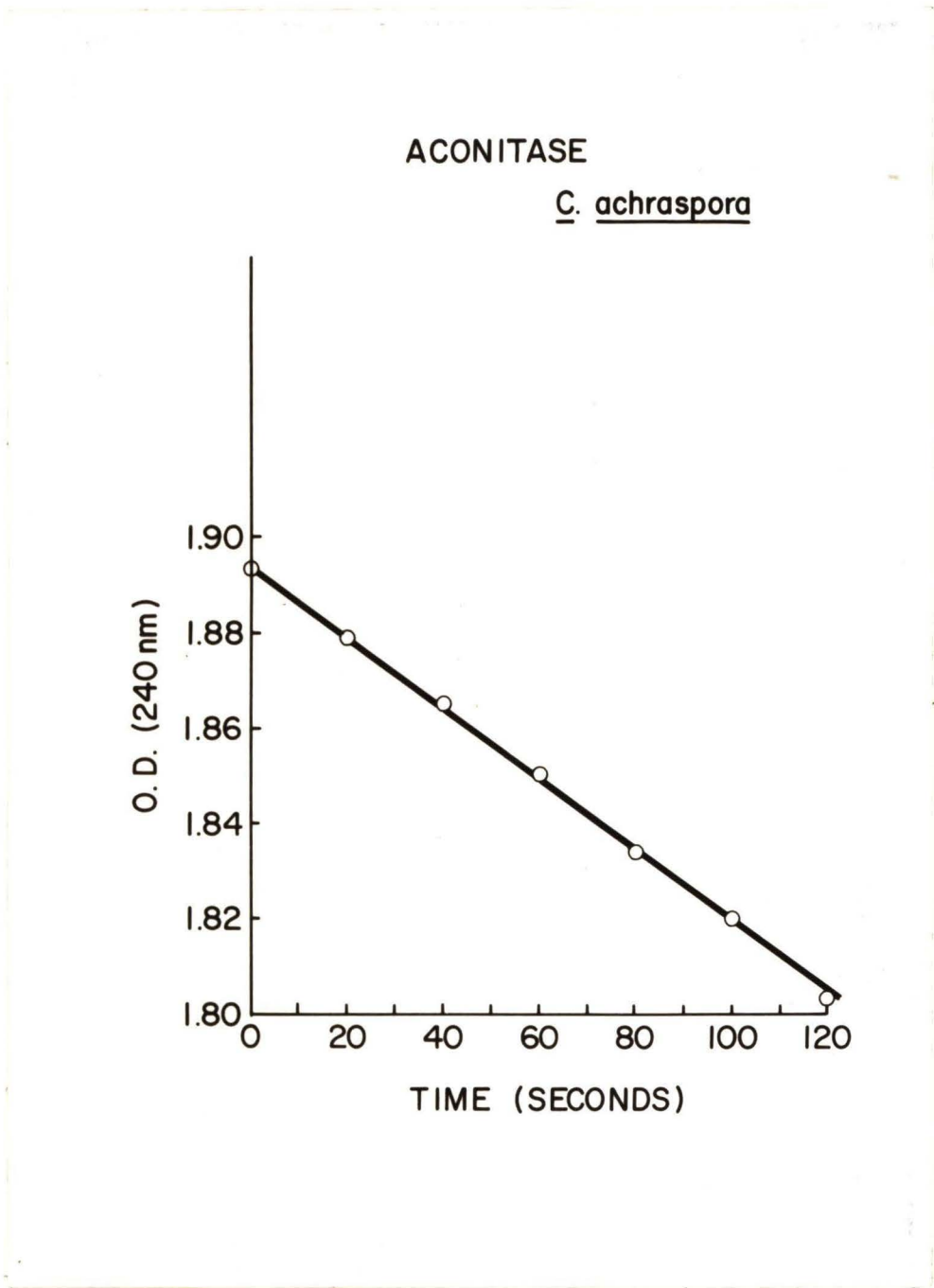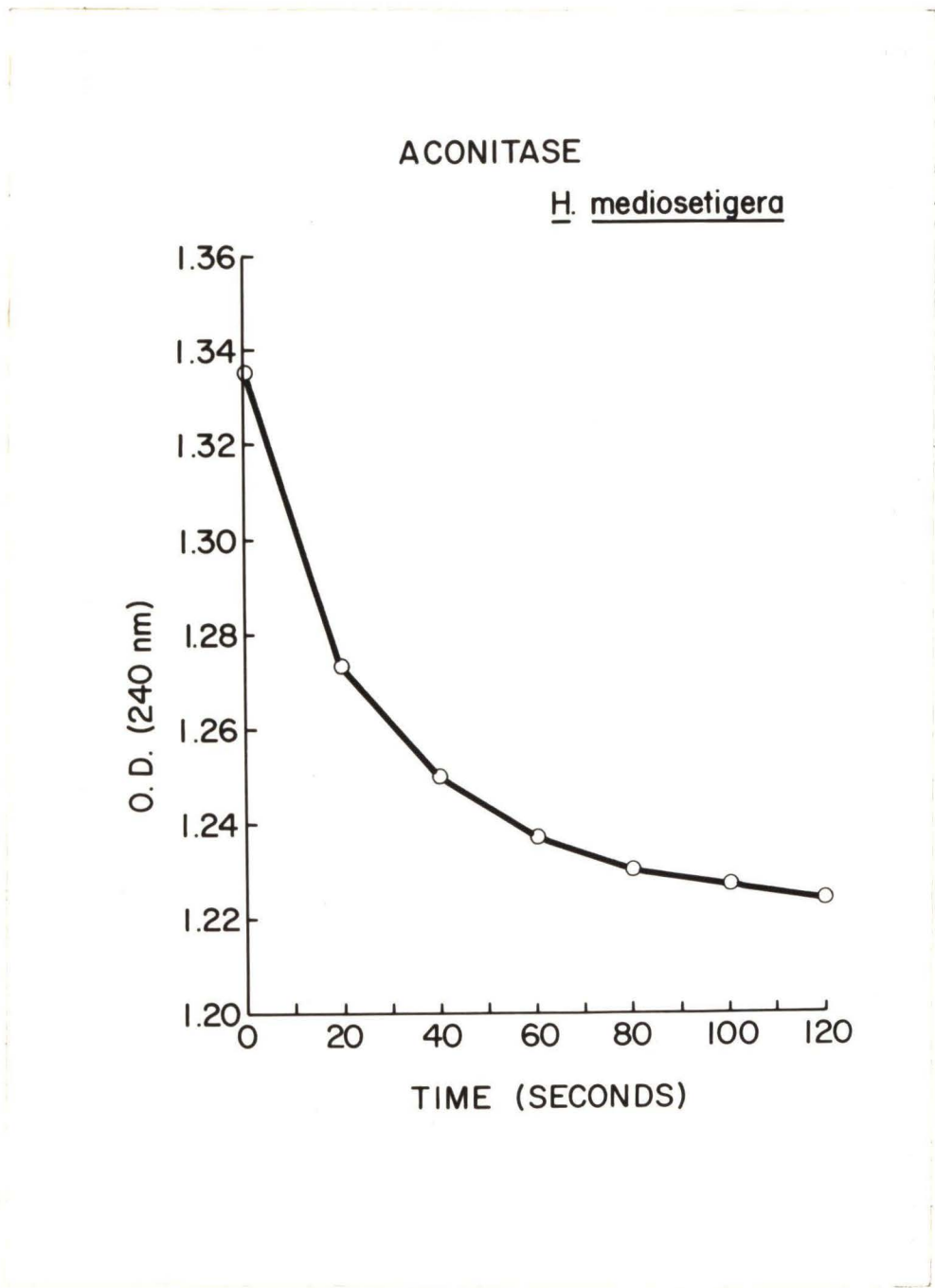
Fig. 5. Aconitase of H. mediosetigera. The cuvette contained (in 3.0 ml) 60 umole of Tris-HCl buffer, pH 7.6, 0.3 mmole of sodium chloride, 0.1 umole of cis-aconitate and 0.79 mg of protein. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

ACONITASE

H. mediosetigera

the activity in H. mediosetigera declined linearly for only

20 sec. The rate of reaction during this time was faster

than that in C. achraspora. Subsequently, the velocity of the

reaction in H. mediosetigera declined progressively.

The presence of NAD-linked isocitrate dehydrogenase

(Fig. 6 and 7) was demonstrated by the reduction of DPIP.

The activity of the enzymes resulted in a decrease in O.D.

which was comparable in both cultures. Initially, a rapid

decline in activity occurred for 20 sec, after which a

diminution in reaction rate was noted.

The increase in absorbance at 340 nm, owing to the

reduction of NADP, indicated the presence of NADP-linked

isocitrate dehydrogenase in C. achraspora (Fig. 8). Enzymatic

activity was linear for a period of 80 sec, after which it

began to level off. However, no enzyme activity was observed

in extracts of H. mediosetigera.

The presence of succinate dehydrogenase was observed by

measuring the reduction of $K_3Fe(CN)_6$. The change in O.D.

shown by extracts of H. mediosetigera was greater than that

observed in those of C. achraspora (Fig. 9 and 10).

The presence of fumarase was demonstrated by an increase

in absorbance, using malate as substrate. Under experimental

Fig. 6. Isocitrate dehydrogenase (NAD-specific) of
C. achraspora. The cuvette contained (in 3.85 ml) 0.48 mmole
of Tris-acetate buffer, pH 7.6, 1.5 umole of NAD, 0.53 mmole
of DPIP, 10 umole of $MgCl_2$, 8 umole of AMP, pH 7.6, 2.5 umole
of trisodium DL-isocitrate, 0.01 ml of lipoamide oxidoreductase
and 5.8 mg of protein. Suitable blanks were included in which
substrate or enzyme was omitted; temperature 25 C.

ISOCITRATE DEHYDROGENASE
(NAD-SPECIFIC)

C. achraspora
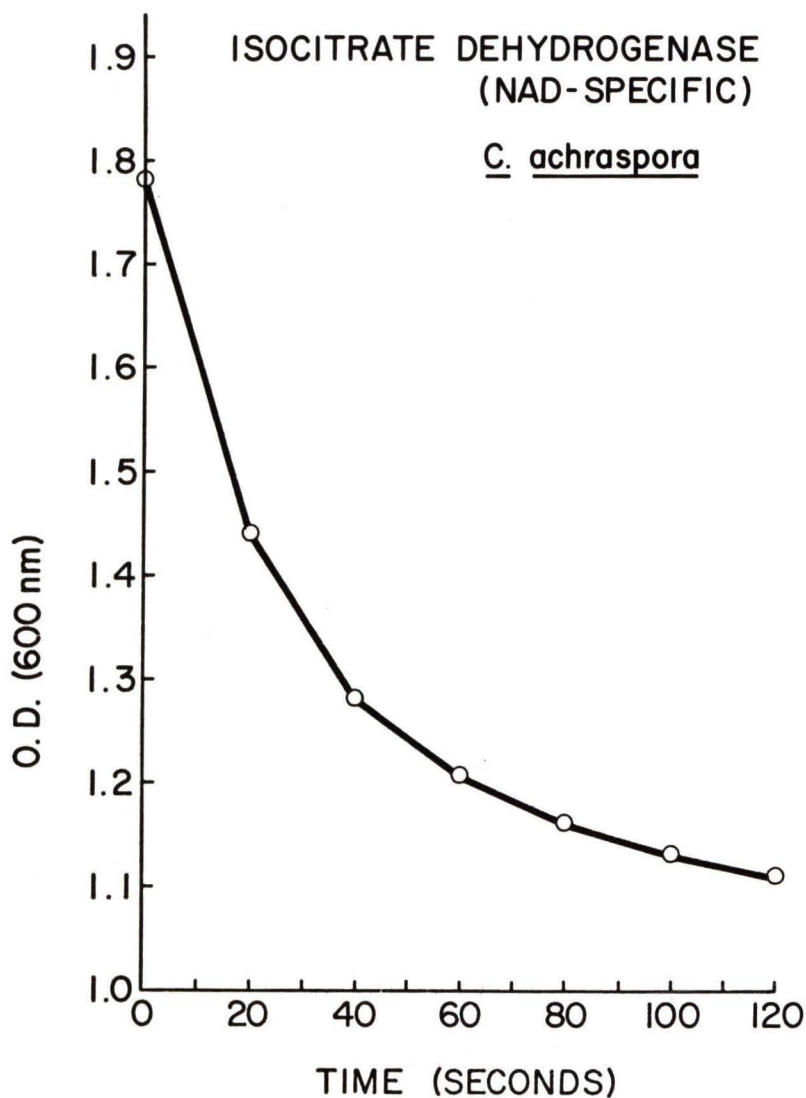
Fig. 7. Isocitrate dehydrogenase (NAD-specific) of
H. mediosetigera. The cuvette contained (in 3.85 ml) 0.48
mmole of Tris-acetate buffer, pH 7.6, 1.5 umole of NAD, 0.53
mmole of DPIP, 10 umole of $MgCl_2$, 8 umole of AMP, pH 7.6,
2.5 umole of trisodium DL-isocitrate, 0.01 ml of lipoamide
oxidoreductase and 9.2 mg of protein. Suitable blanks were
included in which substrate or enzyme was omitted; temperature
25 C.

ISOCITRATE DEHYDROGENASE
(NAD-SPECIFIC)

H. mediosetigera

O. D. (600 nm)

TIME (SECONDS)
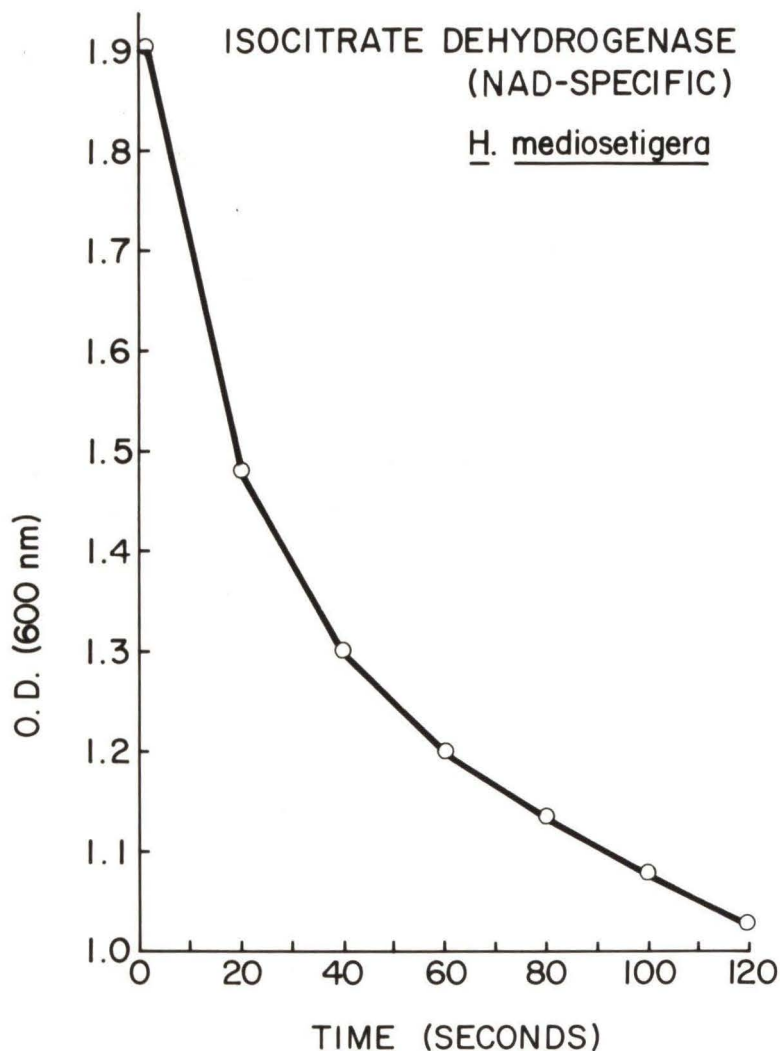
Fig. 8. Isocitrate dehydrogenase (NADP-specific) of
C. achraspora. The cuvette contained (in 3.0 ml) 1 mmole of
EDTA and 0.3 mmole of dithiothreitol in 100 mmole of Tris-HCl
buffer, pH 7.4, 20 mmole of $MnSO_4$, 1.5 mmole of NADP, 80 mmole
of DL-isocitrate and 3.75 mg of protein. Suitable blanks were
included in which substrate, enzyme or NADP was omitted;
temperature 25 C.

ISOCITRATE DEHYDROGENASE
(NADP-SPECIFIC)
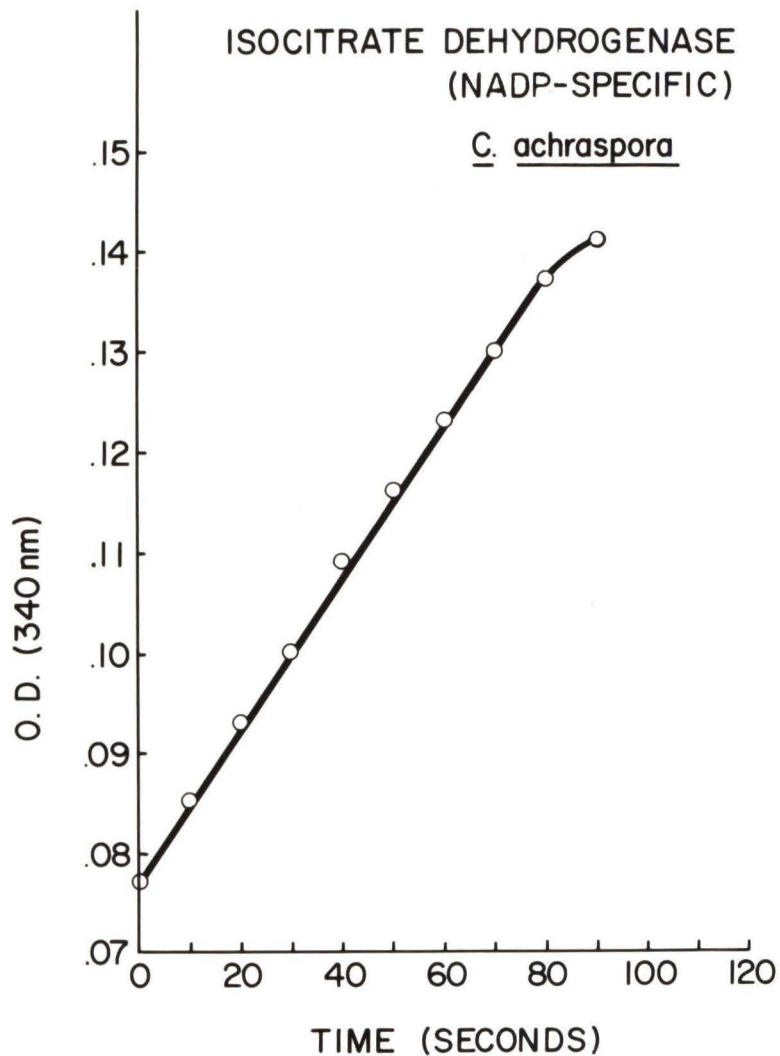
C. achraspora

Fig. 9. Succinate dehydrogenase of C. achraspora. The cuvette contained (in 3.35 ml) 0.3 mmole of sodium phosphate buffer, pH 7.6, 0.3 umole of EDTA, 15 umole of $K_3Fe(CN)_6$, 40 umole of succinate, pH 7.6, 0.1 ml of 3% (w/v) bovine serum, 3 umole of KCN and 2.29 mg of protein in 13.5 umole of deoxygenated buffer. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

SUCCINIC DEHYDROGENASE

C. achraspora
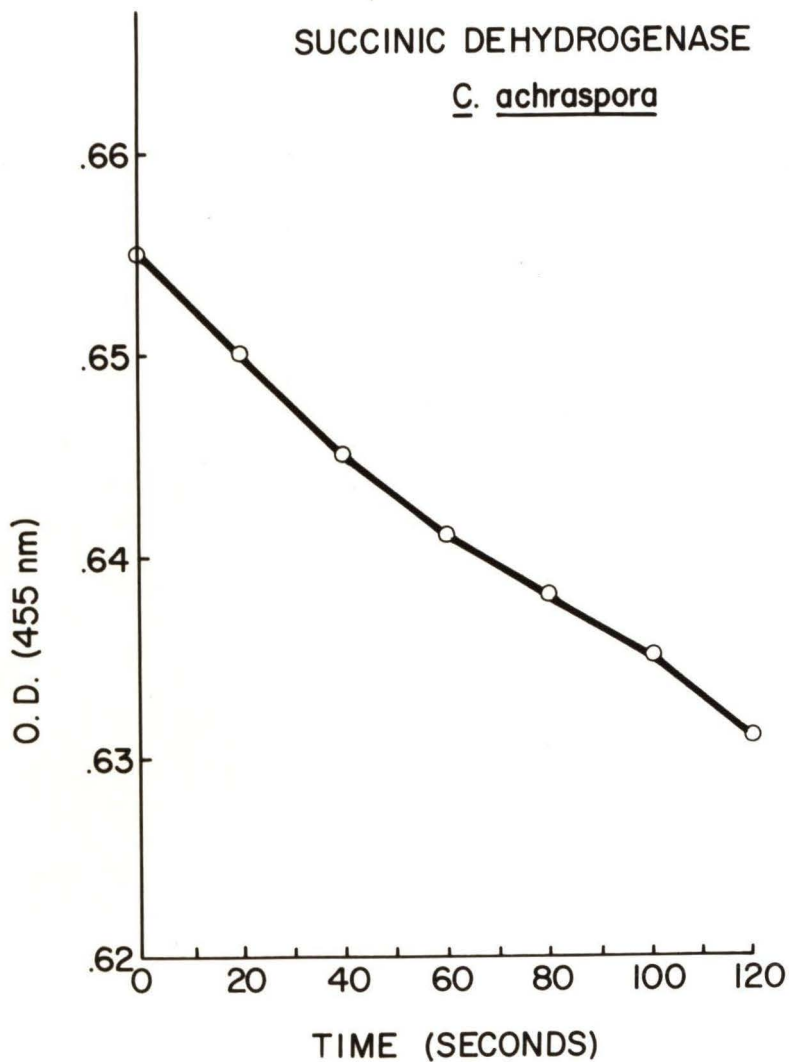
Fig. 10. Succinate dehydrogenase of H. mediosetigera. The cuvette contained (in 3.35 ml) 0.3 mmole of sodium phosphate buffer, pH 7.6, 0.3 umole of EDTA, 15 umole of $K_3Fe(CN)_6$, 40 umole of succinate, pH 7.6, 0.1 ml of 3% (w/v) bovine serum, 3 umole of KCN and 3.55 mg of protein in 13.5 umole of deoxygenated buffer. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

SUCCINIC DEHYDROGENASE

H. mediosetigera

O.D. (455 nm) vs TIME (SECONDS)
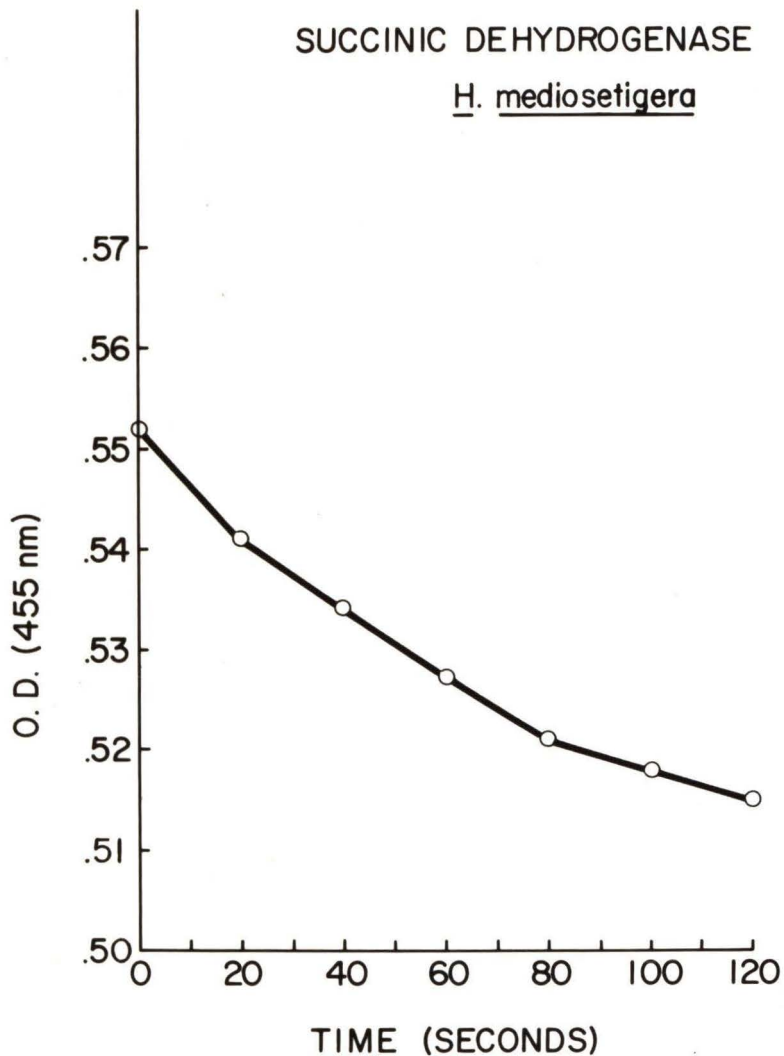
conditions, the reaction was linear for 80 sec in C. achraspora.
In H. mediosetigera, the reaction was linear for 20 sec, during
which time the O.D. increased to 1.06 units. The reaction rate
slowed down after 20 sec (Fig. 11 and 12).

The fall in O.D., owing to the oxidation of NADH, indicated
the presence of malate dehydrogenase. The velocity of this
reaction is proportional to the enzyme concentration, provided
the rate of change in O.D. does not exceed 0.07 (Davies, 1969).
In H. mediosetigera and C. achraspora, the initial rate of
change in O.D. observed was 0.038 units/min and 0.031 units/min,
respectively, allowing the assumption that the velocity of the
reaction was proportional to enzyme concentration (Fig. 13 and
14).

The increase in absorbance due to the formation of
glyoxylate indicated the activity of isocitratase. In H.
mediosetigera, the increase in O.D. was linear up to 80 sec,
whereas in C. achraspora it was almost linear up to 60 sec
(Fig. 15 and 16).

The specific activities of all enzymes assayed in both
cultures are reported in Table 5 as units/mg protein on the
basis of the definition of each individual enzyme. To compare
the various enzymes, specific activities were calculated using
one common formula for all the enzymes based on the definition

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Fig. 11. Fumarase of C. achraspora. The cuvette contained (in 3.075 ml) 50 umole of DL-malate, 50 umole of sodium phosphate buffer, pH 7.3, and 0.443 mg of protein. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.


FUMARASE

C. achraspora
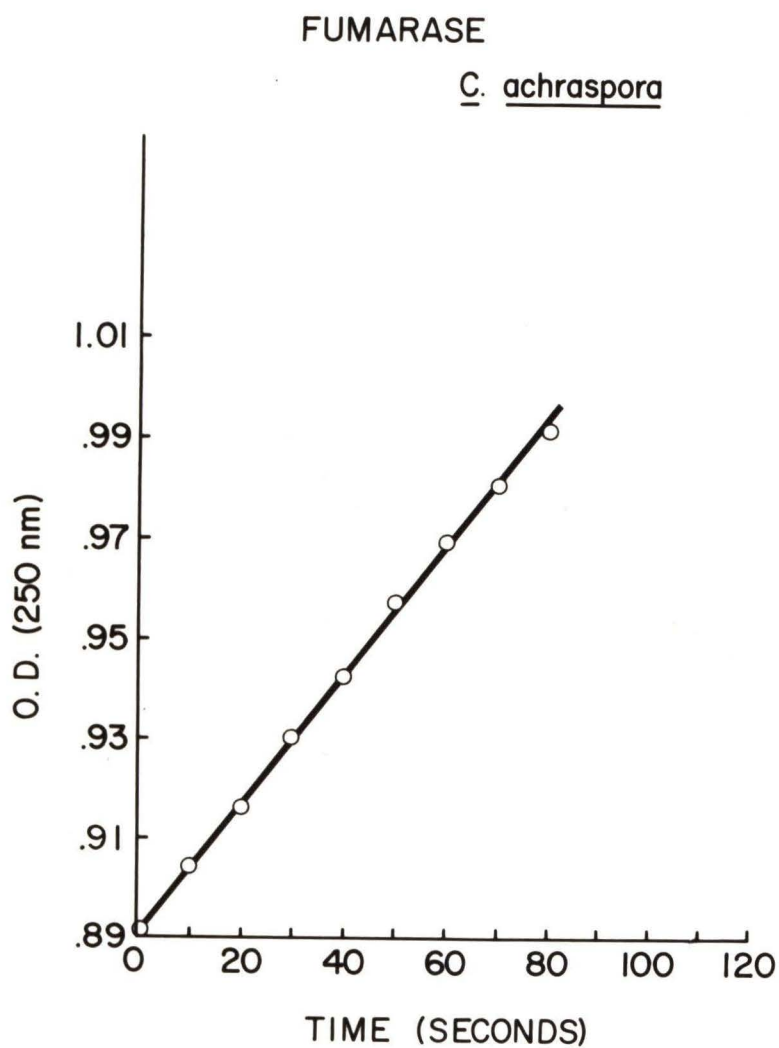
Fig. 12. Fumarase of H. mediosetigera. The cuvette contained (in 3.01 ml) 50 umole of DL-malate, 50 umole of sodium phosphate buffer, pH 7.3, and 0.065 mg of protein. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

FUMARASE

H. mediosetigera
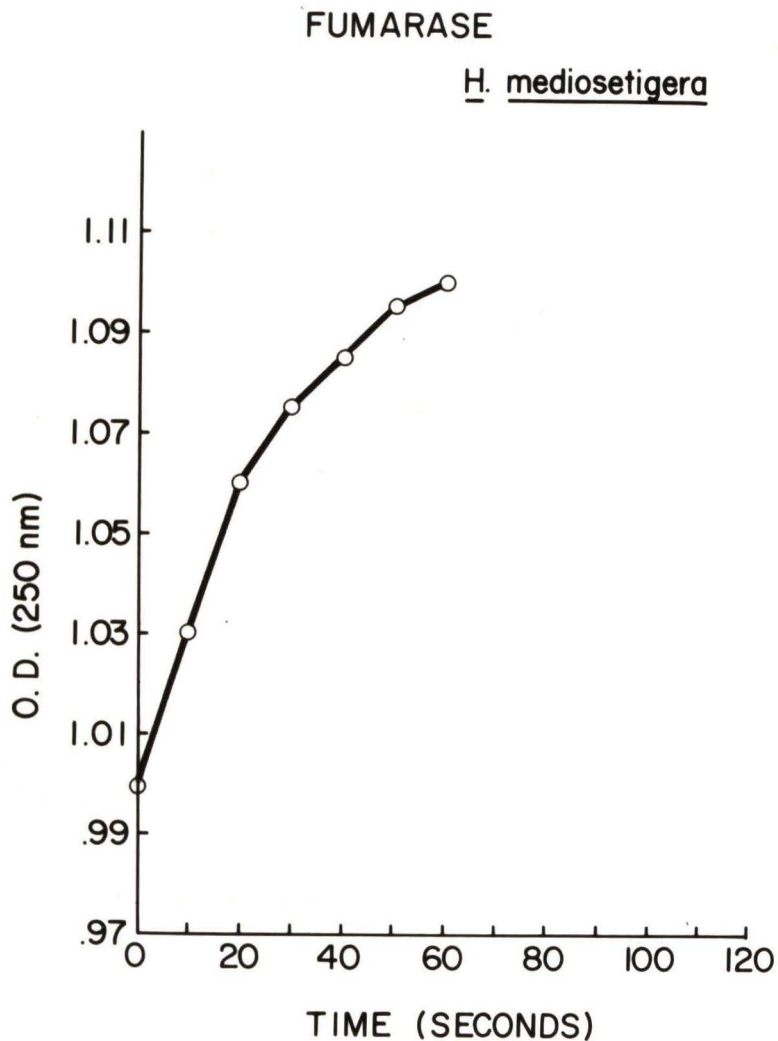
Fig. 13. Malate dehydrogenase of C. achraspora. The
cuvette contained (in 3.0 ml) 0.6 mmole of potassium phosphate
buffer, pH 7.0, 0.4 umole of NADH, 0.02 umole of oxalacetate
in 0.6% EDTA solution and 0.59 mg of protein. Suitable blanks
were included in which substrate, enzyme or NADH was omitted;
temperature 25 C.

MALATE DEHYDROGENASE

C. achraspora
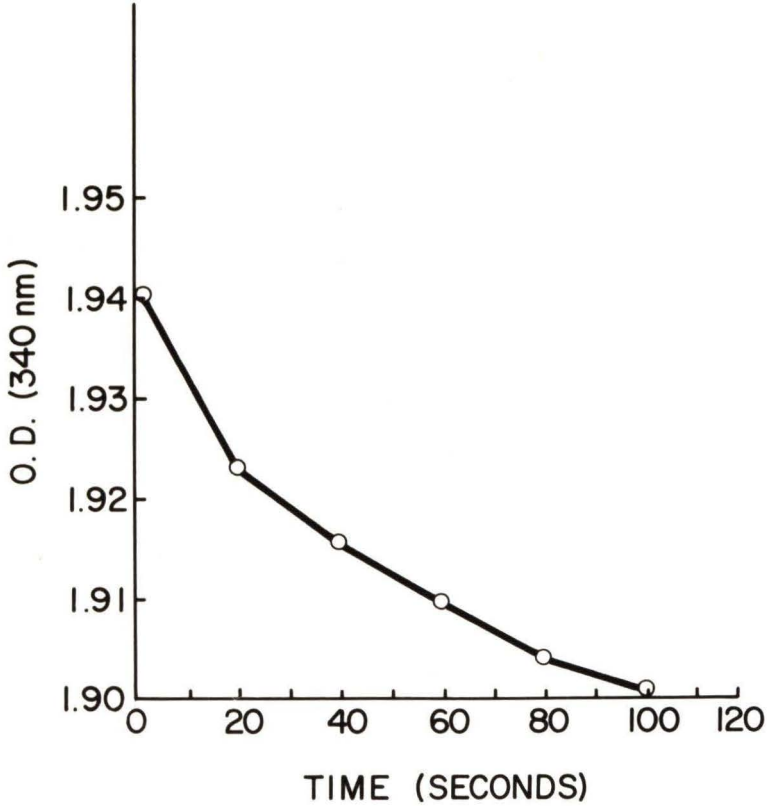
Fig. 14. Malate dehydrogenase of H. mediosetigera. The cuvette contained (in 3.0 ml) 0.6 mmole of potassium phosphate buffer, pH 7.0, 0.4 umole of NADH, 0.4 umole of oxalacetate in 0.6% EDTA solution and 1.3 mg of protein. Suitable blanks were included in which substrate, enzyme or NADH was omitted; temperature 25 C.

MALATE DEHYDROGENASE

H. mediosetigera

O. D. (340 nm) vs TIME (SECONDS)
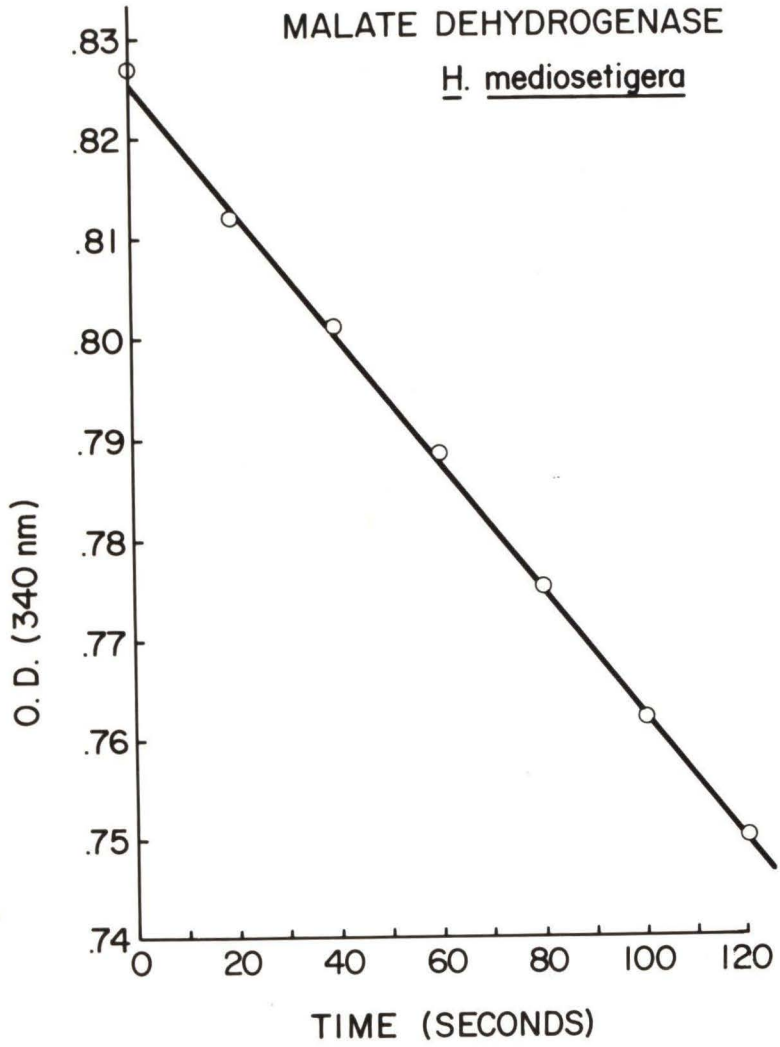
Fig. 15. Isocitratase of <u>C. achraspora</u>. The cuvette contained (in 3.4 ml) 200 umole of potassium phosphate buffer, pH 6.85, 15 umole of $MgCl_2$, 10 umole of phenylhydrazine·HCl, 4.5 umole of cysteine·HCl, 5 umole of DL-isocitrate and 3.4 mg of protein. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

ISOCITRATASE

<u>C.</u> achraspora
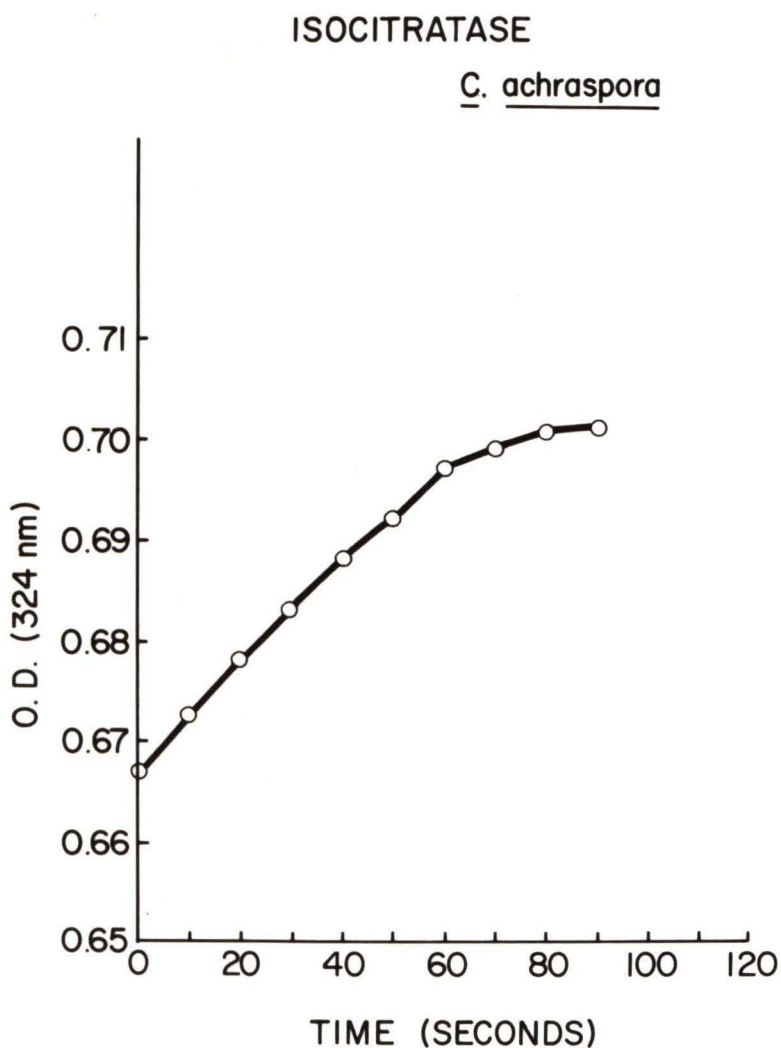
Fig. 16. Isocitratase of <u>H. mediosetigera</u>. The cuvette contained (in 3.1 ml) 200 umole of potassium phosphate buffer, pH 6.85, 15 umole of $MgCl_2$, 10 umole of phenylhydrazine·HCl, 4.5 umole of cysteine·HCl, 5 umole of DL-isocitrate and 1.61 mg of protein. Suitable blanks were included in which substrate or enzyme was omitted; temperature 25 C.

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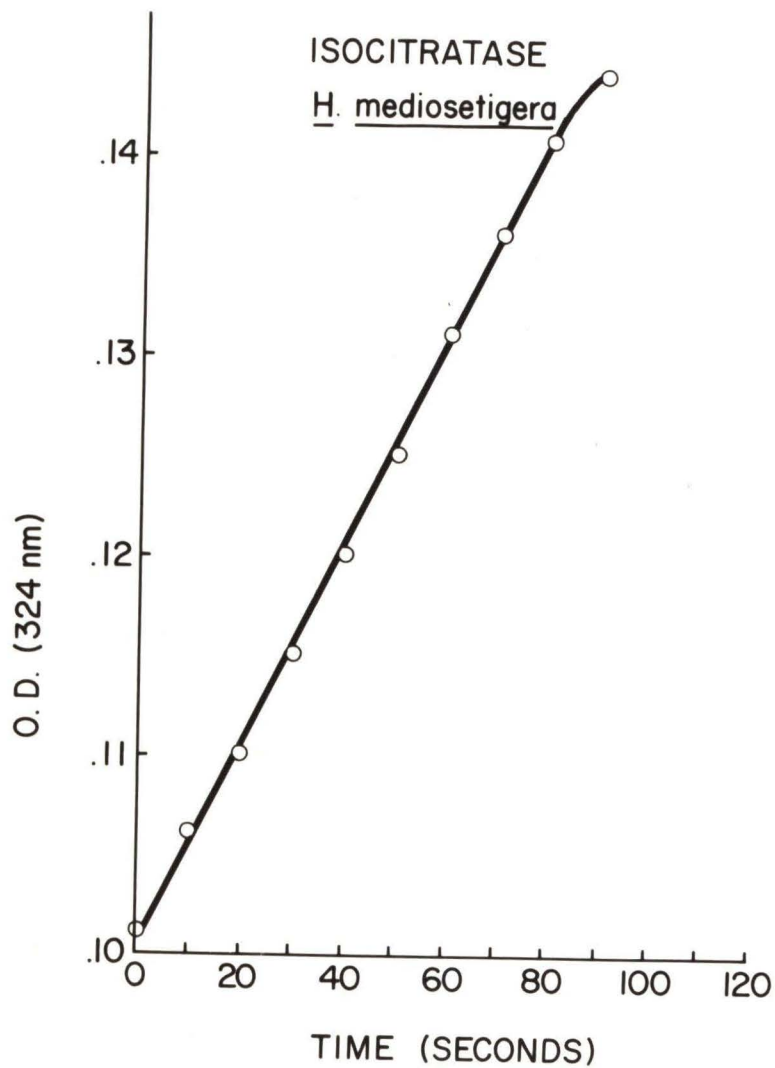
TABLE 5. Comparison of specific activities of selected TCA and glyoxylate pathway enzymes in acetone powder extracts of H. mediosetigera (700) and C. achraspora (230)

Enzyme	Definition of specific activity	Calculated specific activity		Specific activity according to Racker unit. (Change in O.D. of 0.001/min/mg protein)	
		Culture			
		700	230	700	230
Aconitase	Substrate (umole) disappearing/min/mg protein at 24 C	50.62	15.4	248.0	75.0
Isocitrate dehydrogenase (NAD-specific)	Change in O.D. of 0.001/min/mg protein	75.0	98.2	75.0	98.2
Isocitrate dehydrogenase (NADP-specific)	Change in O.D. of 0.001/min/mg protein	-	1.22	-	12.2
2-Oxoglutarate dehydrogenase	Formation of 1 mole of NADH/min/mg protein	-	-	-	-
Succinate dehydrogenase	Change in O.D. of 0.001/min/mg protein	14.1	13.8	14.1	13.8
Fumarase	Initial rate of change in O.D./10 sec X 10^3/ml/mg protein	461.5	27.1	1539.0	176.0
Malate dehydrogenase	Change in O.D. of 1/min/mg protein	0.067	0.59	67.0	590.0
Isocitratase	Substrate (umole) disappearing/min/mg protein	0.0035	0.0018	19.0	9.0

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of the Racker unit (Racker, 1955), as shown in the last column of Table 5. The specific activities of aconitase, fumarase and isocitratase were higher for H. mediosetigera than for C. achraspora, whereas the reverse was true for NAD-linked isocitrate dehydrogenase and malate dehydrogenase. Isocitrate dehydrogenase (NADP) was absent in H. mediosetigera, while succinate dehydrogenase showed approximately the same activity for both cultures. According to the specific activities based on the definition of each individual enzyme, the highest activity was shown by fumarase for H. mediosetigera and NAD-specific isocitrate dehydrogenase for C. achraspora, while the lowest activity was shown by isocitratase for both cultures. These limits, however, were slightly different when specific activities were based on the Racker unit. In this case, the highest activities were shown by fumarase of H. mediosetigera and malate dehydrogenase of C. achraspora, while the lowest activities were shown by isocitratase of both cultures.

To avoid the permeability barriers suspected in intact cells, cell-free extracts were used to obtain manometric responses from terminal oxidation enzymes. The capacity of such preparations to metabolize TCA cycle intermediates was

shown with citrate, cis-aconitate, DL-isocitrate,
2-oxoglutarate, succinate, fumarate and oxalacetate (Table 6).
A glucose control was employed also. The rate of oxidation
of various substrates was different in the two types of
extracts employed, i.e., acetone powder and sonic.

In H. mediosetigera, acetone powder extracts gave higher
oxygen uptakes in the presence of glucose and isocitrate than
were observed for dialyzed extracts from sonically disinte-
grated cells. Citrate did not stimulate oxygen consumption,
while cis-aconitate was oxidized only when sonic extracts
were employed. In C. achraspora, the activity of acetone
powder extracts in the presence of glucose, citrate, cis-
aconitate, isocitrate and fumarate was higher than that of
sonically disrupted cells. Neither culture oxidized
2-oxoglutarate when acetone powder extracts were used.
Succinate did not stimulate oxygen consumption in either
culture under these circumstances. Neither whole cells nor
acetone powder extracts showed oxygen uptakes when the ethyl
esters of citrate, fumarate, malate and oxalacetate were
tested.

TABLE 6. Oxidation of TCA cycle intermediates by acetone powder and sonic extracts.

$$Q_{O_2} \ (N)$$

| Substrate | C. achraspora | | H. mediosetigera | |
	Sonic extracts (dialyzed)	Acetone extracts	Sonic extracts (dialyzed)	Acetone extracts
Glucose	1.7	2.1	3.2	3.54
Citrate	0.9	1.0	-	-
Cis-aconitate	0.66	1.47	1.0	-
Isocitrate	1.60	1.86	4.06	4.41
2-Oxoglutarate	0.54	-	1.28	-
Succinate	-	-	-	-
Fumarate	0.43	1.6	2.24	1.79
Malate	3.2	1.0	2.72	2.51
Oxalacetate	1.28	0.82	0.91	0.62
Endogenous	0.41	0.69	0.32	0.57

DISCUSSION

The filamentous marine fungi have received relatively
little biochemical study. While there is no reason to
suppose that marine fungi are not metabolically comparable to
terrestrial forms, there is always the possibility that some
response to the ocean environment will reveal itself in an
interesting and significant manner. All lines of metabolic
research promote the solution of the numerous problem areas
listed in the introduction to this thesis. The position of
this diverse group of microorganisms in the marine biosphere
and the potentiality of their practical importance will
remain incompletely understood until their biochemistry is
known rather than assumed.

One undeniably useful means of accomplishing this task
lies in the careful examination of enzyme systems operable
under simulated marine environmental conditions in the
presence of natural carbon sources or their degradation
products. With the exception of the marine fungal cellulo-
lytic enzyme complex, which has only recently begun to receive
fundamental study (Jensen and Sguros, 1971), dehydrogenase
rapid test method development (Rodrigues et al., 1970) and

investigations of enzymatic indicators of glycolytic metabolism (Sguros et al., 1971), cogent enzymological data is lacking. Extension of this approach into the area of aerobic terminal oxidation appeared a logical means of continuing the biochemical delineation.

The terminal oxidation of pyruvate in aerobic organisms occurs via the TCA cycle and its offshoot, the glyoxylate pathway (Fig. 17). While many recent studies indicate the utility of terminal oxidation systems in providing carbon skeletons for growth and biosynthesis in fungi, it is now almost axiomatic that TCA cycle dehydrogenases couple to a respiratory process, thereby producing high energy phosphate during electron transfer to molecular oxygen. The sequence of reactions by which the electrons are withdrawn from the substrate and transferred to oxygen is known as terminal respiration. The transfer is mediated by a system in which coenzymes and carriers of increasingly positive oxidation-reduction potential form a continuous pathway. The most common terminal avenue involving the flow of electrons consists successively of pyridine nucleotide, flavoprotein and cytochromes:

Fig. 17. TCA cycle and glyoxylate by-pass indicating the presence of isocitratase (1) and malate synthetase (2) (Niederpruem, 1965).

$$\text{substrate} \longrightarrow \text{pyridine nucleotide} \longrightarrow$$

$$\text{flavoprotein} \longrightarrow \text{cytochromes} \longrightarrow \text{oxygen}$$
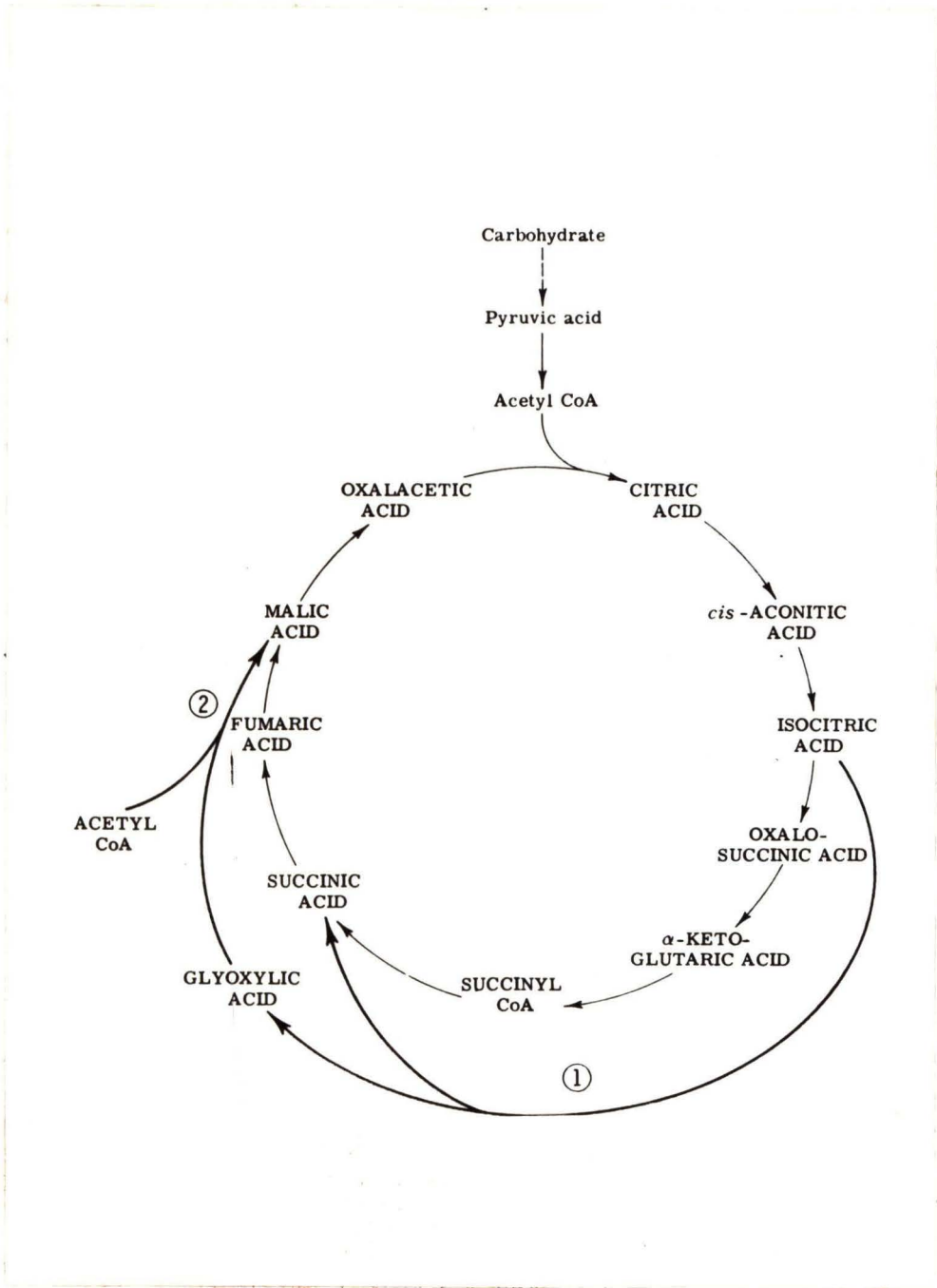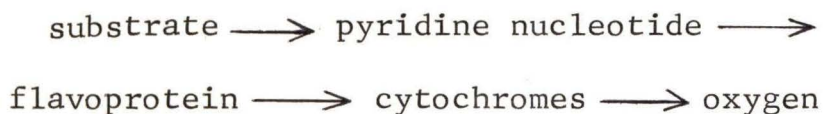
The only effective means known for coupling energy production with energy utilization is by the esterification of phosphate to form ATP. The energy then may be readily transferred to endergonic systems for biosynthesis, membrane maintenance and such physical functions as motility, as appropriate.

Much of the energy available to the aerobic cell, therefore, is due to the oxidation of pyruvate to CO_2 and H_2O. The complete breakdown of one molecule of glucose to CO_2 and H_2O in eucaryotic cells results in the formation of 40 energy-rich phosphate bonds, out of which only two are gained during glycolysis. The remaining 38 energy-rich phosphate bonds are made available to the cell by the TCA cycle. Analyses of the energetics of this system indicate that the free energy change involved in the complete breakdown of one molecule of glucose is -688 kcal. If the terminal phosphate bond represents an F_{hydrol} of -8000 calories, then 688,000/8,000 provides a potential of 85 energy-rich phosphate bonds. Thus an efficiency (38/85 x 100) of 45% is attained in capturing the energy theoretically available. The remaining fraction of the energy is lost, possibly as heat (Neilands and Stumpf, 1958).

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Concerning the role of the TCA cycle in provision of carbon skeletons for growth and biosynthesis, it has been confirmed that aspartic and glutamic acids result from the TCA cycle and, as parent compounds, further serve as precursors of other amino acids (Niederpruem, 1965).

Utilizing few exogenous carbon sources in a natural environment where the organic carbon averages only 10 mg/liter, it has been expected that marine fungi might employ maximal energy gaining mechanisms for survival. Until this work, however, the issue has been conjectural.

As an initial step, vegetative growth curves were correlated with whole cell oxygen consumption for both cultures confirming previous data on the age of maximum endogenous respiration under experimental conditions. Such information allowed the assessment of cultural vigor upon which harvest times for maximal enzymatic activity depended.

While establishing the maximal oxidative capacity of these fungal isolates some divergence was noted in respirometric data, although the range of Q_{O_2} maxima in several experiments for H. mediosetigera at 72 hr and for C. achraspora at 96 hr was approximately the same as reported by Rodrigues et al. (1970). This variation may have resulted

from slight differences in growth rates as mentioned earlier. Anomalies, which might have resulted from the failure of O_2 to adequately penetrate pellet interiors, were minimized by homogenizing mycelia, although it is also possible that mechanical injury could have caused spurious respiratory results (Cochrane, 1958).

The high endogenous respiration rate characteristic of many fungi precludes the use of exogenous substrates without special treatment, and thus complicates experimental study (Cochrane, 1958). Filamentous marine fungi were no exception to this rule. In addition, exogenous substrates may compete with the endogenous metabolism, thus partly suppressing it. Experimentally, the high level of endogenous respiration may be reduced by starving mycelia before the addition of exogenous substrates (Webley and Dekock, 1952). Certain fungi may lose respiratory activity during the starvation period (Bentley, 1953), while others may exhibit morphological changes (Ingraham and Emerson, 1954). Another approach for reducing endogenous respiration levels may be to cultivate these organisms in a medium in which cells do not accumulate reserve materials, such as lipids, in large quantities (Blumenthal, 1965).

In the absence of deliberate attempts to reduce the endogenous respiration of these marine cultures, the oxidation of test substrates by whole cells appeared restricted to glucose and malate. It is difficult to distinguish between the inability of cells to respire exogenous substrates because they are unusable or because they fail to penetrate the cell membrane. Accumulated evidence shows that cells in general are impermeable to certain substrates, such as polar substances, which are ionized at physiological pH. In other cases, it is possible for enzymes in the cell to be inaccessible to substrate owing to subcellular organization. Such a situation is not easily distinguishable from true membrane effects (Cochrane, 1958).

These studies of TCA cycle enzymes required the implementation of extraction procedures. Since the isolation and purification of each individual enzyme in the cycle was beyond the scope of this work, experiments were performed with crude preparations from sonically disintegrated cells and acetone powders. The low yields resulting from the extraction of protein from the dark colored powder may be attributed to moisture which tends to support the inactivation of enzymes by extraneous factors (Umbreit et al., 1964).

After acetone treatment, the white appearance of the filtrates was probably due to the presence of lipids extracted from the mycelium. The removal of fat, coupled with dehydration, probably widened the pores of hyphal walls, facilitating enzyme removal.

One main disadvantage in the use of crude cell-free extracts is the effect of competing enzymes or substrates on the quantitative measurement of specific enzyme activities. The studies of Kaplan, Colowick and Nason (1951) on mycelial extracts and those of Zalokar and Cochrane (1956) on fungal conidia exemplify such interfering actions. The latter, using Neurospora crassa, found interference owing to the presence of a diphosphopyridine nucleotidase. Similarly, Casida and Knight (1954), studying the respiration of P. chrysogenum, noted significant levels of phosphatases which hydrolyzed ATP, FAD, NAD and NADP. Such interference could have presented a problem during the assay of certain enzymes of the TCA cycle in marine fungi, although evidence was insufficient to warrant extensive countermeasures in most cases. Preliminary attempts to assay citrate synthase were negated by the apparent presence of a deacylase, but methods for overcoming this obstacle in crude extracts have not been

devised as yet (Srere, 1969).

Extracts obtained from sonically ruptured cells exhibited higher endogenous respiration than those from acetone powder, and also were prone to inhibition by certain substrates. Both problems were rectified by dialyzing the extracts against cold, distilled water. On the other hand, extracts obtained from acetone powder had to be dialyzed only when the isocitratase of H. mediosetigera and the isocitrate dehydrogenase of both cultures were assayed spectrophotometrically. Dialysis was necessary since undialyzed extract controls showed excessive activity. The high endogenous respiration and control activities shown by undialyzed extracts could have been caused by the destruction of unknown protective factors during exposure to the harsh effects of acetone, even at low temperature.

The detection of aconitase in cell-free extracts was the first definite indication of the existence of the TCA cycle in marine fungi. The assay should have been performed in 0.5 mm light path cuvettes in the presence of 2 mmoles of isocitrate, since Fansler and Lowenstein (1969) have shown that under such conditions the reaction rate is maximal. Lacking suitable quartz spacers, the assay was performed

with 0.1 mmole of substrate and a light path of 1 mm. Under such conditions the reaction rate was considerably below the maximum, but none-the-less discernible.

Aconitase is primarily concerned with the interconversion of the three tricarboxylic acids:

$$citrate \longrightarrow cis\text{-}aconitate \longrightarrow isocitrate$$
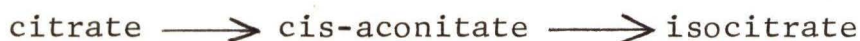
It is believed that there are two components involved which are separable by conventional ammonium sulfate fractionation (Racker, 1950). One of these components, designated aconitic hydrase (Neilson, 1962), is associated with a high molecular weight protein and is responsible for the formation of citrate from cis-aconitate. The other component, aconitase, resembles that of animal tissues and, unlike aconitic hydrase, requires ferrous ions and a reducing agent such as thiomalate for activation.

Morrison (1954) reported that aconitase activity increased considerably in the presence of ferrous ions and a reducing agent. Ferrous ions alone caused some increase in activity, while the reducing agent alone did not. The failure of other metallic ions to stimulate the enzyme indicated that ferrous ion is a specific integral component of aconitase. The relationship between ferrous ion concentration and enzyme

activity is consistent with the idea that ferrous ions react with the enzyme in the ratio of one-to-one, forming a ferrous enzyme complex. This in turn reacts with one molecule of reducing agent to activate the enzyme. Since the substrates of aconitase are also capable of forming complexes with ferrous ions, there is a possibility that the free valencies of the latter may be concerned in linking the substrate to the enzyme (Dickman and Cloutier, 1951).

The isocitrate dehydrogenases are distinguished by co-factor requirements and other important features. Unlike the NAD-specific isocitrate dehydrogenase, which occurs in mitochondria, the activity of the NADP-linked enzyme is confined to the soluble fraction. The function of the two types of enzymes are different, in that the NAD-linked catalyst provides a means for the production of ATP by oxidative phosphorylation, while the NADP-linked enzyme furnishes reducing power to the cell for biosynthesis. Depending on the coenzymes involved, the reactions catalyzed are:

(1) isocitrate + NAD $\longrightarrow$ 2-oxoglutarate + NADH + H^+ + CO_2

(2) isocitrate + NADP $\longrightarrow$ oxalosuccinate + NADPH + H^+
oxalosuccinate + $H^+ \longrightarrow$ 2-oxoglutarate + CO_2

$\overline{}$

isocitrate + NADP $\longrightarrow$ 2-oxoglutarate + CO_2 + NADPH + H^+
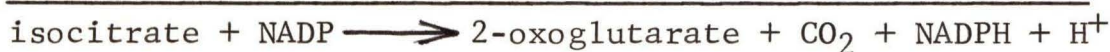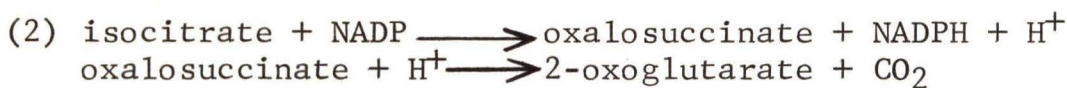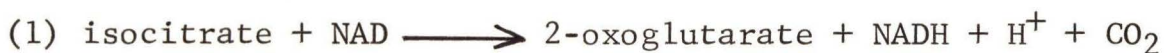
Also, the interconversion between NADPH and NADH may be
catalyzed by yet another enzyme, pyridine nucleotide
transhydrogenase (Lowenstein, 1969):

$$\text{isocitrate} \longrightarrow \text{2-oxoglutarate} + CO_2 + NADH \xrightarrow{\sim P} O_2$$

$$\Big\downarrow \text{Transhydrogenase}$$

$$\text{isocitrate} \longrightarrow \text{2-oxoglutarate} + CO_2 + NADPH \longrightarrow O_2$$
$$\text{No} \sim P$$
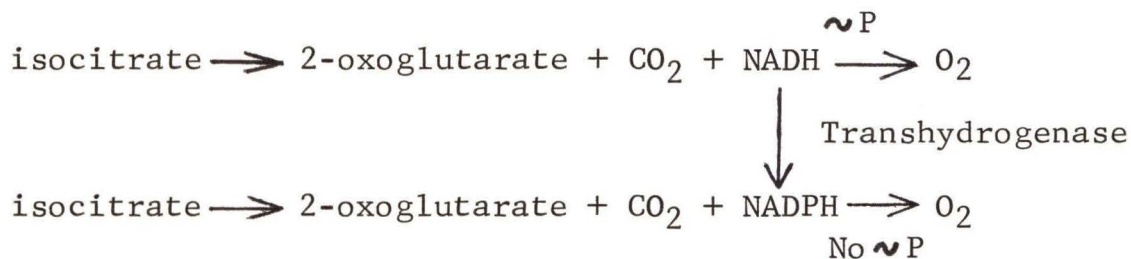
Since the NADP-linked dehydrogenase was absent in H.
mediosetigera, NADPH is probably furnished in this manner from
the NADH produced during glycolysis. C. achraspora, on the
other hand, may be independent of this interconversion.

Purified NAD-specific isocitrate dehydrogenase requires
AMP and Mg++ or Mn++ for maximum activity. According to
Sanwal, Zink and Stachow (1963), AMP probably causes intra-
molecular changes (allosteric activation) conducive to a
more efficient functioning of the enzyme. Enzyme inhibition
caused by high concentrations of AMP can be relieved by
excess NAD. While the exact nature of the inhibition is
unknown, it does not appear to be due to competition at the
NAD site.

In the crude cell-free extracts of many fungal species,
NAD oxidase activity is present requiring the use of a

coupled assay for the NAD-linked isocitrate dehydrogenase. The fungal subjects of this research proved no exception. The NADP-linked enzyme, however, is not vulnerable to such a complication and was readily assayed in C. achraspora.

The failure to detect 2-oxoglutarate dehydrogenase, a key enzyme of the TCA cycle (Khouw and McCurdy, 1969) was puzzling. Although manometric studies on sonically disrupted mycelia showed 2-oxoglutarate oxidation, those on the acetone powder did not. Since the extracts used for the spectrophoto-metric determination of the enzyme were obtained from acetone-treated cells, it is possible that the solvent proved harmful.

Lovett and Cantino (1961), during their studies on Blastocladiella, used a direct chemical method for determin-ing 2-oxoglutarate in a mixture of acetyl CoA, NAD, potassium phosphate buffer and a high protein concentration, but the expected product did not appear. Since fungi, in general, have high intracellular amino acid concentrations and com-mensurately active transaminases, such as those catalyzing the conversion of 2-oxoglutarate to glutamate, the disappear-ance of the keto acid cannot be attributed unequivocally to the action of its specific dehydrogenase.

In these marine isolates, the successful assay for

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succinate dehydrogenase made it reasonable to expect the presence of other related TCA cycle dicarboxylic acids. It is agreed generally that the enzyme is bound to the mitochondrial membranes, being in close physical relationship with the coenzymes of the respiratory chain (Lowenstein, 1969). The reversible oxidation of succinate to fumarate appears as:

$$\text{succinate} \longrightarrow \text{fumarate} + 2H^+ + 2e^-$$
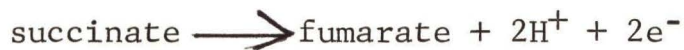
The pure enzyme, obtained from animal tissues and yeast, is dependent upon the presence of sulfhydryl groups and is competitively inhibited by malonate, oxalacetate or pyrophosphate (Niederpruem, 1965).

The choice of electron acceptor for assaying succinate dehydrogenase was problematical, the identity of the natural acceptor being uncertain. In vitro only N-alkylphenazonium salts (phenazine methosulfate), ferricyanide and Würster's blue have been reported to be effective (Lowenstein, 1969). These marine cultures reacted properly to ferricyanide.

Errors owing to inhibition by accumulating oxalacetate, when malate dehydrogenase is present in the same proportion, can complicate the assay of succinate dehydrogenase (Lowenstein, 1969). As in yeast, measured catalytic activity in

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marine cultures could have been lower than the true activity
if the fungal enzyme was somewhat sensitive to acetone
(Niederpruem, 1965).

The use of bovine serum albumin and EDTA was justified
in the primary assay system to remove the harmful effects of
metals and prevent the swelling of mitochondria (Hunter et al.,
1959) as well as the loss of unbound NAD (Lester and Hatefi,
1958).

Fumarase catalyzes the reversible hydration of fumarate
to L-malate:

$$\text{fumarate} + H_2O \longrightarrow \text{malate}$$
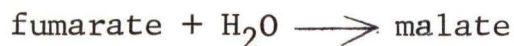
This enzyme possesses an absolute specificity for both
substrate and product and compounds structurally related to
fumarate and L-malate remain unaltered by it. The effect of
pH on the enzymatic activity of fumarase has been discussed
in detail by Alberty (1961). Activity-pH studies yield a
bell-shaped curve, suggesting that the enzyme possesses both
acidic and basic functional groups in its active site.
Studies on the effect of buffer composition are complicated
by the binding of anion impurities. Monovalent anions such
as iodide, bromide and chloride have been observed to inhibit

fumarase, although polyvalent anions such as phosphate and sulfate actually are stimulating in dilute concentrations.

Reactions of the native mammalian enzyme show irreversible inactivation by a variety of sulfhydryl reagents, depending upon the nature and concentration of the latter (Hill and Bradshaw, 1969). In contrast, yeast fumarase actually requires thiol groups for activity, while otherwise closely resembling that of mammalian systems (Niederpruem, 1965). Although the study of such details were outside the scope of this work, it was obvious that the extracts of marine fungi either furnished the thiols or inhibited them as required. The type of enzyme involved remains to be determined.

Hayman and Alberty in 1961 have demonstrated two molecular forms of fumarase in Candida utilis. Although the enzymes have not been crystallized, adequate separation has been achieved by column electrophoresis. The two fumarases differ with respect to their electric charge, pH optima, K_m values and acid ionization constants (Niederpruem, 1965). Again, it is not known whether marine fungi are equipped with more than one kind of fumarase.

The activity of fumarase has been reported to be highly temperature sensitive (Hill and Bradshaw, 1969), and care

was taken to maintain the optimum constant temperature of 25 C during the assay of marine fungal extracts. Within the range of 22-28 C, the activity of the enzyme varies by 8% for every change of one degree on either side of the optimum. Also, in common with many other enzymes, fumarase shows marked changes in activation energy under various conditions. Michaelis and inhibition constants are also markedly temperature dependent. It has been shown that many of the effects of temperature on catalysis are paralleled by changes in the physical properties of the enzyme.

The presence of malate dehydrogenase in these fungi indicates utilization of the last step of the TCA cycle, i.e., the conversion of malate to oxalacetate:

$$\text{malate} + \text{NAD} \longrightarrow \text{oxalacetate} + \text{NADH}^+ + \text{H}^+$$

While earlier studies (Niederpruem, 1965) indicated that only malate could serve as a substrate, it was shown later (Davies and Kun, 1957) that a broader range of substrates could be oxidized, the enzyme appearing to be an α-hydroxy dicarboxylic acid dehydrogenase of only moderate specificity (Davies, 1969). In the present work, oxalacetate was used as a substrate, since the in vitro equilibrium favored the

reduction of oxalacetate by NADH rather than the oxidation of malate by NAD. The marine fungal enzyme must be of the soluble form, since this form alone can accomplish the reverse reaction, where oxalacetate is reduced to malate (Kaplan, 1963).

The detection of isocitratase in both fungal species was presumptive evidence for the operation of the glyoxylate by-pass (Fig. 17), which provided a mechanism for a second point of entry for acetate into the TCA cycle (Niederpruem, 1965). As previously mentioned, the pathway serves to conserve carbon as CO_2 and to replenish C_4 acids by providing succinate, the amount of which may be reduced owing to the continual loss of 2-oxoglutarate to transamination reactions. The key reaction is the conversion of isocitrate to succinate and glyoxylate:

$$\text{isocitrate} \longrightarrow \text{glyoxylate + succinate}$$

Glyoxylate is postulated to then react with acetyl CoA in the presence of malate synthetase to form malate which enters the TCA cycle.

Only the enzyme from Pseudomonas indigofera has been purified to homogeneity and shows a marked specificity for

glyoxylate and succinate. The most effective metallic acti-vator is Mg++, while thiols and EDTA are not always required.

It should be recalled that initially only acetate-grown cells were believed capable of by-pass induction (Krampitz, 1961). According to Gottlieb and Ramachandran (1960), however, the isocitratase activity of acetate-grown cells was merely greater than that of cells grown on glucose. The presence of this enzyme in glucose as well as acetate-grown cells allowed the speculation by these authors that its presence was constitutive, which was contrary to reports on other organisms.

Finding isocitratase in glucose-grown marine fungi may have supported the "constitutive" hypothesis, but since growth on acetate could not be obtained (Sguros et al., 1971) complete comparisons were not possible. Gallakota and Halvorson (1959) postulated the induction of this enzyme due to the accumulation of acetate, and further reported that adaptive formation of this enzyme was inhibited by glucose in the culture medium. Since the marine fungi under study here were harvested in the mid-linear phase, prior to glu-cose exhaustion (Sguros and Dias, unpublished), the constitu-tive theory appears more favorable.

Cell-free extracts were employed during manometric observations to avoid anticipated substrate permeability problems. Specific substrates may be taken across the cell membrane by one or more of a variety of mechanisms. The nature of the substrate is influential, e.g., substances ionized at physiological pH, such as phosphorylated compounds and organic acids, are frequently excluded by a so-called permeability barrier. Assuming that impenetrability is caused by the charge on weakly ionizing acids at physiological pH, lowering the pH of media should prevent dissociation and allow penetration. The employment of inhibitors of enzymes specific for certain acids, causing accumulation of the latter, also has led to permeability difficulties. While inhibition of N. crassa respiration was rapidly obtained with monofluoroacetic acid (Strauss, 1955), Goldschmidt, Yall and Koffler (1956) noted that P. chrysogenum required incubation with this poison for as long as 1 hr before significant inhibition occured. It appeared that here also acidic pH favored the penetration of the inhibiting compound (Black and Hutchens,1948). Since many microorganisms may have a metabolic capability and still fail to use a substrate owing to the lack of a suitable intake mechanism, it is

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frequently necessary to use cell-free preparations in enzyme assay systems.

As the data have shown, in all instances, except for succinate in both cultures and citrate in H. mediosetigera, these fungi showed oxidative capabilities in either acetone powder or sonically disintegrated form, or both (Table 6). Manometric results confirmed those obtained spectrophoto-metrically. H. mediosetigera, unlike C. achraspora, failed to show manometric activity against citrate. Such negative results may have been due to improper concentrations of exogenous substrate used in the tests, despite employment of a substrate range of 7.5-30 umole.

The failure of succinate to stimulate oxygen uptakes in both cultures may have been due to some discrepancy in technique. The absence of components used in the successful spectrophotometric method, e.g., EDTA and bovine serum, may have been partly responsible (cf.p. 61). Also, this inconsistency could have been caused by failure of the dehydrogenase to utilize phenazine methosulfate as an artificial electron acceptor, even though this was not the posture of the other dehydrogenases. Wessels (1959) has reported that it is the NAD-linked formation of oxalacetate

that normally suppresses succinate dehydrogenase. Therefore, inhibition of the marine fungal enzyme was not surprising.

Although spectrophotometric results were negative, the absence of 2-oxoglutarate dehydrogenase in these marine organisms was unlikely, since sonically disintegrated cells did take up oxygen in the presence of 2-oxoglutarate. Negative manometric results with acetone extracts of both cultures may have indicated the destruction of the enzyme by acetone. Externally added oxalacetate, being a competitive inhibitor of succinate dehydrogenase, may have inhibited the succinate-to-fumarate reaction in the cycle, slowing the entire system (Wessels, 1959). This may have accounted for the slow oxidation of oxalacetate by these marine isolates.

Lower endogenous respiration with acetone powder agreed with work reported by Bentley (1953) in which it was brought out that acetone can cause the malfunction of certain respiratory enzymes. The use of non-dialyzed extracts from acetone powder was therefore justified as an effort to retain the protection afforded by dialyzable substances.

According to Beevers et al. (1952), esters should be able to pass through cell membranes over a wide pH range. Studies on intact cells of P. chrysogenum indicated that,

while succinate failed to promote basal respiration, diethylsuccinate, applied at pH 6, significantly increased oxygen consumption. It appeared possible that, in this present work, esterification might preclude the effect of pH on substrate ionization, allowing its penetration at physiological pH, provided the ester per se was acceptable.

The failure of esters of TCA cycle substrates to successfully challenge the permeability barriers of intact marine fungal mycelia was probably due to the latter's inability to break down the alcohol moieties or to hydrolyze the original compounds. Any effect of ethyl alcohol, used as the solvent for all esters, was countered by the use of appropriate controls.

Positive assays for aconitase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase and isocitratase in H. mediosetigera and C. achraspora strongly suggest that the TCA cycle and glyoxylate by-pass are major mechanisms of terminal oxidation by these organisms in the sea. However, since the mere presence of appropriate enzymes is not conclusive evidence of the importance of these pathways, additional approaches will be required before irrefutable confirmation

can be obtained.

One of the most successful means of establishing such a terminal oxidation system has been to incubate mycelia in the presence of radioactive acetate and show its oxidation through the TCA cycle (Demoss and Swim, 1957). The separation and identification of radioactive intermediates by chromatographic techniques would lend further evidence.

It is felt, however, that, with minor exceptions, sufficient evidence has been gathered to demonstrate the operation of the TCA cycle and glyoxylate by-pass in these marine fungi. The general manifestation of this means of terminal oxidation makes it possible now to speculate that all marine fungi probably employ this means of energy production and biosynthesis.

Having stressed the limitations of the particular approach used here, it seems pertinent to discuss the applicability of those standard methods actually employed. The manometric and spectrophotometric methods are general in their application, while dye reduction methods have a more limited value. Spectrophotometric methods are sensitive as well as quantitative and are used whenever a component of the system changes its light absorption characteristics as a

consequence of an enzymatic reaction.

Manometric techniques have been used successfully with bacteria, but quantitative studies with fungi, in general, have been less rewarding. The rate of endogenous respiration among the filamentous marine fungi was high compared to total respiration in presence of exogenous substrates, limiting this approach. Nevertheless, this method was considered a potential means of identifying metabolic intermediates. Because of the technical difficulties mentioned above, manometric techniques must be used in conjunction with spectrophotometric methods to indicate unequivocally the presence of metabolic pathways.

Sguros and Rodrigues (1966) revealed that whole cells of marine filamentous fungi showed no significant oxidation of TCA cycle intermediates, above endogenous rates, despite the employment of various optimal conditions. It was assumed that the lack of substrate utilization was due either to a permeability barrier or to the failure to decrease the endogenous respiration to the point where exogenous substrate activity became discernible.

Rodrigues et al. (1970) suggested that these marine filamentous fungi utilized glucose via the TCA cycle. By

employing a rapid screening method for dehydrogenases, it was found that sonic extracts of H. mediosetigera and C. achraspora showed the presence of NAD-linked isocitrate dehydrogenase and malate dehydrogenase. The data of this present work have confirmed these preliminary results, and, in addition, have provided a more complete picture based on accurate methods.

Sguros et al. (1971), while surveying the effects of carbon sources on the growth of these marine fungi, observed that they were highly selective in the utilization of substrates. Only cellulose, its related products, such as cellobiose and glucose, and the latter's rearrangement products, fructose and mannose, were significantly utilized. TCA cycle intermediates scarcely elicited any responses, thereby suggesting that these organisms were impermeable to such metabolites. Again, this present work confirms and supports previous observations.

The major objective of cellulose degradation by these microogranisms is the acquisition of energy and carbon skeletons for growth and development. These singular marine fungi appear to rely mainly on cellulose and its degradation products in nature and possess a cellulolytic exocellular

enzyme complex to initiate their metabolic endeavors (Jensen and Sguros, 1971). In the littoral zone of the marine environment, conditions favor the provision of richly oxygenated water. This parameter, when combined with the abundance of most inorganic nutrients in the sea and the presence of suitable cellulosic substrates, ensures that marine environments will continue to be a suitable niche for the growth of filamentous marine fungi.

SUMMARY

1. Previous data on fungal culture, inoculum production and respirometry were confirmed as a basis for the production of enzymatically active acetone powder and sonic extracts of selected marine fungi.

2. Acetone powder extracts of glucose-grown H. mediosetigera and C. achraspora were assayed successfully for aconitase, NAD-linked isocitrate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase and isocitratase. In addition, C. achraspora showed NADP-linked isocitrate dehydrogenase activity.

3. Acetone powder extracts of C. achraspora oxidized all TCA cycle intermediates except succinate and 2-oxoglutarate.

4. Sonic extracts of C. achraspora oxidized all TCA cycle intermediates except succinate.

5. Acetone powder extracts of H. mediosetigera oxidized all TCA cycle intermediates except citrate, succinate, cis-aconitate and 2-oxoglutarate.

6. Sonic extracts of H. mediosetigera utilized all TCA cycle intermediates except citrate and succinate.

7. Attempts to overcome possible permeability barriers of whole cells using esters of succinate, fumarate, malate and

oxalacetate failed. Acetone powder extracts of C. achraspora
also did not utilize the esters.

8. The presence of TCA cycle enzymes in these marine fungi,
following growth under simulated marine conditions, has been
established.

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APPENDIX I

CALCULATION FORMULAE

1. Volume required to give 2 mg mycelium/ml of inoculum for culture production:

$$\frac{(\text{mg/ml sample} \times \text{original volume})}{4} \quad \frac{(\text{ml in blendor})}{\text{original volume}} - \text{ml in blendor}$$

2. Volume required to give 4 mg mycelium/ml for whole cell respirometry:

$$\frac{\text{mg growth expected/ml}[1] \times \text{ml culture}}{4 \text{ mg/ml}}$$

3. Volume required to give 10 mg mycelium/ml for sonic disintegration and acetone powder preparations used for respirometry:

$$\frac{\text{ml sample} \times \text{mg/ml suspension}}{10 \text{ mg/ml}}$$

[1]From standard growth curves.

BIOGRAPHICAL ITEMS

Devi Vembu was born in Karachi, India on October 12, 1938. She graduated from Lady Irwin higher secondary school in June, 1955. She received her first Bachelor of Science degree in Physics, Chemistry and Zoology from Delhi University in June, 1958 and her second Bachelor of Science degree in Microbiology and Zoology from Bombay University in June, 1969. She is a candidate for the degree of Master of Science in Microbiology in March, 1972.

ABSTRACT

Author: Devi Vembu
Title: Evidence for the Existence of the Tricarboxylic
               Acid Cycle and Glyoxylate Pathway in Certain
               Filamentous Marine Fungi
Institution: Florida Atlantic University
Degree: Master of Science
Year: 1972

Cellulolytic marine isolates, Culcitalna achraspora and
Halosphaeria mediosetigera, were shaker-grown at 25 C in
artificial seawater containing glucose, NH_4NO_3, tris(hydroxy-
methyl)aminomethane and yeast extract, pH 7.5. Cultures were
harvested in the late linear phase where whole cell endogenous
respiration was maximal. Spectrophotometric determinations on
acetone powder extracts revealed the presence of aconitase,
NAD-linked isocitrate dehydrogenase, succinate dehydrogenase,
fumarase, malate dehydrogenase and isocitratase. Culcitalna
also showed NADP-linked isocitrate dehydrogenase activity.
Manometric examinations of acetone powder and dialyzed sonic
extracts were largely confirmatory. Oxidation of citric acid
cycle intermediates by extracts were observed with Culcitalna
for all but succinate with sonic extracts and for all but
succinate and 2-oxoglutarate in acetone powder extracts. With
Halosphaeria, neither extract could oxidize citrate or succinate,
while acetone powder extracts were unable to degrade cis-aconitate
and 2-oxoglutarate. Native mycelia were active only against
malate. Attempts to permeate cell walls with ethyl esters of
intermediates failed.