# ENERGY AND EVOLUTION IN THE THIOBIOS: AN EXTRAPOLATION FROM THE MARINE GASTROTRICH *THIODASYS STERRERI.*

by

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**Résumé**

*Thiodasys sterreri*, très abondant dans la couche de discontinuité du potentiel d'oxydo-réduction et capable de survivre à des conditions complètement anaérobies, semble apte à fixer le dioxyde de carbone. Des mesures chromatographiques et autoradiographiques indiquent qu'il s'incorpore activement dans une séquence inversée du cycle de Krebs. La cytochrome-oxydase est présente mais sa fonction et le mécanisme lui évitant un empoisonnement par les sulfures restent inconnus. Les mitochondries sont semblables à celles des autres espèces anaérobies. Le métabolisme anaérobie des hydrates de carbone et des amino-acides est discuté en rapport avec la méiofaune. Les auteurs montrent que les composants du cycle de Krebs pourraient avoir évolué originellement vers des fonctions anaérobies puis, plus tard, être utilisés comme base d'un métabolisme oxydatif plus efficace. Ces résultats sont discutés en rapport avec les origines des Métazoaires.

## Introduction

Since the publication of Fenchel and Riedl's important study of the sulphide biome in 1970, much interest has been shown in the meiofaunal community of reduced sediments, which were originally thought to be devoid of life. Fenchel and Riedl (1970) showed that the sulphide biome supported a distinct and complex fauna, which they postulated was adapted at the physiological level to the anoxic environment. Ott and Schiemer (1973) discussed nematodes of anoxic sediments but could not present physiological proof of anaerobic metabolism. Pamatmat and Bhagwat (1973) demonstrated the importance of anaerobic metabolism as a component of the overall energy budget of reduced sediments. Boaden and Platt (1971) described a deep level component of the meiofaunal community and coined the term "thiobios" to describe the biome of sulphide rich zones.

The study of the metabolism of this thiobiotic community is important for two main reasons; firstly to determine the contribution of anaerobic metabolism to the energy and nutrient flow of the whole benthos and secondly to understand the methods and evolutionary implications of physiological adaptation to this mode of life. A survey of the literature on anaerobiosis in invertebrates showed that one pathway occurs frequently in several different phyla. This involves the fixation of $CO_2$ into a 3-carbon intermediate; it can then be used as a source of oxidising power, thus producing reduced end products, which are usually excreted (Gilles, 1970; Hammen and Lum, 1962; Saz, 1971). The existence of a similar pathway in thiobiotic species seemed likely. This paper outlines some physiological work carried out to establish whether this is the case.

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## MATERIALS AND METHODS

The thiobiotic gastrotrich *Thiodasys sterreri* Boaden (1974) was chosen as experimental animal because of its large size (< 3 mm) and relative abundance. It is mostly found in the redox potential discontinuity (R.P.D.) layer of sands. It does not occur in any great quantity in the deeper black layers, although it is quite able to survive in completely anaerobic conditions. Animals have been kept for up to four months in sealed jars of sand by which time the sediment was completely anoxic and smelling strongly of hydrogen sulphide. They have also survived well in a continuous culture apparatus (Gallenkamp CeCa), in seawater covered sand under an atmosphere of nitrogen, for 15 days.

### Incubation experiments

*Thiodasys sterreri* was extracted from sand obtained from Ballyholme beach, Bangor, Co. Down, using 7 p. 100 $MgCl_2$ followed by 10 p. 100 ethanol (Gray and Hulings, 1969). Animals were washed with several changes of millipore filtered anaerobic seawater to which a small quantity of sodium thiosulphate had been added, then stored in this medium in solid watch glasses at 8°C. They remained active for at least 3-4 days under these conditions. Approximately 300-400 gastrotrichs in a volume of 2-3 ml of sterile anaerobic seawater were used for each incubation. Incubation was at 10°C. The tubes containing animals were acclimatized in a water bath for at least 30 minutes before addition of labelled material. An aqueous solution of $^{14}C$-labelled sodium bicarbonate (Radiochemical Centre, Amersham, Bucks), activity 60.3u.Ci. per millimole was used. Aliquots of 50(ACì. or 20uCi. were added by micropipette and the animals incubated for one hour. The incubation was stopped by the addition of a few drops of acetic acid to liberate the excess bicarbonate. *Protodriloides symbioticus* (Giard), an archiannelid found in the same beach but more or less confined to the aerobic zone, was used as a control in one incubation. *Protodriloides* is approximately l1/2 times as long as *Thiodasys* and can be up to twice as wide, so less specimens of this species were used.

A few of the incubated gastrotrichs were used for sectioning and autoradiography. These were washed with millipore filtered seawater, fixed in 4 p. 100 buffered glutaraldehyde, dehydrated with ethylene glycol and embedded in G.M.A. resin. Sections 3u and 5 u. thick were cut with a glass knife and mounted on slides. These were dipped in Kodak L4 emulsion gel. at 45°C and, after drying, were packed in light-tight boxes with a few crystals of silica gel and exposed for at least three weeks. Slides were developed for 10 minutes in Kodak D19 developer, washed with 1 p. 100 acetic acid solution fixed for 10 minutes in Ilford Hypam fixer 10 p. 100 solution. Sections

were then examined under the light microscope for deposited silver grains.

The bulk of the animals were filtered, washed several times with approximately 10 ml filtered seawater and extracted overnight with 80 p. 100 ethanol. A small proportion of each extract was counted directly on a planchette in a Nuclear Chicago low background gas flow counter for 5 minutes to estimate total radioactivity. Ethanol extracts from three incubations were pooled, evaporated to dryness and redissolved in a small volume of methanol. Paper chromatograms were made of this extract using Whatman's number 20; 20 X 20 cm paper. Markers of fumaric, malic, succinic and citric acids in methanolic solution were used, spots containing between 0.5 and 1.0 m. mole being placed on each side of the paper. The *Thiodasys* extract, with a small quantity of all markers added, was spread as a thin line along the middle. The chromatogram was developed with ether-formic acid - water, 18:5:9., (Soldatenkov and Mazurova, 1962, in Hammen 1966) in only one direction for 9 hours. The markers were detected by spraying with Hargreaves mixed indicator (Hammen, 1966) or methyl orange in ethanolic solution.

Radioactivity in the first chromatograms was determined by cutting the paper into strips corresponding to each marker spot, eluting with distilled water and counting directly on planchettes. This was not very satisfactory as counts were not significantly above background and the separation of markers was not complete. Later chromatograms were assessed for radioactivity by autoradiography. They were tightly packed with Kodirex Royal Blue X-ray film and exposed for several months. The film was developed in Kodak D19 developer at 20°C for 5 minutes, washed with water and fixed in Hypam one in ten solution.

### Cytochrome oxidase assay

Cytochrome oxidase (cytochrome $a,a_3$) is the terminal electron carrier in the normal electron transport chain, transferring e^ to molecular oxygen. Graaf's G-Nadi reaction is a standard method for its detection.

Several *Thiodasys* were embedded in a small block of ice and sections of 15 a thickness were cut in a "Pelcool" microtome. Sections of fresh mouse liver, 15 u. thick, were used as a control. The sections were floated off the knife into a water bath, then mounted on slides and air dried. They were incubated in Nadi reagent at 37° for one hour *(Thiodasys)* and 15 minutes (mouse liver) respectively.

*Nadi reagent* 20 ml each of α-Napthol solution and oxidase reagent mixed with 8 ml of 0.1M phosphate buffer immediately before incubation.

(1) *α-Napthol solution* 0.1 gm α-napthol dissolved in 1 ml alcohol and made up to 100 ml with distilled water.

(2) *Oxidase reagent* 0.12 gm dimethyl-p-phenylenediamine hydrochloride dissolved in 100 ml distilled water.

After incubation, the slides were washed with distilled water and mounted in glycerine.

## RESULTS

### Autoradiograms of light microscope sections

Deposition of silver grains over sections was not uniform, occurring above some areas of tissue, but not above others. Where there were silver grains, these were not concentrated along the edge of the sections and so do not appear to be a result of pressure effects or adsorption of radioactive carbon onto the epidermis (Plate I, 1 and 2). It is probable that the patchy development was a result of differing levels of metabolism in different types or areas of tissue.

### Incubation experiments

Radioactivity is expressed as counts per minute (c.p.m.) above background.

1st incubation Approximately 400 *Thiodasys,* 50μCi.
TOTAL EXTRACT 340 c.p.m.

2nd incubation Approximately 150 *Thiodasys,* 50μCi.
TOTAL EXTRACT 100-160 c.p.m.

These results show that incorporation definitely occurred. The eluted material from the paper chromatogram strips did not give counts sufficiently above background to determine which specific components were radioactive, but some radioactivity had travelled up the chromatogram, indicating that there had been true fixation into components and not merely absorption of bicarbonate.

3rd, 4th and 5th incubations Extracts from all three pooled.

Total of 900-1,000 *Thiodasys,* incubated with total 90μCi.

Control of 300 *Protodriloides symbioticus* incubated with total 50μCi.

*Thiodasys* extract 640 c.p.m.

*Protodriloides* extract 32 c.p.m.

Radioactivity was considerably higher in the *Thiodasys* extract, suggesting really active uptake and fixation of labelled carbon dioxide.

Autoradiograms strongly suggest that radioactivity was present in substrates at least moving similarly to fumaric and succinic acids (Fig. 1 ). Clear separation between the four markers was not obtained because of drag and blurring effects. More precise identification of radioactive components would require considerably more starting material and would necessitate preparative treatment and 2-D chromatography.

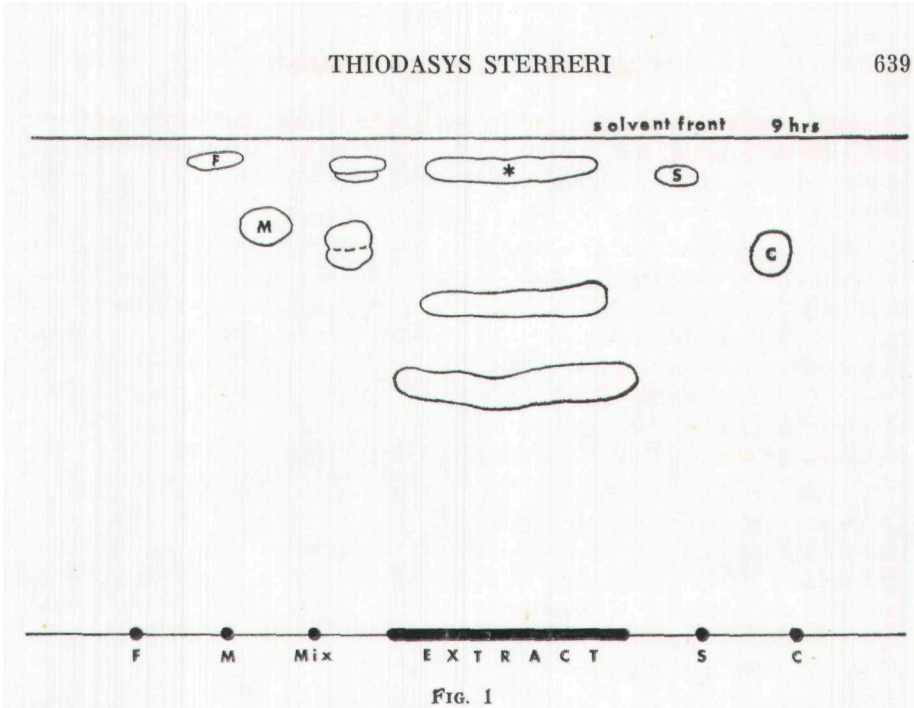


FIG. 1

Tracing from paper chromatogram of extract from incubated *Thiodasys sterreri; F*, fumaric; M, malic; S, succinic; C, citric acid markers; Mix, equal mixture of these. Radioactivity was found in the top band (*) corresponding to fumaric and succinic acids.

### Cytochrome oxidase assay

The reaction was positive in both *Thiodasys* and liver sections, as shown by the formation of indophenol blue in the tissue. This reaction relies on a continued supply of oxidised cytochrome to produce indophenol blue from α-Napthol and the Nadi oxidase reagent. Cytochrome oxidase catalyses the reaction by using molecular oxygen to maintain a supply of oxidised cytochrome.

## DISCUSSION

Anaerobiosis is now known to occur widely amongst invertebrates **(Saz, 1971; Gilles, 1970; Florkin** and Scheer, 1970, 1972 ; Hochachka and Somero, 1972). Many internal parasites exist completely anaerobically (Smyth, 1969) and many intertidal invertebrates e.g. *Crassostrea virginica* (Gmelin) (Hammen and Wilbur, 1958) can survive anoxic conditions for a considerable period of time.

Fixation of carbon dioxide is common to most invertebrate anaerobic metabolism and involves a series of reactions known as the reversed Krebs' cycle sequence. Carbon dioxide is fixed into pyruvate or phosphoenolpyruvate, both products of glycolysis, to form oxaloacetate (for example, see Argosin and Repetto, 1963). Reduction takes place *via* malate and fumarate to succinate, which is frequently

excreted as a reduced end product. Alanine is another fairly common end product, produced by transamination reactions (Awapara and Stokes, 1968). This pathway which occurs in a wide variety of invertebrates is outlined in Fig. 2.

From our results, it seems that a similar pathway is operating in *Thiodasys sterreri.* Some carbon dioxide fixation does occur normally during aerobic metabolism in most living tissues, for example during gluconeogenesis. *Protodriloides symbioticus* was used as a control to determine whether this might be significant. It proved, however, that incorporation by *Thiodasys* was considerably higher. This suggests that the gastrotrich uses $CO_2$ as a means of gaining oxidising power, rather than as an extra carbon source.

Reduction of oxaloacetate to malate regenerates $NAD^+$ which is necessary for continued glycolysis; the hydrogens used for the reduction of fumarate come from a reduced flavoprotein (Fig. 2). Florkin and Scheer (1972) suggest that fumarate acts as a terminal electron acceptor of a modified electron transport chain, mediated by flavins and possibly modified cytochromes; i.e. it is analogous to oxygen in the mammalian electron transport chain.

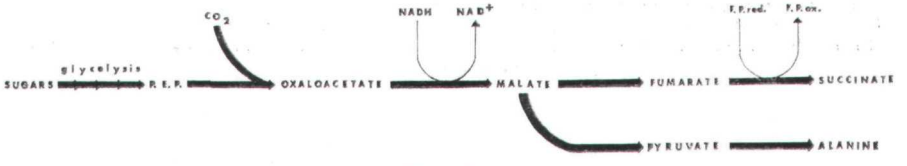


FIG. 2
Route of $CO_2$ fixation common to many invertebrate taxa.

Several parasitic helminths and nematodes have very low levels of cytochromes and those that do occur are often of an unusual type (Bueding and Charms, 1952; Kawai, 1959; Read, 1952). Tappel (1960) examined the cytochrome composition of several marine crustacea and bivalve molluscs; he found some unusual types and some forms were missing altogether in several species. Clearly, the normal aerobic type of electron transport chain present in mammalian mitochondria is not operating but is modified in these species.

The results of the cytochrome oxidase assay on *Thiodasys* suggest that some aerobic respiration may occur within the metabolism. This cytochrome is responsible for the terminal transfer of electrons to molecular oxygen and it is difficult to see why it should be present if molecular oxygen were not used at all. However, there is a bacterium, *Pseudomonas aeruginosa,* which has an unusual type of cytochrome oxidase which can use nitrite as a terminal *e-* acceptor (Massey and Veager, 1963); perhaps *Thiodasys* could be using nitrite, nitrate or sulphate as another source of oxidising power. Cytochrome oxidases are generally sensitive to poisoning by sulphide ions. Presumably, *Thiodasys* has some mechanism protecting its cytochrome oxidase from interference by the high levels of sulphide ions in its environment. It would be of great interest to examine the other cytochromes present in thiobiotic species by spectrophotometry. This

would require a very large number of animals and is probably not feasible until adequate culture methods are developed.

Examination of thiobiotic species for mitochondrial particles could give an indication of their mode of metabolism since anaerobic species have poorly developed mitochondria with few cristae. Electron microscope pictures of *Thiodasys* show mitochondria of the typical anaerobic type (Plate I, 3).

Gnathostomulida of the genera *Austrognathia* and *Gnathostomula* are known to have mitochondria (Graebner, 1968; Riedl, 1969, 1970). Like *Thiodasys*, these genera extend into the R.P.D. layer and above and are not limited to completely anoxic sediments.

## Energetic and evolutionary implications of anaerobiosis

If the pathway outlined in Fig. 2 is widespread amongst the anaerobic meiofauna, then it must have considerable effect on the whole energy budget and nutrient flow of the psammon. Hochachka and Somero (1972), using data from helminthes, annelids and intertidal bivalves, have produced a metabolic map of anaerobic metabolism which shows a great degree of integration between carbohydrate and amino-acid metabolism (Fig. 3). This system shows how a variety of amino-acids can serve as energy sources by deamination and transamination reactions. It is already well-established that this occurs in insects and many marine bivalves and the high level of free amino-acids present in crustaceans suggests that the same system operates in this group too (Gilles, 1970). This system is particularly interesting in view of the high levels of dissolved amino-acids and carbohydrates present in reduced interstitial water (e.g. Brown et al, 1972) which could be available to meiofauna *via* active carrier mediated absorption.

Experiments to determine uptake of labelled amino-acids and occurrences of label in excretory products in the thiobios should prove interesting, but again there are difficulties in dealing with organisms of such small biomass. Uptake of amino-acids may require a specialized epidermis. Riedl (1971) found microvilli on the epidermis of *Gnathostomula jenneri* Riedl which may be one such adaptation.

Special structures may also be required for excreting reduced end products. The dorsal glands of *Thiodasys sterreri* (Plate I, 4) may well be involved with excretion. These glands are fairly transparent in juveniles but become more green-brown and increase in number with age; in fully mature individuals, the glands appear reddish and very granular and can often be observed emerging through the epidermis.

There is now increasing speculation that the type of anaerobic respiration outlined in Fig. 3 is primary and that it produced the preconditions necessary for the evolution of the Krebs' cycle and aerobic respiration (Hochachka and Somero, 1972). The sequence from oxaloacetate to succinate is already present, serving an oxidativc function, in anaerobiosis. Both oxaloacetate and pyruvate can be produced from amino-acids and, in helminthes at least, pyruvate can then serve as a substrate for acetyl CoA which is used as a building

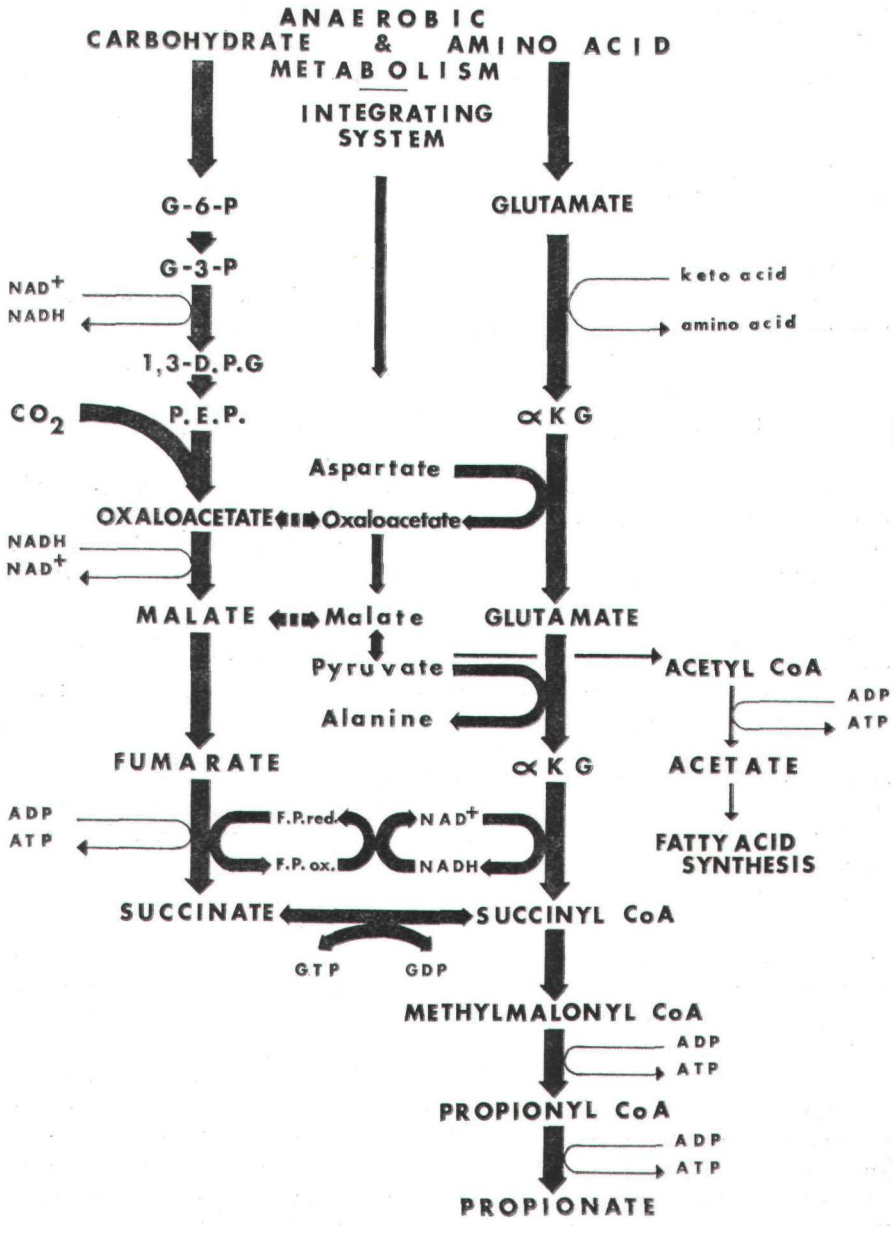


**FIG. 3**

**Map** showing integrating system between anaerobic metabolism **of** carbohydrates and amino-acids. Adapted from Hochachka and Somero **(1970).**

block for fatty acid synthesis. We can speculate that acetyl CoA and oxaloacetic acid, both derived from amino-acids in an anaerobic system, could then combine to produce citrate and ultimately e-keto-glutarate, *via* isocitrate. Any pathway producing a-ketoglutarate is likely to be evolutionarily advantageous, since this substrate can give rise to several alternative routes of ATP or GTP production. Thus

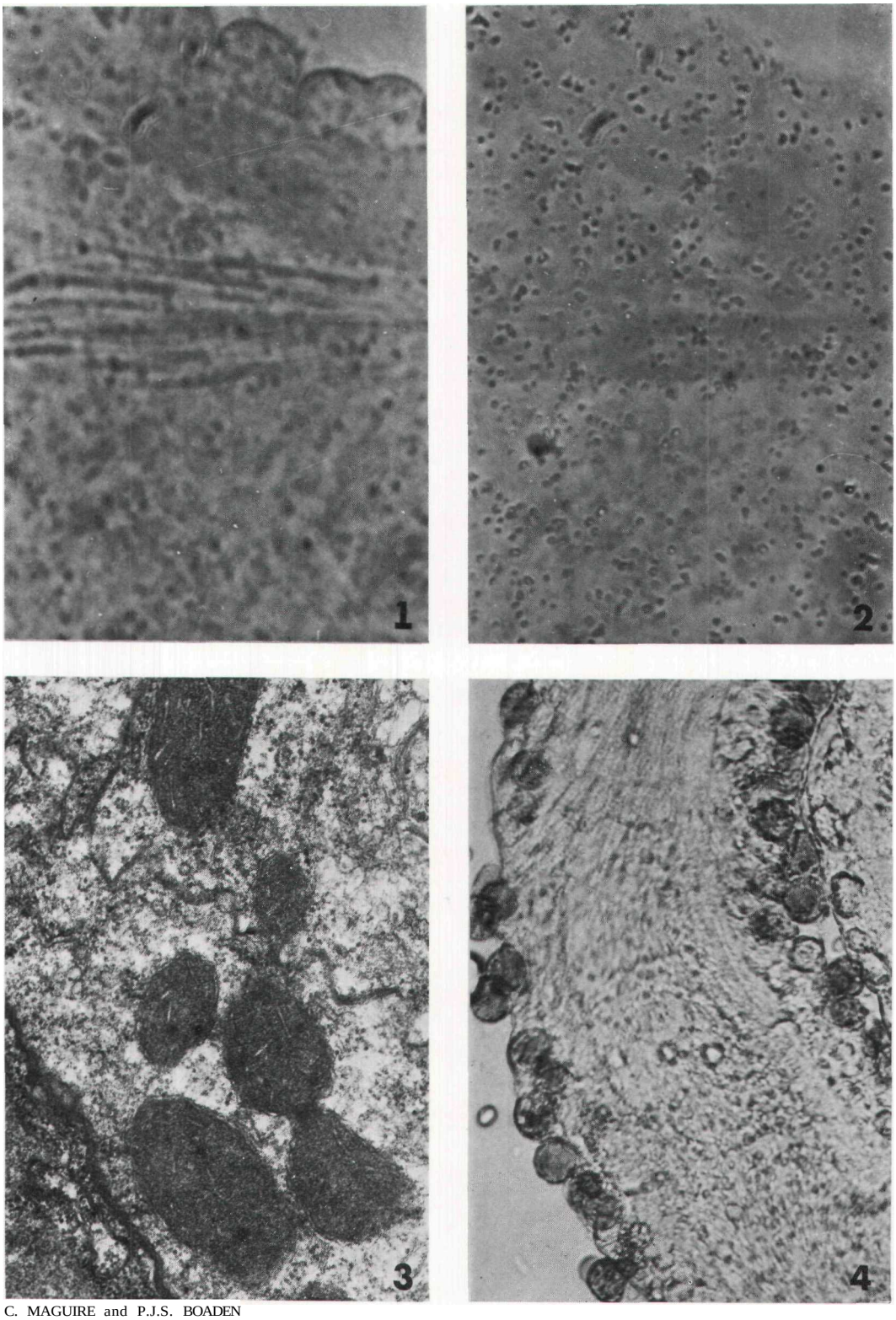


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PLATE I

1 and 2. Autoradiograms of thin sections of *Thiodasys sterreri* incubated with $C^{14}$ labelled $NaHC0_3$; 1: focussed on section, 2: focussed on emulsion.

3. Mitochondria from *Thiodasys sterreri* showing typical anaerobic structure. E.M. photograph by J. McCrea.

4. *Thiodasys sterreri* showing well developed dorsal glands. Low power micrograph of live specimen.

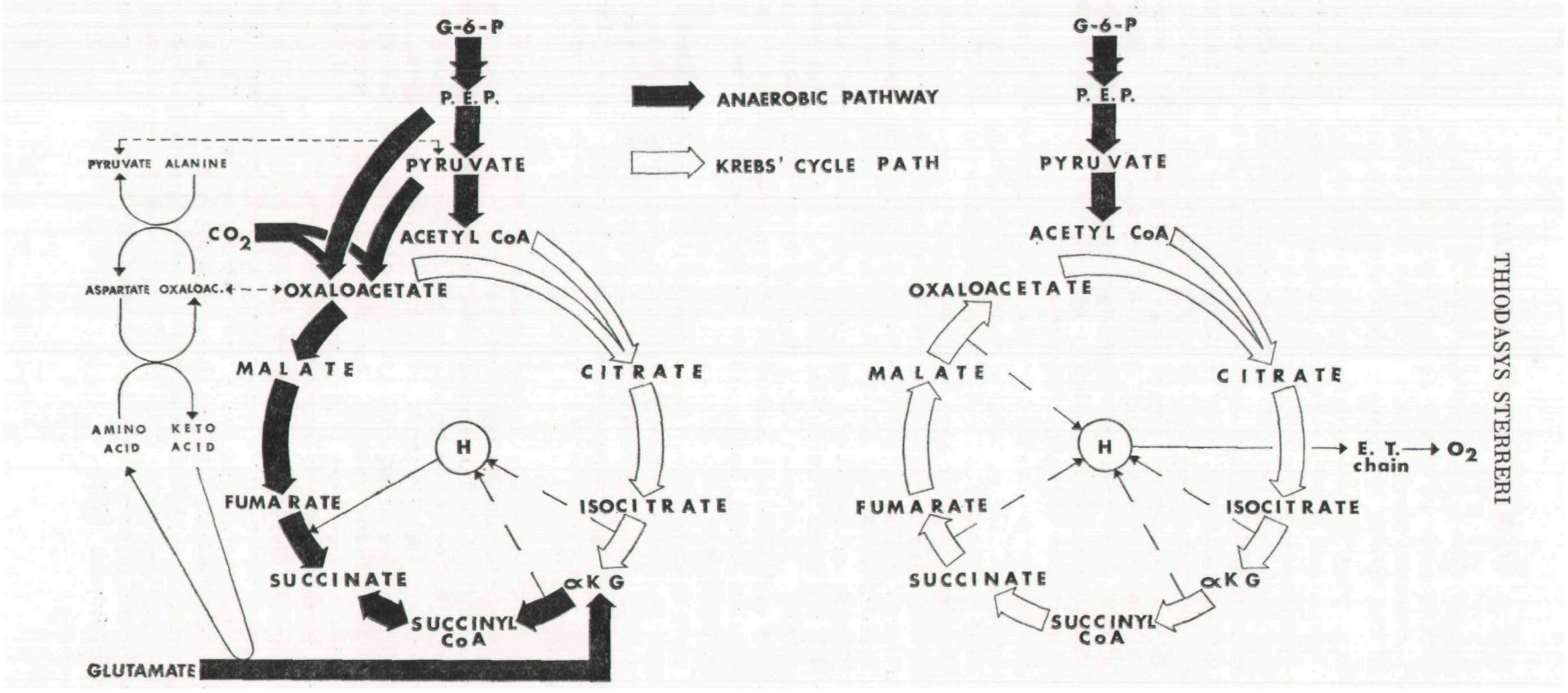


**FIG. 4**

Anaerobic pathways as possible precursors of the Krebs' cycle.

theoretically, all the enzymes and reactions of the Krebs' cycle could have evolved to serve an anaerobic function in primitive life forms and, later, become linked up in a new way (Fig. 4) once the cytochromes of the aerobic electron transport chain had evolved.

Any organisms living in environments where oxygen was freely available would gain great selective advantage by using the Krebs' cycle/electron transport chain system (rather than an anaerobic system) since this is much more efficient for energy production. However, in environments lacking oxygen the pathway in Fig. 3 would be more efficient than, for example, fermentation or lactate production. The high wastage of carbon in end products such as succinate, alanine, etc., would not be disadvantageous because carbon would be freely available from $CO_2$ and dissolved organic matter. Under these conditions, there would be no pressure to evolve any alternative pathway, thus anaerobic invertebrates may still be using the same metabolic systems as their distant ancestors.

The thiobios is dominated by organisms of apparently humble evolutionary origin. The integration of the Bacteria with the physical and chemical parameters is well known (Baas-Becking and Wood, 1955). The Cyanophyta are an important floral component though euglenoids, diatoms, dinoflagellates and lower fungi are also important. The fauna is almost entirely comprised of Protozoa, Gnathostomulida, Turbellaria, Nematoda and Gastrotricha. As Fenchel and Riedl (1970) state, among these groups there are particularly primitive forms which are to be regarded as Lebensort - Typen (in the sense of Riedl, 1963). This means that they represent taxa which have originated in and remained faithful to this particular environment. The turbellarian family Retronectidae Sterrcr and Rieger may be cited as an example.

The vertical distribution of meiobenthic fauna and flora within the sediment also indicates that anoxybiosis is primary in the Metazoa (Boaden, 1975). This author believes that several of the lower phyla had arisen within the thiobios by the Middle Precambrian and that the Metazoa were therefore well established before the advent of oxyaerobiosis.

The classical theories of early metazoan evolution have assumed an aerobic origin of the Metazoa and that eukaryotes evolved from prokaryotes in the Late Precambrian (see Schopf, 1974). The symbiotic theory of eukaryote origin also derives the Metazoa from aerobic forms in the very Late Precambrian or Early Cambrian, indeed Margulis (1970), in discussing this theory, states that all eukaryotic forms are aerobic and that the exceptions are clearly secondary modifications. Our evidence is apparently in conflict with both these viewpoints as far as aerobic respiration is concerned.

According to Meyer and Meyer (1972), the Platyhelminthes are unable to manufacture their own sterols or polyunsaturated fatty acids; these biosynthetic pathways both require oxygen. This deficiency occurs in species whether living anaerobically or aerobically thus suggesting the phylum was established before fully oxidative metabolism had evolved. This and other possible corroborative evidence for the primary thiobiotic origin of the Metazoa is further discussed by Boaden (1975).

There is therefore an increasing amount of evidence using taxonomic, ecological and biochemical criteria that the present day thiobios could represent a partial relict of earth's earliest metazoan biosphere. Further investigation of the biochemistry and physiology of the marine sulphide system community may throw considerable light on the origin of oxyaerobic metabolism.

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## Summary

*T. sterreri*, which occurs extensively in the redox potential discontinuity layer of marine sands and survives completely anaerobic conditions, seems able to fix carbon dioxide. Chromatography and autoradiography indicate its active incorporation into a reversed Krebs' cycle sequence. Cytochrome oxidase is present but its form and mechanism of avoiding sulphide poisoning are unknown. Mitochondria are similar to those of other anaerobic species. Anaerobic carbohydrate and amino-acid metabolism is discussed in relation to meiofauna. It is shown how Krebs' cycle components could have originally evolved for anaerobic functions and later been used as the basis for more efficient oxidative metabolism. These results are discussed in relation to metazoan origins.

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