ACETOACETATE OXIDATION BY HEART MITOCHONDRIA

To

my wife SADEQA

and our children

JALEEL and SHABAÑA

ACETOACETATE OXIDATION BY HEART MITOCHONDRIA

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Submitted to the Department of Experimental Medicine, Faculty of Graduate Studies and Research, McGill University in partial fulfilment of the requirement for the degree of Master of Science.

May, 1976

Work done in the Laboratory of Intermediary Metabolism (Director: Dr. S.V. Pande) of Clinical Research Institute of Montreal (Director: Dr. J. Genest).

Acetoacetate oxidation by heart mitochondria

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ABSTRACT

Acetoacetate-dependent oxygen consumption of mitochondria required presence of both 2-oxoglutarate and malate for maximal rates of respiration. Under these conditions, it was observed that (i) Pi and arsenate were stimulatory, (ii) parapyruvate and arsenite were inhibitory and this inhibition was not reversed by ATP, and (iii) formation of $^{14}CO_2$ from ^{14}C-acetoacetate was not detectable. Experiments with differentially labeled pyruvate suggest that the effect described under (iii) resulted from the efflux of intramitochondrial intermediates of the citric acid cycle in exchange for the entry of added malate.

When acetoacetate-dependent respiration was followed without 2-oxoglutarate and malate, Pi, arsenate and malate were found to be inhibitory. The malate-inhibited respiration was not reversed by ATP.

Similar stimulatory and inhibitory effects of Pi and arsenate were also observed on mitochondrial oxidation of pyruvate and acetylcarnitine.

The results suggest that the activation of acetoacetate for mitochondrial oxidation proceeds mainly if not exclusively through the succinyl-CoA route.

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RESUME

La consommation d'oxygène dépendant de l'acetoacetate requiert la présence simultanée du 2-oxoglutarate et du malate pour l'obtention de taux de respiration maximum. Dans ces conditions, nous avons observé que (i) Pi et arsenate étaient stimulatuers, (ii) parapyruvate et arsenite étaient inhibiteurs et que cette inhibition n'était pas renversée par l'ATP, et (iii) la formation de $^{14}CO_2$ à partir de ^{14}C-acetoacetate n'était pas détectable. Des expériences avec des pyruvates marqués différemment suggèrent que l'effet décrit en (iii) résulte de l'efflux d'intermédiaires intramitochondriaux du cycle de l'acide citrique en contrepartie de l'entrée du malate ajouté.

Lorsque la respiration dépendant de l'acetoacetate était suivie sans 2-oxoglutarate ni malate, Pi, arsenate et malate étaient inhibiteurs. La respiration inhibée par le malate n'était pas rétablie par l'ATP.

Des effets similaires, stimulateurs et inhibiteurs, du Pi et de l'arsenate ont été aussi observés avec l'oxidation mitochondriale du pyruvate et des acylcarnitines.

Les résultats suggèrent que l'activation de l'acetoacetate pour l'oxydation mitochondriale se fait principalement sinon exclusivement par la voie du succinyl-CoA.

ACKNOWLEDGMENT

I wish to express my sincere thanks to Dr. J. Genest, director of the Clinical Research Institute of Montreal, for founding and maintaining this excellent research institute and for his kindness and encouragement during the period of my studies.

I wish to express my deepest gratitude to my research director, Dr. S.V. Pande, for his help and encouragement he has given me during the course of this work and the preparation of this manuscript.

I am grateful to Dr. Yves Marcel, for the translation of the Abstract.

I am indebted to Miss Lucie Paul for assuming the strenous task of typing this manuscript and to Miss Johanne Lalancette for preparing the photocopies of the thesis.

I am thankful to all the technical staff of the "Clinical Research Institute", who in one way or another was helpful in the realization of this work.

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ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CoASH	Coenzyme A
DNP	2,4-Dinitrophenol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
NAD^+	Nicotineamide adenine dinucleotide
NADH	Dihydronicotineamide adenine dinucleotide
$NADP^+$	Nicotineamide adenine dinucleotide phosphate
NADPH	Dihydronicotineamide adenine dinucleotide phosphate
Pi	Orthophosphate
PPi	Pyrophosphate
Tris	Tris (hydroxymethyl) aminomethane

1. INTRODUCTION

1.1 GENERAL CHARACTERISTICS OF MITOCHONDRIA

Mitochondria were first described by Altman (1) in 1894 and were called "bioblast" at that time. Mitochondria are the subcellular organelles present in the cytoplasm of all aerobic eucaryotic cells. Under aerobic conditions most of the energy yielding reactions of the cell proceed in mitochondria. The number of mitochondria per cell appears to be constant and characteristic for any given cell type but may change with the state of development or with functional activity. The size and shape of mitochondria vary from one cell type to another. In general mitochondria are 3-5 μ long by 0.2-0.5 μ wide (1).

The most characteristic feature of mitochondria is the presence of a double membrane system; a smooth outer membrane and an inner membrane that infolds to varying degrees forming "cristae". In liver mitochondria the cristae are sparse and irregular, but in kidney or heart mitochondria they are numerous and nearly fill the entire lumen of the mitochondria. The inner membrane divides the mitochondria into two spaces. The space between the outer and inner membrane is called intermembrane space and the space inside the inner membrane is called matrix. Mitochondria with many cristae have relatively small matrix and those with fewer cristae have large matrix. The cristae have numerous mushroom-like subunits which project into the matrix and are called "inner membrane spheres" or the elementary particles (2).

The primary function of membranes is to separate compartments. Mitochondrial membranes not only divide the mitochondria into compartments but also participate in other biological functions by virtue of the acquisition of numerous secondary components. Isolation and examination of the physical and chemical properties of highly purified outer and inner membranes of mitochondria (2) have revealed that the two membranes contain numerous enzymes e.g. monoamine oxidase and ATP-dependent long chain fatty acid activating enzymes are localized in the outer membrane. Respiratory chain enzymes, ATP synthesizing enzymes, 2-oxoglutarate dehydrogenase, succinate dehydrogenase, carnitine fatty acyl transferase and D-3-hydroxybutyrate dehydrogenase are present in the inner membrane. Adenylate kinase represents enzyme of the intermembrane space. The matrix contains citrate synthase, isocitrate dehydrogenase, fumarase, malate dehydrogenase, aconitase, glutamate dehydrogenase and enzymes involved in fatty acid oxidation (2).

Aerobic cells obtain most of their energy by the transfer of electrons from organic fuel molecules to molecular oxygen. In these cells, the citric acid cycle is the final common pathway in the degradation of acetyl groups derived from the catabolism of carbohydrates, lipids and amino acids. The reducing equivalents, produced by the oxidation of citric acid cycle intermediates and those formed in the cytoplasm (e.g. during glycolysis) are fed into a series of electron carriers before being utilized for the reduction of oxygen. The ensuing process of electron transport to molecular oxygen proceeds with a large decline in free energy, much of which is conserved as ATP by the coupled oxidative phosphorylation of ADP. A simplified scheme of the respiratory chain is shown in Figure 1.

Fig. 1 A simplified scheme of the mammalian mitochondiral respiratory chain and the points of entry of electrons from various substrates.

The flow of electrons is shown from substrates to oxygen which in coupled mitochondria accompanies conservation of energy. However many of the reactions shown can proceed in the reverse direction. The fluted lines represents loci of action of various inhibitory agents. Cyt, cytochrome. FP, various flavoprotein dehydrogenases. CoQ, coenzyme Q. Abridged from Lehninger (3).

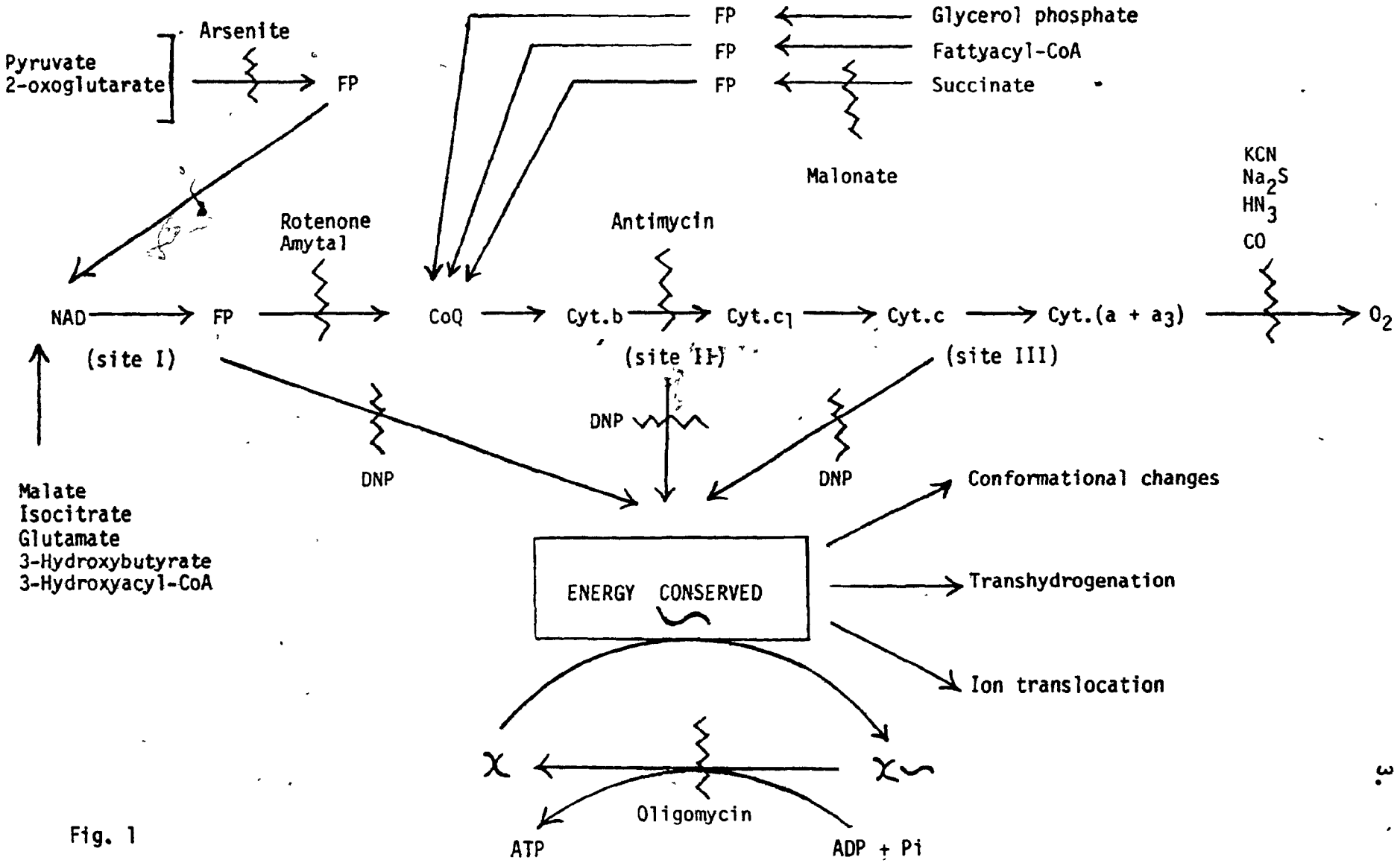

Arsenite
Pyruvate
2-oxoglutarate
FP
FP
FP
Glycerol phosphate
Fattyacyl-CoA
Succinate
Malonate
KCN
Na_2S
HN_3
CO
Rotenone
Amytal
Antimycin
NAD
FP
CoQ
Cyt.b
$Cyt.c_1$
Cyt.c
Cyt.(a + a_3)
O_2
(site I)
(site II)
(site III)
DNP
DNP
DNP
Malate
Isocitrate
Glutamate
3-Hydroxybutyrate
3-Hydroxyacyl-CoA
Conformational changes
ENERGY CONSERVED
Transhydrogenation
Ion translocation
X
X~
Oligomycin
ATP
ADP + Pi

Fig. 1

Oxidative phosphorylation

Respiration of mitochondria with intact membranes requires not only a substrate but also ADP and Pi. This is because of the coupling of oxidation to phosphorylation as a result of which ATP is formed. It has been shown that during the oxidation of substrates that feed reducing equivalents at the level of NAD of the respiratory chain, the observed P/O or ADP/O ratio is approximately 3. The corresponding ratio is approximately 2 and 1 when reducing equivalents are fed into the chain at the level of flavoproteins and cytochrome c, respectively (4). It is generally accepted that there are three phosphorylation sites, located at the span between NADH and flavoprotein (site I), between cytochrome b and c_1, (site II), and between cytochrome c and oxygen (site III), (3,4). It is unclear however whether the primary energy conserving act is the formation of a high-energy compound (5), the development of a membrane potential (6), or a conformational change in the membrane (7).

Metabolite transport systems of mitochondria

Since oxidation of citric acid cycle intermediates and oxidative phosphorylation occurs within the mitochondria, the membranes must be permeable to a variety of ions and substrates. Unlike the inner membrane, the outer membrane of mitochondria is freely permeable to molecules of up to a molecular weight of 10,000. Although the inner membrane allows only much smaller (molecular weight < 150) neutral molecules to permeate readily, it is impermeable to many ions, and is considered to be the mitochondrial osmotic barrier.

However, transport of certain ions across the inner membrane is made possible because of the presence of specific translocators in the inner membrane (2). These translocators catalyze a 1:1 exchange between specific ion pairs (8,9). Exchange reactions have been demonstrated for ADP and ATP and for most of the intermediates of the citric acid cycle (8,9).

For certain molecules it has been suggested that only a portion of a molecule is transported into mitochondria by what has been described as group translocation mechanism (8). Such a mechanism of translocation has been implicated in the transport of aspartate and fatty acylcarnitine esters. At least for the latter, it has been shown recently that acylcarnitine entry involves a 1:1 exchange with the intramitochondrial carnitine and that this process is catalyzed by the presence of a carnitine acylcarnitine translocase in mitochondria (10).

Transport of reducing equivalents across mitochondrial membrane

Unlike the reducing equivalents generated within the mitochondria, the reducing equivalents generated in cytoplasm are not directly transported into the mitochondria for oxidation via the electron transport chain. The reducing equivalents in cytoplasm appear as reduced pyridine nucleotides which cannot penetrate the mitochondrial inner membrane. However electrons derived from NADH can enter the mitochondrial respiratory chain by indirect routes called shuttles. The best known are the glycerol phosphate and malate shuttles (9). The glycerol phosphate shuttle is unidirectional and is operative in liver and insect muscle. In other tissues as well as in liver, the malate shuttle, which is bi-directional appears to be the major shuttle. In liver

malate shuttle functions to carry reducing equivalents from the mitochondrial matrix to the reductive biosynthetic reactions in the cytoplasm (9).

Control of respiration: the respiratory states

Apart from oxygen and the respiratory chain itself, mitochondrial respiration and oxidative phosphorylation depend on the presence of substrates, Pi and ADP. The general conditions affecting the respiratory rate of intact mitochondria as described by Chance and Williams (11) are classified as follows.

(i) State 1 respiration is the condition in which both ADP and respiratory substrates are lacking.

(ii) State 2 respiration is the condition in which respiratory substrate only is lacking.

(iii) State 3 respiration is the condition in which all required components are present (active respiration)

(iv) State 4 respiration is the condition in which only ADP is lacking.

When ADP is added under conditions of state 4 respiration, the respiration increases markedly (state 3 respiration) and added ADP is phosphorylated to ATP. The dependence of respiratory rate on ADP concentration is called respiratory control or acceptor control. The rate of respiration in the presence of ADP divided by the rate in its absence (state 3/state 4) is defined as the "respiratory control ratio". A high respiratory control ratio is generally observed with tightly coupled intact mitochondria. The phenomenon of respiratory control is also seen in living cells. A relaxed muscle respires slowly, but when ATP breakdown is accelerated such as during exercise, the ADP formed immediately accelerates respiration (4).

Various substances can affect the general metabolism of mitochondria and in coupled intact mitochondria, the effect may be on the transport of electrons, energy conservation, ATP synthesis or transport of metabolites.

(a) Inhibitors of electron transport (respiratory) chain

The flow of electrons between NADH dehydrogenase and Coenzyme Q is inhibited by rotenone, amytal, piericidin; between cytochrome b and c by antimycin; between cytochrome oxidase ($a + a_3$) and oxygen by cyanide, azide, sulfide, and carbon monoxide.

(b) Inhibitors of energy-conserving mechanism - the uncouplers

Uncouplers are substances which cause respiratory chain-linked phosphorylation of ADP and Pi to ATP to become dissociated from respiration with the result that the maximal respiration no longer requires ADP and Pi. Under conditions such as in state 4 respiration, addition of uncouplers bring about stimulation of respiratory rates. Some common uncouplers are 2,4 dinitrophenol (DNP), dicumarol, carbonyl cyanide m-chlorophenylhydrazone (m-Cl-CCP) and p-trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP). Uncouplers stimulate ATPase activity i.e. hydrolyze ATP to ADP. The exact mode of action of uncouplers is unknown.

(c) Inhibitors of energy-trapping mechanism for ATP synthesis

Oligomycin and aurovertin inhibit coupled respiration induced by ADP and Pi, but have no effect on uncoupled (non-phosphorylating) respiration. They also have no effect on substrate-linked phosphorylation (12). Arsenate

readily uncouples substrate-linked phosphorylation (12) but is relatively ineffective against respiratory chain-linked phosphorylation. Arsenate does not induce ATPase activity.

(d) Inhibitors of the transport system

Respiration and oxidative phosphorylation is inhibited when transport of ADP, Pi and substrates are inhibited. Atractyloside and bongkrekic acid inhibit phosphorylation of externally added ADP, but do not inhibit phosphorylation of intramitochondrial ADP (8). Mersalyl and other SH-reagents inhibit phosphorylation by inhibiting the transport of Pi (8). n-Butylmalonate, an inhibitor of the transport system of the dicarboxylates (13) effectively inhibits mitochondrial respiration dependent on added malate and succinate.

(e) Inhibitors of citric acid cycle

Oxygen consumption dependent on the oxidation of acetyl group is markedly inhibited by substances that terminate the cyclic operation of citric acid cycle e.g. malonate inhibits succinate oxidation by inhibiting the succinate dehydrogenase. Arsenite inhibits the oxidation of 2-oxoglutarate by inhibiting 2-oxoglutarate oxidase system (12).

1.2 Metabolism of ketone bodies

Acetoacetate, D-3-hydroxybutyrate and acetone, collectively known as ketone bodies, were discovered in urine of diabetics between 1857 and 1884 (14) and were considered to be useless products of a pathological process or of an abnormal metabolism. However, it was later established that ketone

bodies can be utilized by extra-hepatic tissues to serve as a source of energy (14). More recent elucidations of the metabolic reactions involved in the metabolism of ketone bodies have led to the present view that ketone bodies are specially generated by the liver to supply tissues with an alternate fuel of respiration when carbohydrate is short in supply.

Carbohydrate, aminoacids and fat are the three major fuels of respiration. Glucose and fat both are oxidized by tissues. But when glucose is short in supply, the contribution of fat as fuel increases. The three forms in which fat is available to tissues are free fatty acids, triglycerides and ketone bodies. The proportion of energy derived from the oxidation of the three forms described above depends on the presence of the requisite enzymes in the tissues (15). Although fatty acids are an important substrate for myocardial and skeletal muscle (16), the supply of fatty acids is restricted by their relatively low solubility and toxicity at high concentration (17). Therefore ketone bodies are viewed as supplementary means of transporting the energy contained in free fatty acids (18). It has been well documented that ketone bodies can be effectively utilized by extra-hepatic tissues to serve as a source of energy (19) and together with fatty acids play an important part in the caloric homeostasis (20).

Whenever the glucose level in the blood plasma is low, as in starvation or on a low carbohydrate (or high fat) diet, or when glucose is not utilizable, as in diabetes, the concentration of fatty acids in plasma rises. This rise is roughly paralleled by an increase in the concentration of ketone bodies. The moderate ketosis which occurs under a variety of circumstances such as during starvation (21,22) and after prolonged exercise (23) is a normal physiological process that supplies tissues with a ready utilizable

fuel of respiration. Thus ketosis (a state in which ketone bodies accumulate in the extracellular fluid in concentrations greater than during conditions of plenty of carbohydrate) is a physiological phenomenon reflecting metabolic alterations that characterize a change from carbohydrate to fat oxidation. In contrast to the physiological ketosis, the severe ketosis of the diabetic, e.g. ketoacidosis of diabetic coma (18) is obviously a harmful pathological condition in which the quantities of ketone bodies formed grossly exceed possible needs.

1.2.1 Formation of ketone bodies: General considerations

Ketone bodies are formed mainly in liver. Ketone bodies production is also known to occur in mammary gland (24), kidney (14) and by the walls of rumen and omasum (25-27), but the relative contribution is negligible in the regulation of ketone bodies metabolism. The two main ketone bodies acetoacetate and D-3-hydroxybutyrate are interconverted by tissues. Acetone is usually a minor component of the ketone bodies: it is thought not to be utilized to any great extent by animals. It is generally believed that acetone in the body and in the urine arises from a spontaneous, non-enzymatic decarboxylation of acetoacetate (28).

Piror to 1953, it was generally believed that ketone bodies were normal intermediates in the oxidation of fatty acids. In 1953, two new decisive discoveries concerning the metabolism of ketone bodies made it clear that acetoacetate and D-3-hydroxybutyrate are not direct intermediates of the fatty acid oxidation. The fatty acid oxidation does produce ketone bodies but their formation proceed through acetyl-CoA. The two discoveries showed that (i) the intermediates formed during oxidation of fatty acids are the

coenzyme A derivatives of ketone bodies, rather than the free ketone bodies (29) and that (ii) the intermediate formed from long-chain fatty acids is L(+)-3-hydroxybutyryl-CoA, whereas the free 3-hydroxybutyric acid appearing in the body fluids has the D (-) configuration (30). It is now known that fatty acids have to undergo β-oxidation to produce acetyl-CoA which serves as a precursor of ketone bodies

1.2.1.1 Biosynthesis of Acetoacetate

Acetoacetate is considered to be the parent compound from which both acetone and D-3-hydroxybutyrate are derived. Normally acetoacetate formation proceeds when fatty acid oxidation is active in liver mitochondria (14).

Acetyl-CoA serves as the immediate precursor for the formation of acetoacetyl-CoA. Acetoacetyl-CoA arises from the thiolase catalyzed condensation of acetyl-CoA (31,32).

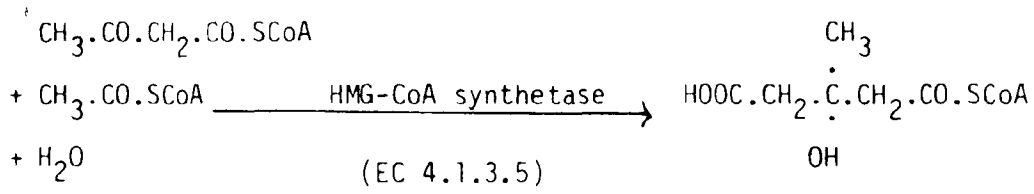
$$2CH_3.CO.SCoA \xleftrightarrow[(EC\ 2.3.1.9)]{\text{Acetoacetyl-CoA thiolase}} CH_3.CO.CH_2.CO.SCoA + CoASH$$

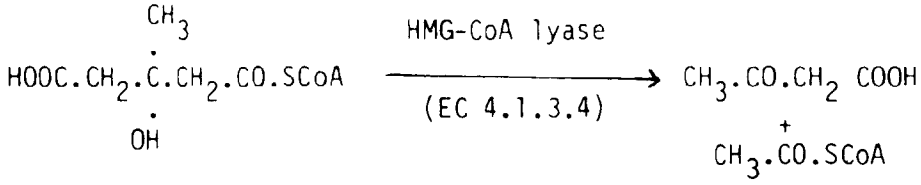
For the production of acetoacetate from acetoacetyl-CoA, two enzymatic pathways have been implicated

(a) Direct deacylation (33 - 35)

$$CH_3.CO.CH_2.CO.SCoA + H_2O \xrightarrow{\text{Acetoacetyl-CoA deacylase}} CH_3CO.CH_2.COOH + CoASH$$

(b) Hydroxymethylglutaryl-CoA (HMG-CoA) pathway (36,37)





There has been considerable controversy as to which of these two pathways play the major role in the production of acetoacetate in liver (19). Recent studies have concluded that HMG-CoA pathway is the major route of acetoacetate formation (38).

Although the major site of acetoacetate production is within mitochondria, an extramitochondrial pathway of acetoacetate formation via the cytoplasmic HMG-CoA route has also been proposed (38,39). Low but significant activities of the enzymes of HMG-CoA pathway have been reported to be present in the cytoplasmic fraction of guinea-pig and rat liver (38). The activities of the cytoplasmic HMG-CoA pathway enzymes are increased in starvation and alloxan diabetes (38). The main source of the cytoplasmic acetyl-CoA is the citrate transported from the mitochondria into the cytoplasm and cleaved by the cytoplasmic citrate lyase (EC 4.1.3.6) (40).

1.2.1.2 Biosynthesis of D-3-hydroxybutyrate

It is generally accepted that D-3-hydroxybutyrate synthesis results from the reduction of acetoacetate according to the following equation.

$$CH_3.CO.CH_2.COOH + NADH + H^+ \xleftarrow[\text{(EC 1.1.1.30)}]{\text{D-3-hydroxybutyrate dehydrogenase}} CH_3.CHOH.CH_2.COOH + NAD^+$$

Acetoacetate — D-3-hydroxybutyrate

This enzyme is present in many tissues, with the highest activity being found in liver (41). D-3-hydroxybutyrate dehydrogenase is tightly bound to the mitochondria and is closely associated with the electron transport and oxidative phosphorylation system (41). Though the reduction of acetoacetate to D-3-hydroxybutyrate as shown in the above equation is reversible, its link to the electron transport chain and oxidative phosphorylation system suggests that its physiological role may be in an oxidative pathway, rather than in the formation of D-3-hydroxybutyrate (41).

1.2.2 Regulation of ketogenesis

Ketosis develops whenever the availability of carbohydrate is deficient in relation to the amount of fat being catabolized. Thus, the relationship between carbohydrate and fat utilization rates are important determinants of ketosis. Under any given condition, the utilizability of the available carbohydrate is determined by the amount of insulin (42). Thus the metabolic key to ketosis is utilizable carbohydrate and the endocrine key to carbohydrate utilizability is insulin.

Ketosis is initiated by a relative or absolute deficiency of insulin which in turn results in mobilization of free fatty acids from peripheral fat stores. The accompanying increased concentration of free fatty acids in plasma leads to an increased delivery and uptake of fatty acids by the liver (43).

There are three major pathways for free fatty acid utilization within the liver (a) esterification of triglycerides (and some phospholipids) where they can be stored in the liver or released as lipoprotein, (b) complete oxidation to CO_2 and water in the citric acid cycle, and (c) partial oxidation to ketone bodies (44). Moreover, these three pathways are interrelated and could act competitively. If pathway (a) or (b) or both are reduced, then fatty acids being presented ot the liver.

There is a decreased output of triglycerides by livers of starved rats (45) and this lower output means that a higher proportion of the plasma free fatty acids are diverted to the oxidation pathway. In states of increased free fatty acid utilization, such as fasting or alloxan diabetes, fatty acid synthetis is also decreased (46). Thus increased free fatty acid utilization associated with a decreased triglyceride output and decreased fatty acid synthesis favour the formation of ketone bodies. In these conditions citric acid cycle activity shows little change (46,47).

When free fatty acids become the predominant oxidative substrate they produce increased levels of acetyl-CoA and increase the redox state of the mitochondria and cytoplasmic compartments of the cell (48,49). With an increase of the redox state of the cell, the substrate pair malate-oxaloacetate, which is in enzymatic equilibrium with NAD-NADH, shifts toward the formation of increased malate and decreased oxaloacetate (48,50,51). The rate of entry of acetyl-CoA into the citric acid cycle is controlled by available concentrations of oxaloacetate (18,48). Oxaloacetate is an intermediate in the synthesis of glucose via the extramitochondrial enzymes of gluconeogenesis and of citrate

via an intramitochondrial condensation reaction, catalyzed by citrate synthase. The increased rates of gluconeogenesis occuring when free fatty acid utilization is augmented bring about a relative and absolute depression of intramitochondrial levels of oxaloacetate (18). The decreased activity of a number of citric acid cycle enzymes and the lower levels of oxaloacetate effect an impaired rate of oxidative utilization of the increased acetyl-CoA. This increased steady-level of acetyl-CoA results in the formation of ketone bodies. Thus, both over production (obligatory) and underutilization (non obligatory) of acetyl-CoA for oxidative purposes in the liver, participate in ketogenesis.

1.2.3 Utilization of ketone bodies

That animal tissues except liver can oxidize ketone bodies has long been known, but the extent to which ketone bodies can be utilized as fuel has become known only recently (18). Apart from heart, kidney, skeletal muscle and brain, utilization of ketone bodies has also been shown to proceed in mammary gland (52, 53) skin (54) submaxillary gland (55) adipose tissue (56-58) and in malignant hepatocyte (59). Moreover, certain tissues utilize ketone bodies in preference to other available substrates. Heart muscle, for example, can use acetoacetate preferentially even when glucose and insulin or lactate are available (60). Ketone bodies are also the preferred substrates over free fatty acids in heart (60, 61-63) and resting skeletal muscle (64). Kidney cortex also uses acetoacetate in preference to glucose and lactate (18). Ketone bodies are the major metabolic fuel for the brain of the suckling rat under normal conditions (65).

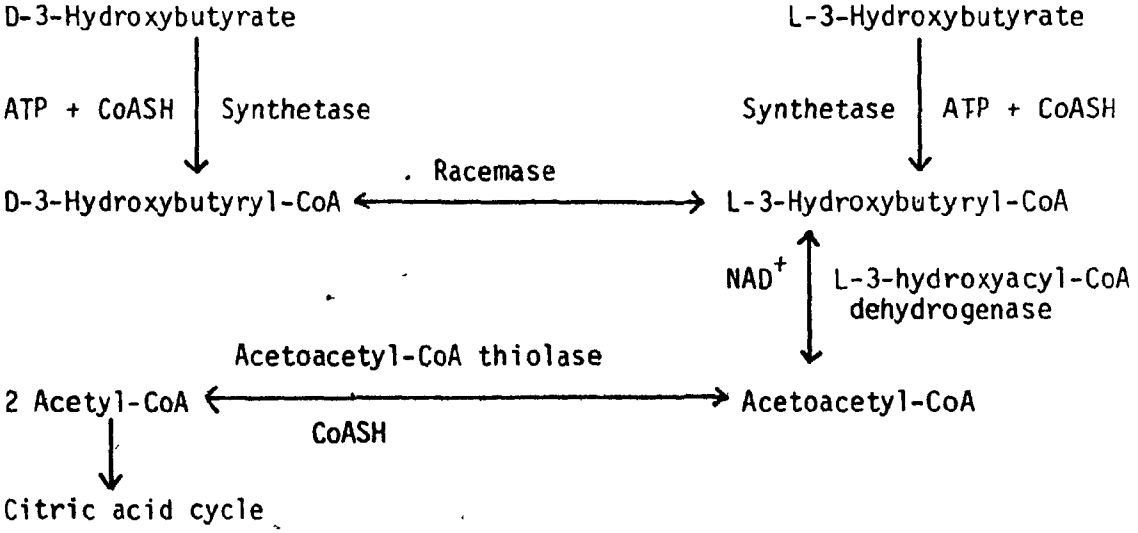
1.2.3.1 Enzymes involved in the utilization of ketone bodies

It is generally accepted that D-3-hydroxybutyrate is initially converted to acetoacetate before being degraded by the extra-hepatic tissues.

Unlike acetoacetate, liver can activate D-3-hydroxybutyrate to D-3-hydroxybutyryl-CoA (66). The D-3-hydroxybutyryl-CoA is converted to L-3-hydroxybutyryl-CoA by a racemase (67) which is then oxidized to acetoacetyl-CoA by L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (29, 68). The acetoacetyl-CoA formed is cleaved by acetoacetyl-CoA thiolase to give two molecules of acetyl-CoA which then enter the citric acid cycle.

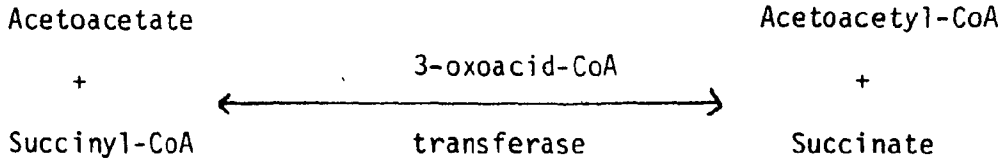
It has been shown that both dextrorotatory and levorotatory isomers of 3-hydroxybutyrate are oxidized by liver, kidney and heart mitochondria (30, 69).

The metabolism of D and L-3-hydroxybutyrate discussed above is summarized below

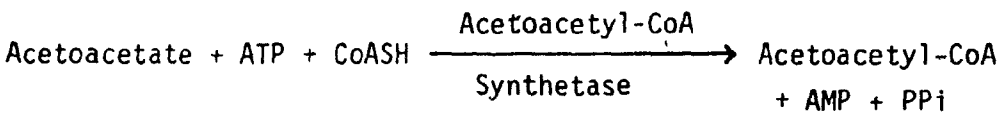


The first obligatory step in the utilization of acetoacetate is its enzymatic conversion to acetoacetyl-CoA. Two different enzymes have been described for the activation of acetoacetate to acetoacetyl-CoA

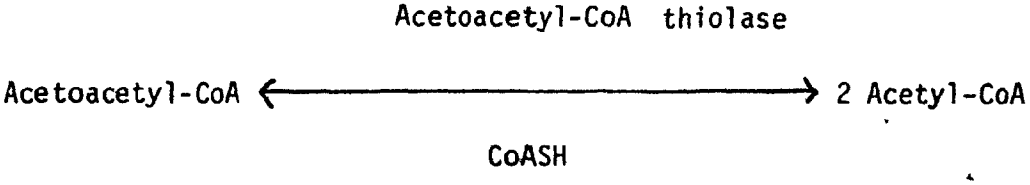
(a) 3-oxoacid CoA transferase (succinyl-CoA:3-oxoacid-CoA Transferase, (EC 2.8.3.5) (19, 70).



(b) Acetoacetyl-CoA synthetase (71-76)



The succinyl-CoA linked activation of acetoacetate has been considered to be the major route for the oxidative utilization of acetoacetate by the extrahepatic tissues (19,70). Unlike 3-oxoacid-CoA transferase, no direct evidences are available to demonstrate the involvement of acetoacetyl-CoA synthetase in the oxidative utilization of acetoacetate. The acetoacetyl-CoA is cleaved to two molecules of acetyl-CoA which then enters the citric acid cycle



The activities of the enzymes of ketone bodies utilization (D-3-hydroxybutyrate dehydrogenase, 3-oxacid-CoA transferase and acetoacetyl-CoA thiolase) present,in many mammalian tissues, have been reported by Williamson et al (77). These authors have shown that the enzyme activities,

found in various rat tissues are more than sufficient to account for the observed rate of ketone bodies oxidation (77). The activities of D-3-hydroxybutyrate dehydrogenase and 3-oxoacid-CoA transferase are not affected by ketonemic conditions such as starvation, alloxan diabetes or fat-feeding i.e. conditions where the rate of ketone bodies utilization are increased (77, 78). However, the acetoacetyl-CoA thiolase activity has been reported to increase in kidney, heart and liver on fat feeding (77, 79).

As compared to the adult tissues, developing tissues show higher enzyme activities. In rat mammary gland, the low activities of the enzymes of ketone bodies utilization before parturition, increase four to eight-fold after suckling commenced and reach peak activities between 11th and the 20th day of lactation, and finally return to the pre-partum values within five days of weaning (52). In immature rat brain, the D-3-hydroxybutyrate dehydrogenase activity is three times higher (80, 81), and the 3-oxoacid-CoA transferase activity is two to four-fold higher, than in adult brain (81-82). The acetoacetyl-CoA thiolase activity is twice as high at birth than in adulthood and declines slowly from birth to weaning (80-83). Neither starvation, (20, 83) nor fat feeding (20, 79) appears to alter 3-oxoacid-CoA transferase activity of brain at any age after birth, although a maternal high fat diet has been reported to increase both 3-oxoacid-CoA transferase and acetoacetyl-CoA thiolase activities of fetal brain (84). In developing heart and kidney, in contrast to developing brain, there is a slow rise in the activities of the ketone bodies utilization to reach maximum values after weaning, but never exceed the adult values (81).

The D-3-hydroxybutyrate dehydrogenase and 3-oxoacid-CoA transferase act directly on free ketone bodies, and their developmental pattern in brain closely follows the changes in blood ketone bodies levels. The new born rat is transiently hypoglycemic after birth, and its blood ketone bodies level are initially very low, as low as those of the normally fed adults (20) With the onset of suckling the blood ketone bodies levels rise as much as ten-fold, and a true ketosis ensues (20). This ketosis is of nutritional origin, it reflects the ketogenic nature of maternal rat milk (84). The ketosis lasts until about 20-22 days of age when the rat is weaned onto standard high carbohydrate diet, the ketosis then gradually disappears (20). The greater utilization of ketone bodies by immature rat brain slices (85) and higher cerebral arterio-venous differences in suckling rats (65) parallel the increased blood concentration and the increase in enzyme activities in developing brain. Thus the rate of utilization of ketone bodies in the brain of the suckling rat is determined by both greater amount of the key enzymes and higher concentration of ketone bodies.

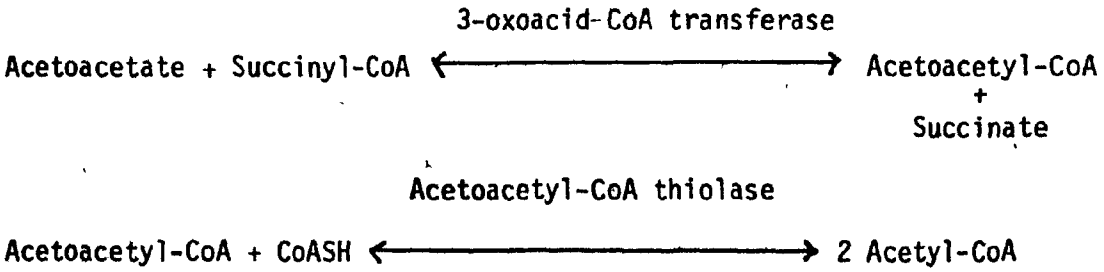
1.2.3.2 (ii) Control of the utilization of ketone bodies

The utilization of ketone bodies depends on the presence of the requisite enzymes. Liver is unable to utilize ketone bodies because of the absence of the key enzyme 3-oxoacid-CoA transferase. Recently Cornblath et al (86) have reported a unique genetic syndrome of ketoacidosis in infancy which was shown to be due to the deficiency of 3-oxoacid-CoA transferase (87).

The difference between the utilization of ketone bodies by various organs, and the difference between the suckling and adult rats, are due to the

different amounts of the enzymes in the tissues. The activities of the enzymes involved in the utilization of ketone bodies are fairly constant and do not alter in ketonemic conditions. On the other hand, the concentration of ketone bodies in the blood varies with the ketonemic conditions. Under conditions such as severe exercise, fasting, the ketone bodies concentration can increase 10-30 fold and this range of concentration change far exceeds that of any other blood metabolite (19). Thus in ketonemic conditions, the activities of the enzymes of ketone bodies utilization are not the major controlling factor of the rate of ketone bodies utilization. Under these conditions, the controlling factor is the concentration of ketone bodies, in particular acetoacetate, in plasma and tissues. The concentration of acetoacetate in turn depends on the rate of ketogenesis by the liver. The rate of ketogenesis is determined by the plasma concentration of free fatty acids. The plasma free fatty acid concentration in turn depends mainly on the release of free fatty acids from adipose tissues by the hydrolysis of triglycerides. The degradation of triglycerides to release free fatty acids is prompted by several hormones such as glucagon, epinephrine, norepinephrine and corticosteroids (20).

The rate of acetoacetate oxidation also depends on the rate of formation and removal of acetyl-CoA



Although the equilibrium of the 3-oxoacid-CoA transferase reaction favours the formation of acetoacetate, that of acetoacetyl-CoA thiolase favours the formation of acetyl-CoA (19). It is the coupling of the thiolase with transferase that overcomes the unfavourable equilibrium of the transferase thereby allows the formation of acetyl-CoA from acetoacetate to proceed readily. Thus an increase in acetoacetate results in a reciprocal increase in acetyl-CoA. Conditions which favour rapid removal of acetyl-CoA, e.g. oxidation via citric acid cycle, favour acetoacetate utilization.

Succinyl-CoA is required for the activation of acetoacetate, but other substrates also compete with acetoacetate for succinyl-CoA. Hence the rate of formation and utilization of succinyl-CoA may influence the rate of ketone bodies utilization. For succinyl-CoA utilization, both the activities of succinyl-CoA hydrolase and synthetase have been implicated (19).

Succinyl-CoA hydrolase

Succinyl-CoA + H_2O ⟷ Succinate

(EC 3.1.2.3)

Succinyl-CoA synthetase

Succinyl-CoA ⟷ Succinate + GTP

\+ GDP(ADP) + Pi

(EC 6.2.1.4 and 5)

\+ GTP(ATP) + CoASH

Because substrate-level phosphorylation involving succinyl-CoA synthetase is freely reversible (88), it has been suggested that changes in the concentration

of Pi, GDP and ADP could affect the concentration of succinyl-CoA (19). In agreement with this view, it has been reported that high levels of Pi and nucleotides capable of accepting high energy phosphate "~P", inhibit acetoacetate oxidation (89).

1.2.4 Role of ketone bodies

In ketonemic conditions, ketone bodies are the major source of energy production. At birth and during the suckling period, ketone bodies are the major metabolic fuels utilized by the brain of the rat (85,65) and other species (90,91). It has been estimated that 48-76% of the energy requirement of the brain of the young rat could be accounted for by the uptake of ketone bodies from the blood (65). The suckling period in the rat is a time of rapid lipogenesis (92) and active cholesterol synthesis in the central nervous system (93). It has been postulated that the ketone bodies are not only major matabolic fuels for energy production, but are also sources of carbon for the synthesis of sterol and fatty acids in the central nervous system of the suckling rat (93). The acetyl-units, required for the cytoplasmic lipogenesis and cholesterol synthesis have to be transported across the mitochondria. In rat liver, citrate is an effective carrier of acetyl-CoA (94). Citrate produced in the mitochondria enters the cytoplasm and is cleaved by the citrate lyase (EC 4.1.3.8) to acetyl-CoA and oxaloacetate (95).

$$\text{Citrate} + \text{ATP} + \text{CoASH} \xrightarrow[\text{-lyase}]{\text{Citrate-}} \text{Acetyl-CoA} + \text{Oxaloacetate} + \text{ADP} + \text{Pi}$$

It has been reported that, in mice liver as well as in adipose tissue of rat and mice, acetoacetate could substitute for citrate in the transfer of acetyl-units outside the mitochondria (96). Buckley and Williamson (75) and Stern (73)

have reported the existence of acetoacetyl-CoA synthetase in rat brain and liver cytosol respectively. They have suggested that the coupling of this enzyme (acetoacetyl-CoA synthetase) with cytosolic acetoacetyl-CoA thiolase could provide acetyl-CoA for cytoplasmic biosynthetic processes without the need for the transport of C_2-units from the mitochondria. It is speculated that, acetoacetate coupled to cytoplasmic acetoacetyl-CoA synthetase would be a more efficient carrier of acetyl-CoA than citrate, because, as a result of cytoplasmic thiolase activity, twice as much acetyl-CoA would be formed per mole of ATP utilized than results from the cleavage of citrate to acetyl-CoA 973,75).

1.3 Statement of the problem

The first step in the oxidative utilization of acetoacetate is its activation to acetoacetyl-CoA. For this two different mechanisms have been described (19,49). One of these is the succinyl-CoA-linked activation involving the enzyme 3-oxoacid-CoA transferase. The second is a synthetase mediated ATP-and CoA-dependent conversion of acetoacetate to acetoacetyl-CoA. The 3-oxoacid-CoA transferase has been well characterized and the qualitative involvement of succinyl-CoA route in the activation of acetoacetate is fairly certain (19,70). Although an ATP-dependent acetoacetyl-CoA synthetase has recently been identified (73,75), the evidence used to implicate such a synthetase in the oxidative utilization of acetoacetate is indirect only (71, 72, 74, 76, 97).

The inference that mitochondrial oxidation of acetoacetate proceeds both via the succinyl-CoA route and via ATP-dependent route rests mainly on the following observations.

(i) Mitochondrial oxidation of acetoacetate has been reported to be inhibited by Pi. It is believed that Pi directs succinyl-CoA towards substrate-level phosphorylation whereby the succinyl-CoA-linked activation of acetoacetate is impaired (98). Thus Pi inhibition of acetoacetate oxidation has been considered as an evidence for the succinyl-CoA-dependent route of acetoacetate activation.

(ii) The succinyl-CoA-linked oxidation of acetoacetate involving the enzyme 3-oxoacid-CoA transferase has been reported to be inhibited by arsenate, because arsenate directs succinyl-CoA towards arsenolysis. Accordingly arsenate-insensitive oxidation of acetoacetate has been considered to provide evidence for succinyl-CoA-independent route (s) of acetoacetate activation (97).

In the above studies, no attempts were made to examine the specificity of the effects of Pi and arsenate. I have done this using substrates other than acetoacetate and under a variety of experimental conditions such as:

- in the presence or absence of the sparkers of the citric acid cycle
- time of prior incubation of mitochondria,
- concentration dependence of Pi or arsenate.

Further, to determine if acetoacetate oxidation can proceed via an ATP-dependent activatory route, effect of ATP on acetoacetate-dependent oxygen consumption was determined under conditions in which generation of succinyl-CoA was specifically suppressed.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Acetoacetate (Lithium salt), amytal (amobarbital), dithiothreitol (DTT), 2,4 dinitrophenol (DNP), diketene, DL-3-hydroxybutyrate (sodium salt), L-malic acid, NADP, 2-oxoglutarate (monopotassium salt), oligomycin, cis-oxaloacetic acid, pyruvic acid (potassium salt), rotenone, succinic acid and Trizma-base were purchased from Sigma Chemical Co. St. Louis, Missouri. ATP (disodium salt), ADP (sodium salt), acetoacetyl-CoA (trisodium salt). Coenzyme A (lithium salt), NAD and NADH were obtained from P.L. Biochemicals, Milwaukee, Wisconsin. Sodium arsenite, sodium arsenate, EDTA, ferric chloride, ethyl acetate, hydroxylamine-HCl, D-mannitol, manganous sulphate, potassium floride, potassium cyanide and sodium nitrite ($Na\ NO_2$) were purchased from Fisher Scientific Co. Acetone, butyric acid, citric acid, ferrous ammonium sulphate [$Fe(NH_4)_2SO_4.6H_2O$], malonic acid, magnesium chloride, methanol, perchloric acid, potassium phosphate (mono and dibasic), sucrose, sodium acetate and Triton X-100 were supplied by J.T. Baker Chemical Co. Phillipsburg N.J. The following chemicals were obtained from the sources mentioned against their names. Trifluoroacetic acid and p-nitroaniline (Eastman Organic CHemicals), L-carnitine (General Biochemicals), albumin (bovine) Fatty acid poor (Mann Research Labs), parapyruvate or pyruvate aldol (Calbiochom), cysteine-HCl (Nutritional Biochemicals Co.) Hydroxide of Hyamine (Packard),

sodium pentabarbital (Abbott), p-Trifluoro methoxyphenylhydrazone of carbonyl cyanide (FCCP) was a gift from Dr. P.G. Heylter of Du Pont. L(-) Acetylcarnitine and L(-) palmitylcarnitine were synthesized by Dr. S.V. Pande.

2.1.2 Enzymes

Nagarse (EC 3.4.4.16) was obtained from Nagase & Co. Osaka Japan (U.S. Distributor : Enzyme Development Corp., 2 Penn Plaza, New York). Aconitase (EC 4.2.1.3) 30-70 U/g and isocitrate dehydrogenase (EC 1.1.1.42.) 10 mg/ml, 5 U/mg were purchased from Sigma Chemical Co. Carnitine acetyl transferase (EC 2.3.1.7) 5 mg/ml, 80 U/mg; D-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) 5 mg/ml, 3 U/mg; L-3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) 2 mg/ml, 125 U/mg; and citrate synthase (EC 4.1.3.7) 2 mg/ml, 110 U/mg were obtained from Boehringer Mannheim.

2.1.3 Radioactive Chemicals

Ethyl [$^{14}C_3$] acetoacetate, Sodium [$^{14}C_1$] pyruvate, Sodium [$^{14}C_2$]pyruvate were obtained from New England Nuclear. Ethyl [$^{14}C_4$] acetoacetate and [U-^{14}C] pyruvic acid were purchased from Amersham/Searle.

All solutions, made with deionized double distilled water, were neutralized to pH 7.0 unless otherwise mentioned.

2.1.4 Synthesis of acetoacetylcarnitine

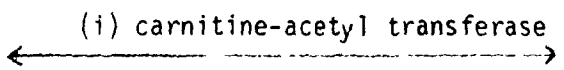
Acetoacetylcarnitine was synthesized according the method described by Bohmer and Bremer (99). , 500 mg L-carnitine was weighed in a 10 ml centrifuge tube and 0.5 ml trifluoroacetic acid and 0.225 ml Diketene were added and the tube was placed in ice for six hours. The tube was then kept at room temperature overnight. 5 ml of acetone were then added and the tube was kept in ice for four hours. The tube was centrifuged in cold to remove the undissolved material. The supernatant was transferred into a 150 ml centrifuge bottle kept in ice and 50 ml diethyl ether was added to precipitate acetoacetylcarnitine. After the precipitation of acetoacetylcarnitine as a sticky mass, the diethyl ether was removed and the precipitates were dissolved in 0.5 ml ethanol. Acetoacetylcarnitine was again precipitated by the addition of 50 ml diethyl ether. The precipitates were dried under reduced pressure and a crisp, foamy and extremely hygroscopic acetoacetylcarnitine was thus synthesized. That the product synthesized was acetoacetylcarnitine, was confirmed by estimating the product enzymatically.

$$\text{Acetoacetylcarnitine} + \text{CoASH} \xleftrightarrow[\text{acetyl transferase}]{\text{Carnitine}} \text{Acetoacetyl-CoA} + \text{Carnitine}$$

$$\text{Acetoacetyl - CoA} + \text{NADH} \xleftrightarrow[\text{-CoA dehydrogenase}]{\text{L-3-hydroxyacyl}} \text{L-3-hydroxybutyryl - CoA} + \text{NAD}^+$$

The sum:

Acetoacetylcarnitine
+
CoASH
+
NADH



L-3-hydroxybutyryl-CoA
+
Carnitine
+
NAD^+

The following reagents were added to a cuvette with 10 mm light path.

60 µl 0.2 M Potassium phosphate buffer pH 7.4

10 µl 10 mM CoASH

10 µl 6 mM NADH (freshly prepared in 10 mM Tris-Cl) pH 8.0

10 µl 125 mM DTT

0-20 nmoles of acetoacetylcarnitine solution.

The reaction was started by the addition of 5 µl carnitine acetyl transferase. Total assay volume was 250 µl. The oxidation of NADH was followed by a decrease in extinction at 340 nm as a result of the conversion of acetoacetylcarnitine to L-3-hydroxybutyryl-CoA. The product synthesized was 85% pure acetoacetylcarnitine and was found to be free of acetoacetate.

2.1.5 Preparation of [^{14}C]acetoacetate

[^{14}C]Acetoacetate was prepared by the hydrolysis of Ethyl [^{14}C] acetoacetate according to the procedure as described by Hall (100). Equimolar amounts of ethyl [$^{14}C_3$]acetoacetate or ehtyl [$^{14}C_4$]acetoacetate and LiOH were incubated at 40^{o}C for four hours. The solution was neutralized to pH 7.0. Acetoacetate was estimated enzymatically and stored frozen in small portions.

2.1.6 Preparation of mitochondria from rat heart

Rat heart mitochondria were isolated according to the procedure described by Pande and Blanchaer (101). The rats weighing 150-200 g were sacrificed by decapitation and the hearts were quickly removed, and placed in chilled homogenizing medium, which consisted of 0.21 M mannitol, 70 mM sucrose and 0.1 mM EDTA. The hearts were cut into pieces and repeatedly washed with several portions of chilled homogenizing medium to remove the external blood. The procedure for preparation of mitochondria from one rat heart is described below. The volumes of the homogenizing medium and the amount of Nagarse etc. were proportionately increased according to the number of rats hearts used. The heart, free from external blood was finally chopped and suspended in 8 ml of the homogenizing medium containing Nagarse a proteolytic enzyme [subtilopeptidase A, (EC 3.4.4.16)], and made 10 mM with respect to Tris-Cl (pH 7.4). Following 8 minutes of incubation at $0^{o}C$ with occasional stirring, 12 ml of homogenizing medium was added and the tissue was homogenized with a Potter-Elvehjem glass-Teflon homogenizer (size C, A.H. Thomas Company, Philadelphia) having a loosely fitting pestle (clearance about 0.66 mm) until large tissues no longer remained. The contents were again incubated at $0^{o}C$ for 8 minutes. Following incubation 15 ml of homogenizing medium was added and the process of homogenization repeated but this time with pestle that gave clearance of 0.20 mm until the resistance to homogenization was no longer felt (four to six passes were generally found sufficient). The resulting homogenate was centrifuged at 400 x g for five minutes at $0^{o}C$ using a Sorvall RC-2B refrigerated centrifuge. The supernatant containing mitochondria and microsomes was again centrifuged at 12,000 x g for ten minutes to sediment mitochondria.

The mitochondrial pellet obtained was rinsed with homogenizing medium to remove any loosely adhering upper layer of white material. The pellet was washed by suspending in 15 ml homogenizing medium and recentrifuged at 12,000 x g for eight minutes. The resulting pellet was once again rinsed with homogenizing medium and then uniformly suspended using a vortex mixer in 0.6 - 0.8 ml of homogenizing medium which had been made 10 mM with respect to Tris-Cl (pH 7.4). The mitochondrial suspension was stored at $0^{o}C$ when not in use. Total time for isolation was about 1 1/2 hours.

Only freshly isolated mitochondria were used for the measurement of oxygen consumption. The isolated mitochondria were used in these experiments within 2-3 hours after their isolation, and were stored at $0-4^{o}C$. Freshly prepared mitochondria exhibiting respiratory control ratio above 5 were used. The substrate pair used were either pyruvate and malate or acetoacetate, malate and 2-oxoglutarate. The respiratory control ratio declined when mitochondria were stored at $0-4^{o}C$ for 4 - 6 hours.

Preparation of mitochondria from rabbit heart

The method used for the isolation of rabbit heart mitochondria were essentially similar to the one described above for rat heart mitochondria. The rabbit weighing 2.5 kg was anesthesized by intravenously injecting pentabarbital (25 mg/kg body weight) in the marginal ear vein and the heart was quickly removed. One gm portion of the heart was used and the mitochondria were isolated according to the procedure which was followed for one rat heart.

2.1.7 Preparation of homogenate from rat heart

White male Sprague-Dawley rats weighing 150-200 gm were used. The rats were decapitated with guillotine, their hearts quickly removed and placed in cold 0.25 M sucrose. The hearts were cut into pieces and washed 2-3 times with chilled 0.25 M sucrose. The hearts were chopped using medium (5ml/gm heart) that contained 0.25 M sucrose, 20 mM Tris-Cl pH 7.4. A Potter-Elvehjem homogenizer with a tightly-fitting pestle was used. The pestle was motor-driven and about six up and down movements of the homogenizing tube were required for complete homogenization. The homogenate was centrifuged at 400 x g at 0-2^{o}C in Sorvall RC-2B refrigerated centrifuge for one minute to sediment only unbroken cells etc. The resulting supernatant was used as heart homogenate.

Preparation of homogenate from rat kidney

Rat kidney homogenate was prepared according to the method described by Johnson and Lardy (102). The kidney capsules were removed by gently squeezing the kidneys through the thumb and fingers. The kidneys were then cut sagittally and the medullary portions were removed and discarded. The kidney cortexes were chopped and then homogenized in 0.25 M sucrose made 20 mM with respect to Tris-Cl pH 7.4 as described for rat heart homogenate.

Dialyzed homogenate

Whenever required, the homogenate prepared as above was poured into dialyzing bag and dialyzed against water containing 1 mM DTT and 20 mM Tris-Cl pH 7.4 at 0-4^{o}C with four changes for two hours.

Sonicated mitochondria

Sonicated mitochondria were prepared by exposing the freshly isolated mitochondria to high frequency sound using a sonic dismembrator manufactured by Quigley-Rochester, Inc. Rochester, N.Y. Sonication was done generally three times at 0-4^{o}C for 30 seconds duration each time, with 30 seconds intervals in between successive sonication.

Sonicated heart homogenate

Sonicated heart homogenate was prepared by the procedure as described above for sonicated mitochondria.

2.2 ANALYTICAL METHODS

2.2.1 Measurement of oxygen consumption by polarographic method

Mitochondrial oxygen consumption was measured at 28^{o}C using a Clark-type oxygen electrode (Yellow spring Instrument Co. Ohio) having a teflon membrane. The electrode was fitted in a glass reaction chamber of 1.6 to 1.8 ml in volume with a temperature jacket. The reaction chamber contained a magnetic stirring bar which was driven by a magnetic stirrer positioned directly underneath the chamber. The use of the magnetic stirrer permitted continual mixing of the reaction medium and facilitated establishment of the equilibrium between the oxygen dissolved in solution and the gas diffusing through the teflon membrane of the oxygen electrode. The reaction chamber was first filled with known volume of the reaction medium without

trapping any air bubble to avoid back diffusion of oxygen and then closed with a glass stopper which had a small capillary opening. Additions to the system were made via the capillary opening using Hamilton microsyringes.

The oxygen electrode consists of a platinum wire sealed in glass as the cathode and a silver wire immersed in a chloride-containing solution as reference anode. When a voltage is imposed across the two electrodes immersed in an oxygen-containing solution, oxygen undergoes an electrolytic reduction. With a polarizing voltage of 8 mV, current is directly proportional to the oxygen concentration of the solution. The current generated was measured with the Gilson recorder which permitted determination of the decrease in oxygen consumption as a function of time. The oxygen content of the air-saturated reaction medium was taken as 472 natoms oxygen/ml at 28°C (103).

2.2.2 Protein estimation

Protein concentrations of the mitochondrial suspension or homogenate were determined by the method of Lowry et al (104) using crystalline BSA as standard.

2.2.3 Estimation of acetoacetate

2.2.3.1 Enzymatic method

D-3-Hydroxybutyrate dehydrogenase catalyzes the reduction of acetoacetate to D-3-hydroxybutyrate according to the following equation

$$\text{Acetoacetate} + \text{NADH} + \text{H}^+ \xleftarrow[\text{dehydrogenase}]{\text{D-3-hydroxybutyrate}} \text{D-3-hydroxybutyrate} + \text{NAD}^+$$

The quantitative measurement of acetoacetate was made by following the oxidation of NADH spectrophotometrically (105). The decrease in extinction at 340 nm due to the oxidation of NADH was proportional to the amount of acetoacetate present.

The following reagents were added to a cuvette having 10 mm light path 85 µl 0.1 M Tris-Cl pH 7.0, 10 µl 5 mM NADH (prepared in 10 mM Tris-Cl pH 8.0 and acetoacetate solution (0-20 nmoles)pH 7.0.

The reaction was started by the addition of 10 µl D-3-hydroxybutyrate dehydrogenase. Total assay volume was 250 µl.

2.2.3.2 Non-enzymatic method

The colorimetric method of Walker (106) as modified by Allred (107) was used. This colorimetric reaction depends upon the coupling of acetoacetate with an excess of p-nitrobenzene-diazo hydroxide. The colored product formed was then extracted with ethylacetate and the absorbance was measured at 450 nm.

Preparation of diazo reagent:

This reagent was prepared immediately before use by adding the following in the order mentioned.

3.0 ml $NaNO_2$ (0.5% w/v, prepared fresh)
20.0 ml p-nitroaniline (0.05% in 0.05 N HCl),
kept in ice bath and then
7.0 ml 0.2 M Sodium acetate was added.

For acetoacetate determination the following components were added to a 5 ml screw capped tube:

250 µl 1 M Sodium acetate buffer pH 5.2

750 µl Diazo reagent (prepared fresh)

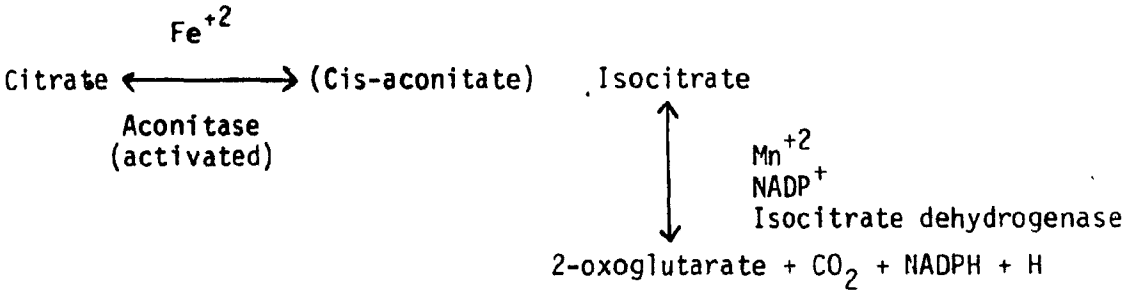
20 µl 0.25 M $HgCl_2$

Acetoacetate solution at neutral pH and water to a final volume of 1250 µl. Following incubation at room temperature for 30 minutes, 250 µl of 5 N HCl was added and the colored product formed was extracted with 1.0 ml of ethyl acetate. The absorbance of the ethyl acetate phase was measured at 450 nm in a Gilford spectrophotometer against a reagent blank. The blank received treatment identical to that of experimental except that acetoacetate solution was substituted by water.

For estimation of acetoacetate in the presence of acetoacetyl-CoA the procedure outlined above was found to be unsatisfactory because in the presence of Hg^{+2} ions, acetoacetyl-CoA also contributed to the color due to acetoacetate formation. In the above procedure, Hg^{+2} ions were included to eliminate the interference (if any) by thiol groups (107). Therefore it is not possible to estimate acetoacetate in the presence of acetoacetyl-CoA by the above mentioned procedure. No such difficulty was encountered by the Walker's method (106) in the presence of acetoacetyl-CoA.

2.2.4 Estimation of citrate

Citrate was estimated according to the method described by Williamson and Corkey (108). The method described measures total of citrate, Cis-aconitate and isocitrate according to the following reaction.



Activation of aconitase

This was carried out as described by Williamson and Corkey (108) To 9.8 mg $Fe(NH_4)2\ SO_4.6H_2O$ and 17.6 mg of cysteine. $HCl.H_2O$ in a 10 ml graduated centrifuge tube was added 4.5 ml of water and after mixing nitrogen was bubbled through the solution for 10 minutes at room temperature. The pH of the solution was then adjusted to 7.4 with 1N NaOH and the volume of the solution was adjusted to 5.0 ml using water. The final concentration of the clear amber solution of $Fe(NH_4)_2\ SO_4.6H_2O$ and cysteine $HCl.H_2O$ at this stage, was 5 and 20 mM respectively. When the activation solution was purple or brown, it was discarded and a new solution was prepared. The brown or purple color is caused by the oxidation of Fe^{+2} to Fe^{+3}, and the solution becomes turbid due to cystine formation (108).

Aconitase (3 mg) was dissolved in 0.15 ml water and then 0.15 ml of activating solution was added and nitrogen was passed above the surface of the solution for a brief period at 0-4°C. The enzyme solution was then incubated for exactly five minutes at 30°C (without nitrogen). The aconitase thus activated was stored in ice until used. (The volume of the activated aconitase solution was made to 0.3 ml with ice cold water if necessary).

Citrate was estimated spectrophotometrically by adding the following reagents to a cuvette with 10 mm light path.

20 µl 1 M Triethanolamine, pH 7.4 (pH adjusted with NaOH)

10 µl $NADP^+$ (Sodium salt) 10 mg/ml

5 µl 2.5 mM $MnSO_4$

200 µl citrate solution (0-20 nmoles) at neutral pH.

2 µl Isocitrate dehydrogenase

The reaction was started by the addition of 10 µl of activated aconitase. Total assay volume was 250 µl. The increase in optical density was followed with a Gilson spectrophotometer equipped with recorder (Model 240).

Under these conditions, it was observed that:

(a) Oxaloacetate interfered with citrate estimation. Concentration of oxaloacetate above 0.66 mM were inhibitory and complete inhibition was observed when oxaloacetate concentration reached 1.66 mM. It was found that the interference by oxaloacetate can be eliminated by boiling the citrate solution for 10 minutes prior to the estimation of citrate.

(b) Free ATP, 1 mM and above also interfered with citrate estimation. The interference by 3 mM ATP was eliminated partially but not completely by the presence of 0.8 mM $MgCl_2$.

(c) It has been reported that presence of high concentration of K^+ ions interfere with the estimation of citrate (108). Our experiments contained K^+ ions and it was observed that K^+ ions up to 30 mM did not interfere with the estimation of citrate.

2.2.5 Estimation of 2-oxoglutarate

2-oxoglutarate was estimated according to the method described by Bergmeyer and Bernt (109).2-Oxoglutarate is quantitatively converted to glutamate in the presence of an excess of ammonium ions, NADH and glutamate dehydrogenase (L-Glutamate : NAD(P) oxido-reductose, deaminating, EC 1.4.1.3).

$$\text{2-oxoglutarate} + \text{NADH} + NH_4^+ + H^+ \;\underset{\text{Glutamate dehydrogenase}}{\longleftrightarrow}\; \text{Glutamate} + NAD^+ + H_2O$$

The following components were added to a cuvette with 10 mm light path and the fall in absorbance at 340 nm was recorded against a blank in which 2-oxoglutarate solution was replaced by water.

25 µl 0.1 M Phosphate buffer pH 7.4

10 µl 1 M Ammonium chloride

10 µl 5 mM NADH

0-20 nmoles 2-oxoglutarate solution

The reaction was started by the addition of 2 µl of glutamate dehydrogenase. Total assay volume was 250 µl.

2.2.6 Collection of $^{14}CO_2$ for liquid scintillation counting

In experiments where mitochondrial oxidation of [^{14}C]aceotacetate or [^{14}C]pyruvate to $^{14}CO_2$ was followed, the tube containing the incubation system

was closed with rubber serum stopper. A hanging plastic cup (inserted through the rubber stopper) containing 0.2 ml of 1 M Hyamine and a filter paper-wick served to trap evolved $^{14}CO_2$. At a predetermined time, the reaction was terminated by injecting methanol to a final concentration of 50% (v/v) This followed separate injectiors of aqueous NaHCO3, to provide 2 μmoles of NaHCO3 to act as a carrier for CO_2 (110)and a predetermined volume (between 1-1.5 ml) of 1 M potassium phosphate buffer (pH 5.5) to bring final pH of the solution to 6.0 (111). The lower part of the tube was kept at 37°C. After two hours, the Hyamine containing cup was transferred to a counting vial containing 15 ml Aquasol (New England Nuclear Corporation) and the radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer system (Model 3375).

3. RESULTS

3.1 INHIBITION OF ACETOACETATE OXIDATION BY MALATE

It is known that respiration of mitochondria with substrates that require operation of citric acid cycle is generally stimulated by the addition of small amounts of "sparkers", i.e. intermediates, of citric acid cycle. Szent-Györgyi (112) had shown that addition of small amounts of fumarate, malate or succinate to minced-muscle suspensions greatly stimulated the oxidation of endogenous substrates in tissues and that this effect of sparkers was catalytic. In line with this, the mitochondrial oxidation of pyruvate, acetylcarnitine, acetoacetylcarnitine (Fig. 2, curves A, B and C), and palmitylcarnitine (curve E) was stimulated by 0.2 mM malate. Unlike the mitochondrial oxidation of these substrates (Fig. 2, curves A, B and C), oxidation of acetoacetate was markedly inhibited by malate (Fig. 2, curve D).

Respiratory studies with isolated mitochondria are influenced by the amount and nature of the endogenous substrate (113), because the endogenous substrates of mitochondria are altered in nature and are slowly depleted during storage of mitochondria at $0^{o}C$ (114). Depletion of endogenous substrates was reported to occur much more rapidly by preliminary incubation at $28^{o}C$ for a few minutes than the depletion which occured during storage of the mitochondria at $0^{o}C$ (115). In line with this, the degree of stimulation or inhibition by malate on the mitochondrial oxidation of substrates (Fig. 2) varied with the age of mitochondria after isolation. Thus the stimulatory effect of malate on the mitochondrial oxidation of pyruvate, acetylcarnitine, and acetoacetyl-carnitine was more marked with mitochondria that were aged for 4-6 hours at

Fig. 2 Effect of malate and of subsequently added 2-oxoglutarate on the mitochondrial oxidation of various substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 3 mM ADP, 2.5 mM Pi and freshly isolated mitochondria (0.59 mg protein). Other additions were made as shown. Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria, 2-OG, 2-oxoglutarate, AcAc, acetoacetate, AcAc-C, acetoacetylcarnitine. Pyr, pyruvate. Acetyl-C, acetylcarnitine. The substrates, malate and 2-oxoglutarate were added respectively at 2, 4 and 6 minutes after the addition of mitochondria.

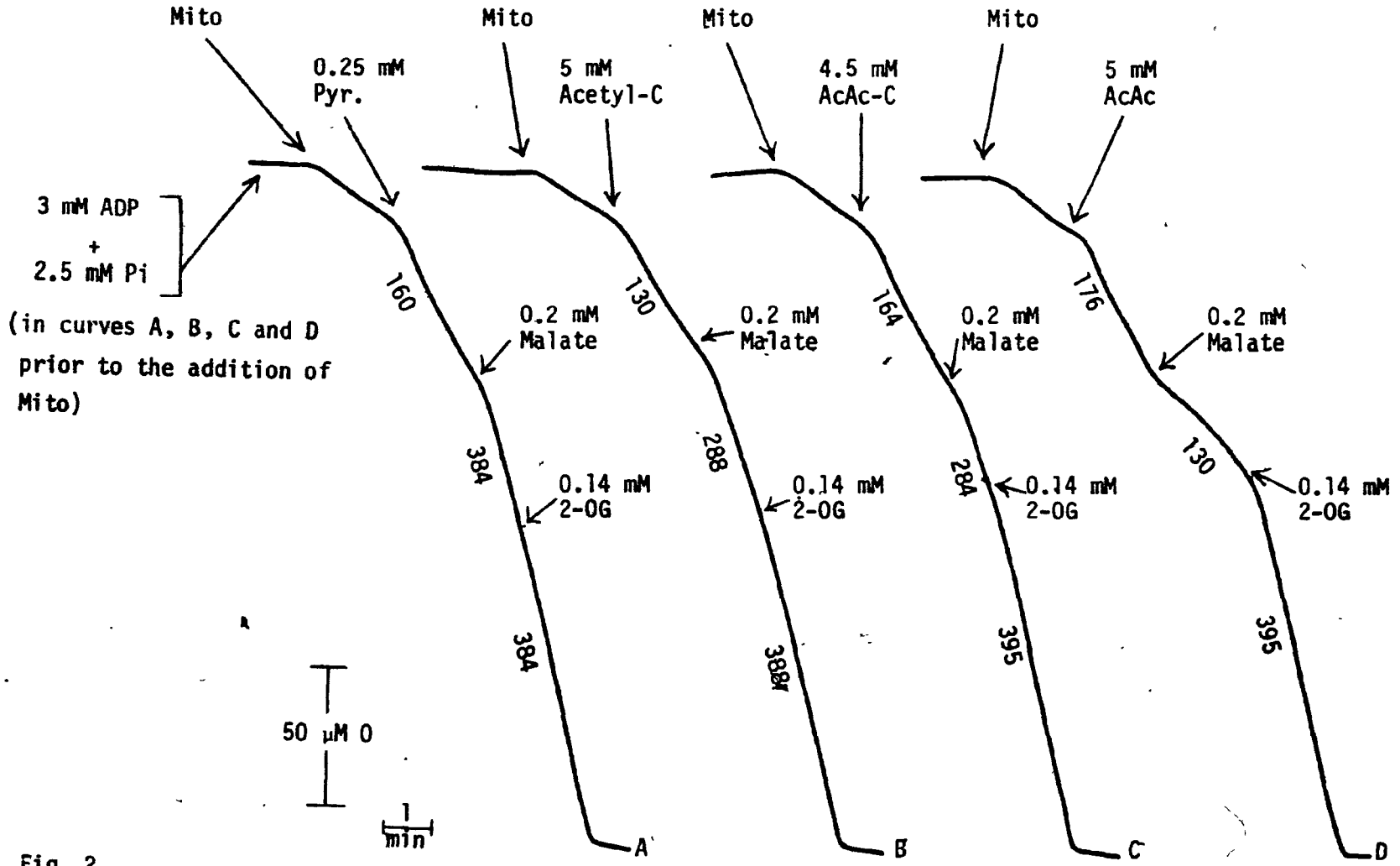


Fig. 2

E

(see next page)

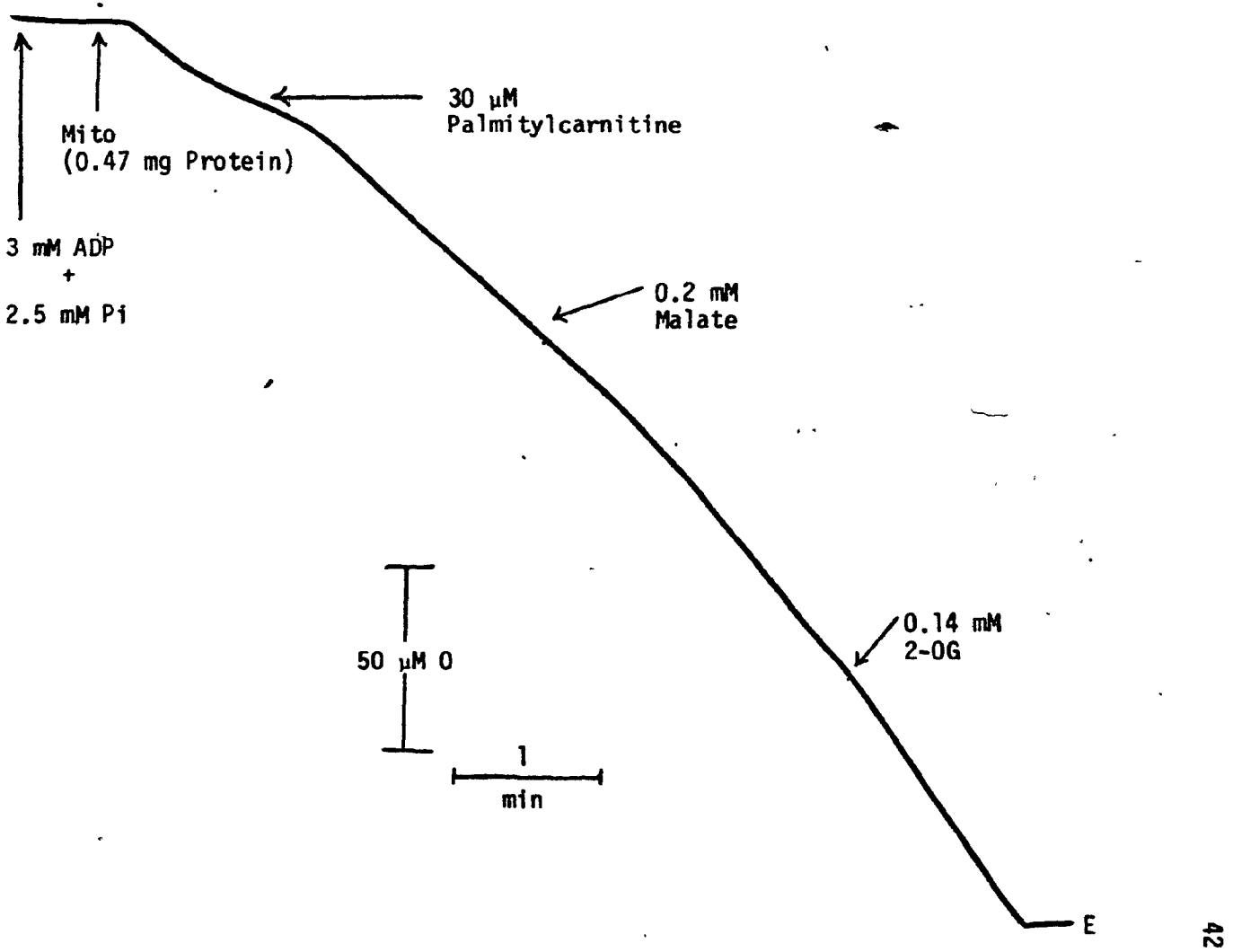

30 µM
Palmitylcarnitine
Mito
(0.47 mg Protein)
3 mM ADP
+
2.5 mM Pi
0.2 mM
Malate
0.14 mM
2-OG
50 µM O
1
min
E

Fig. 2 (cont'd)

$0^{o}C$ (data not shown) or incubated for a few minutes at $28^{o}C$ (Fig. 2, curves A, B and C). On the other hand the inhibition of acetoacetate oxidation by malate was more marked with freshly isolated mitochondria than with mitochondria which were aged for a few hours at $0^{o}C$ (data not shown) or for a few minutes at $28^{o}C$ (Fig. 2, curve D).

The inhibition of acetoacetate-dependent respiration by malate was reversed by added 2-oxoglutarate (Fig. 2, curve D). Although the respiratory rates of other substrates were also stimulated by added 2-oxoglutarate, the 2-oxoglutarate stimulation of respiration was more marked with acetoacetate as compared to that with other substrates (Fig. 2). This greater stimulatory effect of 2-oxoglutarate on acetoacetate oxidation resulted most likely because of the convertibility of 2-oxoglutarate to succinyl-CoA because succinyl-CoA in conjunction with 3-oxoacid-CoA transferase leads to the activation of acetoacetate. Oxidation of pyruvate, acetylcarnitine, and acetoacetylcarnitine - i.e. of substrates which do not require succinyl-CoA for initiation of their oxidative utilization - was not as much stimulated by 2-oxoglutarate.

It is possible that the inhibition of acetoacetate-dependent respiration by malate resulted from the exchange between added malate and intramitochondrial 2-oxoglutarate. It is known that heart mitochondria contain a translocator (2-oxoglutarate translocator) which catalyzes such an exchange (116). Inhibition could thus result from depletion of the precursor of succinyl-CoA. If this were so then respiration with acetoacetylcarnitine (which does not require succinyl-CoA for activation) should not be inhibited by malate. Curve C of Fig. 2 shows that this was indeed so; instead of being

inhibitory malate was now stimulatory for respiration.

It has been suggested that acetoacetate can also be activated by an ATP-dependent activating enzyme (71-76,97). The possibility of such a reaction proceeding in the experiment of Fig. 2 (curve D) cannot be ruled out because under these conditions well coupled-mitochondria oxidizing acetoacetate in the presence of ADP and Pi would synthesize ATP and ATP so generated could activate acetoacetate. In order to test this possibility, oxidation of acetoacetate was followed in the presence of an uncoupler 2,4 dinitrophenol (DNP) and in order to deplete the mitochondria of their endogenous ATP a brief prior incubation of mitochondria with DNP was allowed prior to the initiation of respiration. As is evident from Fig. 3, addition of malate caused inhibition of acetoacetate-dependent respiration, but further addition of ATP did not restore the malate-inhibited respiration inspite of the fact that oligomycin was present to prevent ATP hydrolysis (curve A, Fig. 3). Thus no ATP-dependent activation of acetoacetate proceeded under these conditions. On the contrary, added 2-oxoglutarate markedly stimulated the malate-inhibited respiration (curve B, Fig. 3). Under these conditions the possibility of a direct activation of acetoacetate by an ATP-dependent activating enzyme is remote. Comparison of curve B of Fig. 3 and curve D of Fig. 2 shows that when mitochondrial oxidation of acetoacetate is followed either in the presence of DNP (where no ATP is available) or in the absence of DNP (where ATP is generated), 2-oxoglutarate stimulated the respiration dependent on acetoacetate oxidation. Therefore for the oxidation of acetoacetate succinyl-CoA-linked activatory pathway involving the enzyme 3-oxoacid-CoA transferase appears to be the only

Fig. 3 Effect of ATP and 2-oxoglutarate on the malate-inhibited oxygen consumption of mitochondria with acetoacetate as a substrate.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP and freshly isolated mitochondria (0.59 mg protein). Other additions made as shown. Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria. AcAc, acetoacetate. Oligo, oligomycin. 2-OG, 2-oxoglutarate.

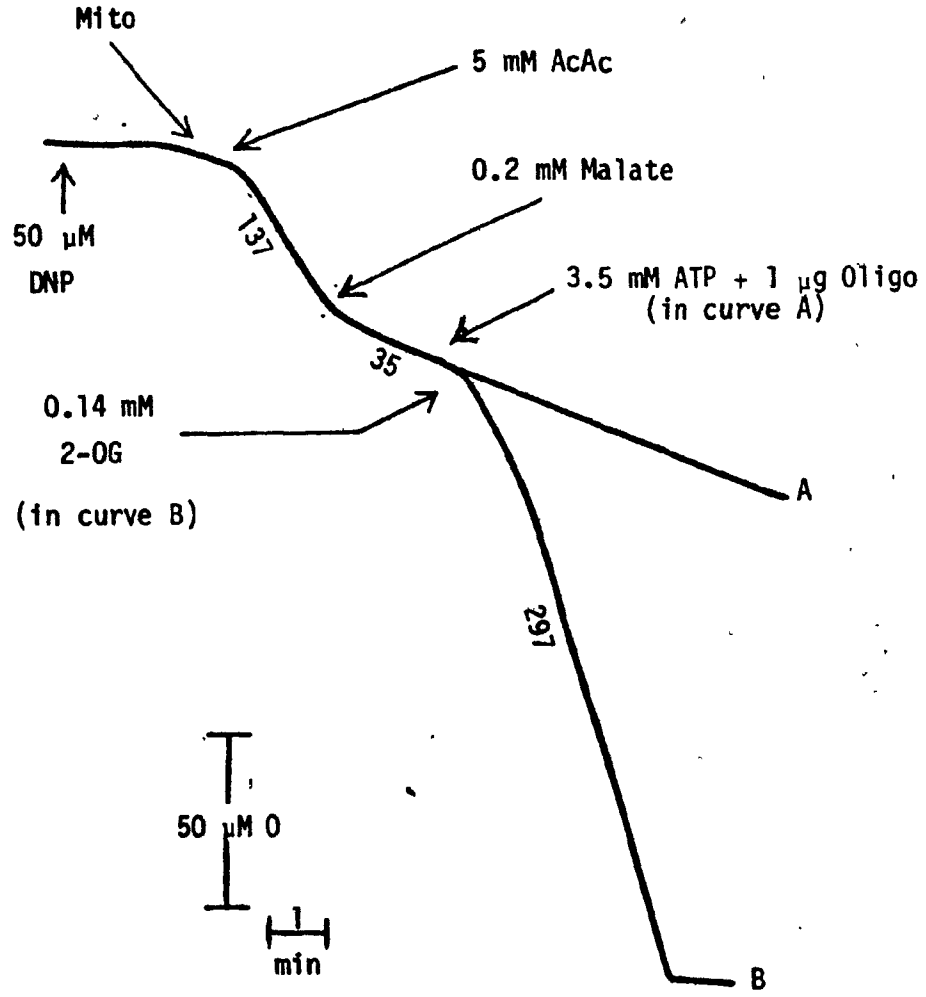

Mito
5 mM AcAc
0.2 mM Malate
50 µM
DNP
137
3.5 mM ATP + 1 µg Oligo
(in curve A)
35
0.14 mM
2-OG
(in curve B)
A
297
50 µM O
1
min
B

Fig. 3

route. If this is so then inhibition of 2-oxoglutarate oxidation should result in the inhibition of acetoacetate-dependent respiration and addition of ATP should not accelerate the respiratory rates. In agreement with this Fig. 4 and 5 show that the mitochondrial oxidation of acetoacetate in the presence of 2-oxoglutarate and malate was markedly inhibited by prior incubation of mitochondria with arsenite (Fig. 4) or parapyruvate (Fig. 5) i.e. by compounds that are known to inhibit the oxidation of 2-oxoglutarate (12,117). The effect of parapyruvate or of arsenite on the acetoacetate-dependent respiration was similar irrespective of whether respiration was stimulated by DNP plus Pi (Fig. 4 and 5) or by ADP plus Pi (Fig. 6). In similar experiments with malate present no such marked inhibitory effect of arsenite or of parapyruvate was observed on the oxidation of substrates - such as acetoacetylcarnitine (Fig. 7), pyruvate (Fig. 8), acetylcarnitine (Fig. 9) and palmitylcarnitine (Fig. 10) - that do not require succinyl-CoA for initiation of their oxidative utilization. The effect of arsenite or of parapyruvate on the oxidation of acetoacetylcarnine, pyruvate, acetylcarnitine and palmitylcarnitine was similar irrespective of whether respiration was stimulated by ADP plus Pi (Fig. 7, 8, 9 and 10) or by DNP plus Pi (data not shown). The fact that arsenite - or parapyruvate-induced inhibition of acetoacetate oxidation was not reversed by added ATP plus oligomycin (curve C, Fig. 4 and 5) strongly suggest that the succinyl-CoA-linked activatory pathway for the oxidation of acetoacetate involving the enzyme 3-oxoacid-CoA transferase may be the only route of acetoacetate activation.

In these and other experiments (to be shown) acetoacetate oxidation was followed in the presence of sparkers of the citric acid cycle 2-oxoglutarate

Fig. 4 Effect of arsenite and of subsequently added ATP on the oxygen consumption of mitochondria with acetoacetate, 2-oxoglutarate and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP, 2 mM Pi, 2 mM arsenite (in curves C and D only) and freshly isolated mitochondria (0.48 mg protein). Substrates added at 3 minutes after the addition of mitochondria are:

Curve A: 5.8 mM acetoacetate + 0.14 mM 2-oxoglutarate + 0.2 mM malate.

Curve B: 0.14 mM 2-oxoglutarate + 0.2 mM malate.

Curve C: 5.8 mM acetoacetate + 0.14 mM 2-oxoglutarate + 0.2 mM malate (2 mM arsenite was present prior to the addition of mitochondria). After following respiration for 2 minutes, 1 μg oligomycin and 3 mM ATP was added.

Curve D: 0.14 mM 2-oxoglutarate + 0.2 mM malate (2 mM arsenite was present prior to the addition of mitochondria).

Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria. AcAc, acetoacetate. 2-OG, 2-oxoglutarate. Oligo, oligomycin.

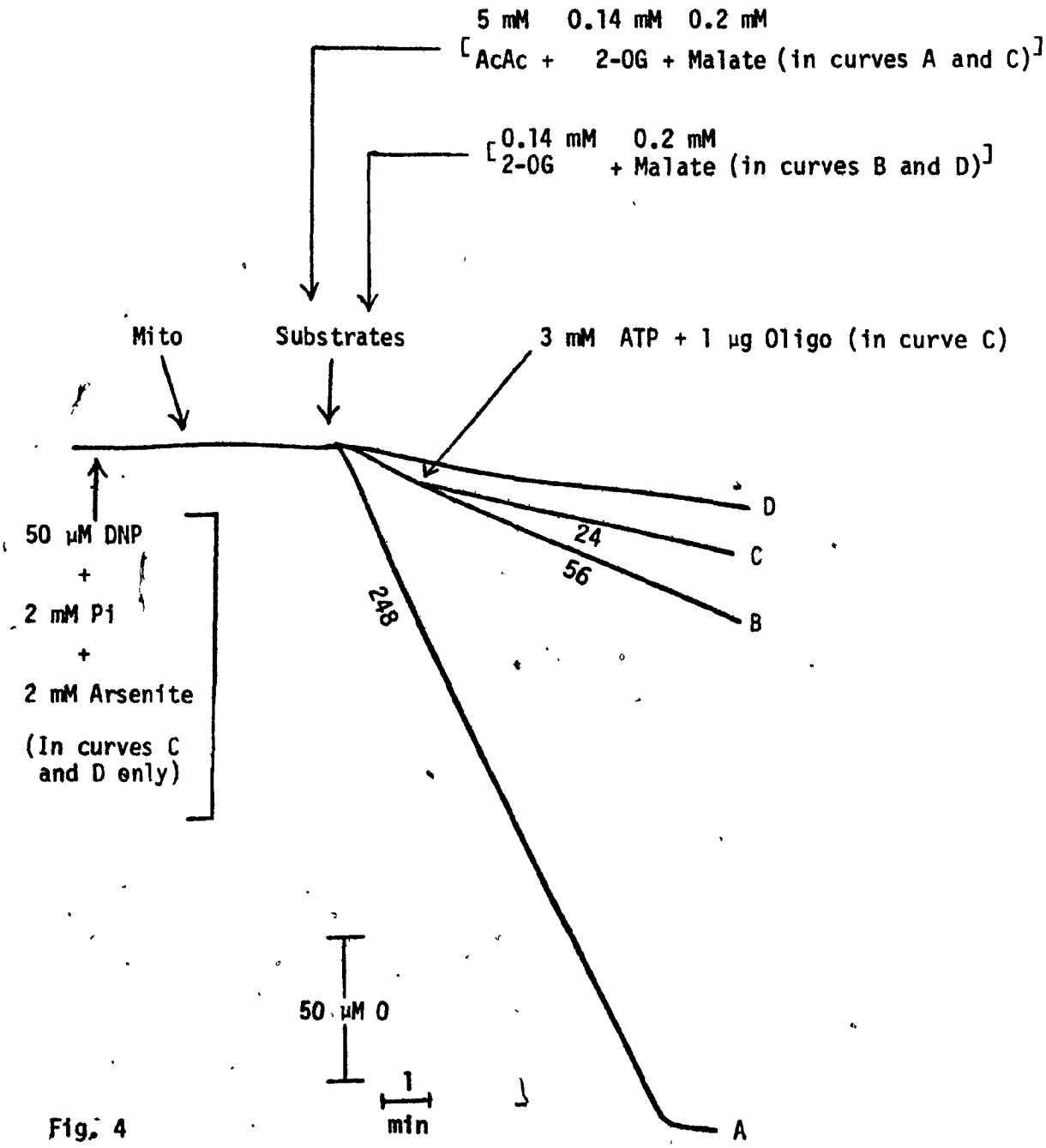

5 mM 0.14 mM 0.2 mM
[AcAc + 2-OG + Malate (in curves A and C)]
[0.14 mM 0.2 mM
2-OG + Malate (in curves B and D)]
Mito
Substrates
3 mM ATP + 1 µg Oligo (in curve C)
50 µM DNP
+
2 mM Pi
+
2 mM Arsenite
(In curves C and D only)
D
C
B
A
24
56
248
50 µM O
1
min

Fig. 4

Fig. 5 Effect of parapyruvate and of subsequently added ATP on the oxygen consumption of mitochondria with acetoacetate, 2-oxoglutarate and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP, 2 mM Pi, 2 mM parapyruvate (in curves C and D only) and freshly isolated mitochondria (0.48 mg protein). Substrates added at 1 minute after the addition of mitochondria are:

Curve A: 5.8 mM acetoacetate + 0.14 mM 2-oxoglutarate + 0.2 mM malate.

Curve B: 0.14 mM 2-oxoglutarate + 0.2 mM malate.

Curve C: 5.8 mM acetoacetate + 0.14 mM 2-oxoglutarate + 0.2 mM malate (2 mM parapyruvate was present prior to the addition of mitochondria). After following respiration for 3 minutes, 1 μg oligomycin and 3 mM ATP was added.

Curve D: 0.14 mM 2-oxoglutarate + 0.2 mM malate (2 mM parapyruvate was present prior to the addition of mitochondria.

Numerals by the side of the tracing, the rate of the oxygen consumption in natoms per min per mg of protein. Mito, mitochondria, P-Pyr, Parapyruvate. 2-OG, 2-oxoglutarate. AcAc acetoacetate. Oligo, oligomycin.

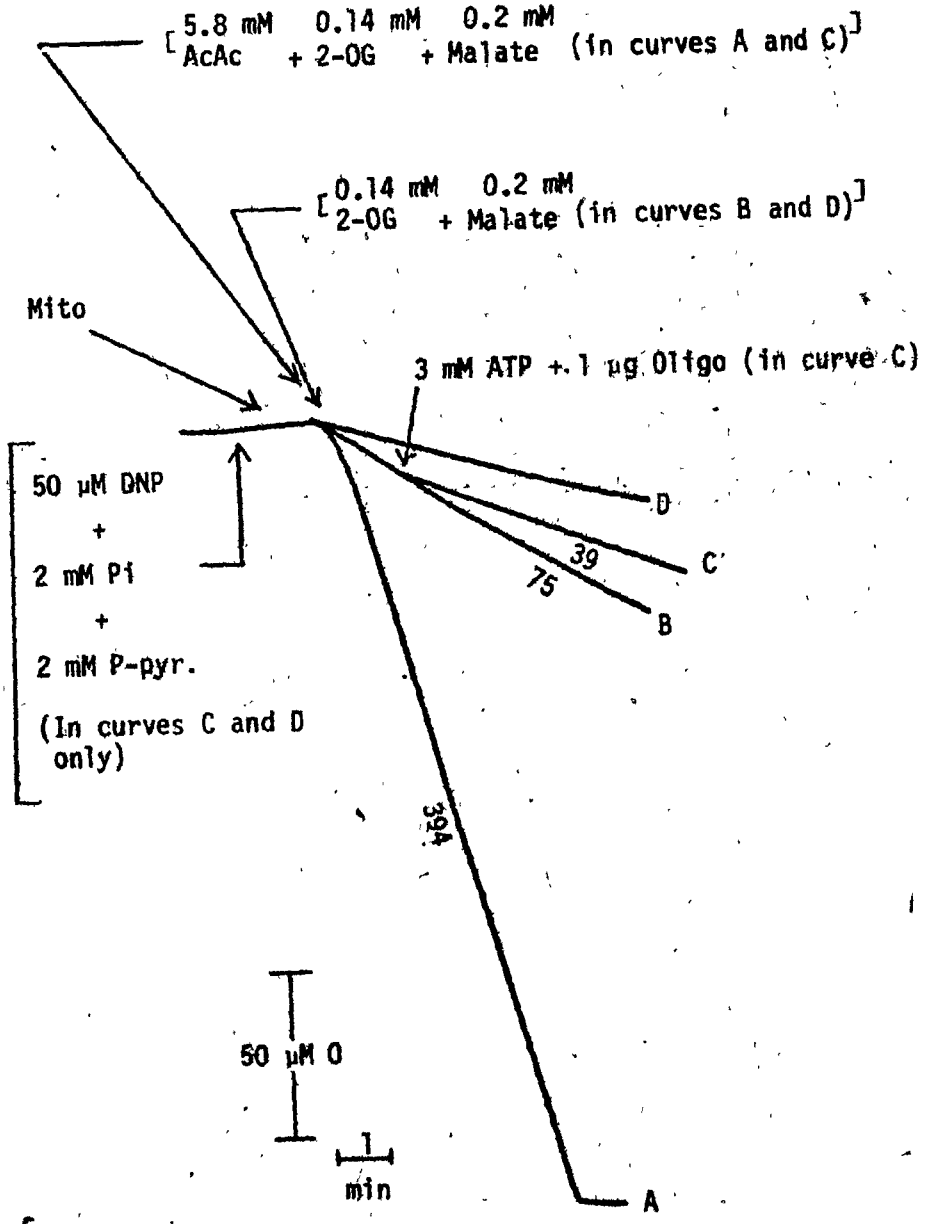

5.8 mM AcAc + 0.14 mM 2-OG + 0.2 mM Malate (in curves A and C)
0.14 mM 2-OG + 0.2 mM Malate (in curves B and D)
Mito
3 mM ATP +.1 µg Oligo (in curve C)
50 µM DNP
+
2 mM Pi
+
2 mM P-pyr.
(In curves C and D only)
D
C
B
A
39
75
394
50 µM O
1 min

Fig. 5

Fig. 6 Effect of arsenite or of parapyruvate on the oxygen consumption of mitochondria with acetoacetate, 2-oxoglutarate and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 3 mM ADP, 2.5 mM Pi, 2 mM arsenite (in curve A only), 2 mM Parapyruvate (in curve C only) and freshly isolated mitochondria (0.51 mg protein). 5.8 mM Acetoacetate, 0.14 mM 2-oxoglutarate and 0.2 mM malate were added at 1 minute (in curves C and D) or at 3 minutes (in curves A and B) after the addition of mitochondria. Mito, mitochondria. 2-OG, 2-oxoglutarate. P-pyr, parapyruvate. AcAc, acetoacetate.

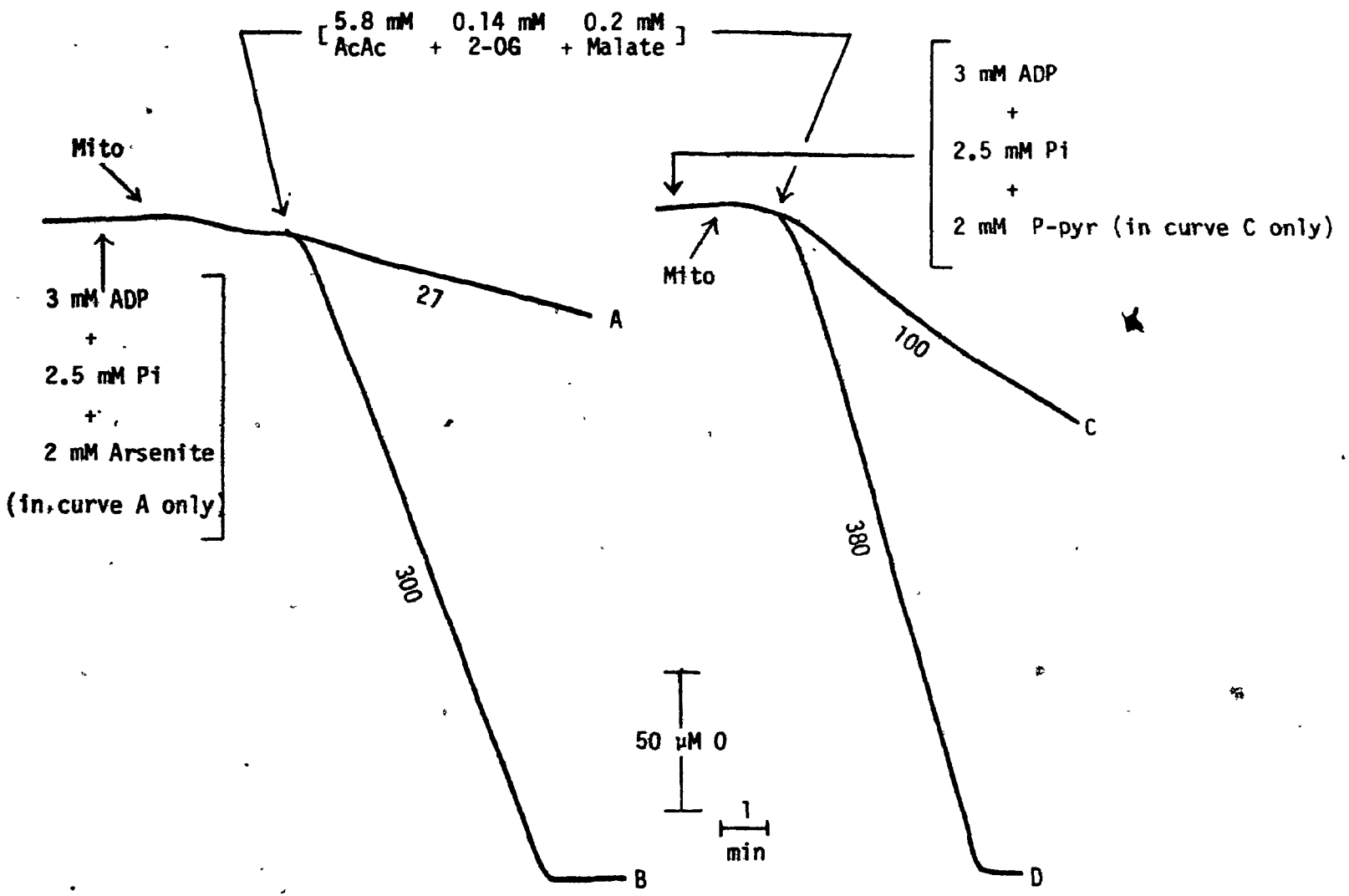

[5.8 mM AcAc + 0.14 mM 2-OG + 0.2 mM Malate]
Mito
3 mM ADP
+
2.5 mM Pi
+
2 mM Arsenite
(in curve A only)
27
A
300
B
3 mM ADP
+
2.5 mM Pi
+
2 mM P-pyr (in curve C only)
Mito
100
C
380
D
50 µM O
1
min

Fig. 6

Fig. 7 Effect of arsenite or of parapyruvate on the oxygen consumption of mitochondria with acetoacetylcarnitine and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 3 mM ADP, 2.5 mM Pi, 2 mM arsenite (in curve A only), 2 mM parapyruvate (in curve C only) and freshly isolated mitochondria (0.51 mg protein). 4.5 mM Acetoacetylcarnitine and 0.2 mM malate were added at 1 minute (in curves C and D) or at 3 minutes (in curves A and B) after the addition of mitochondria. Mito, mitochondira. AcAc-c, Acetoacetylcarnitine. P-pyr, Parapyruvate.

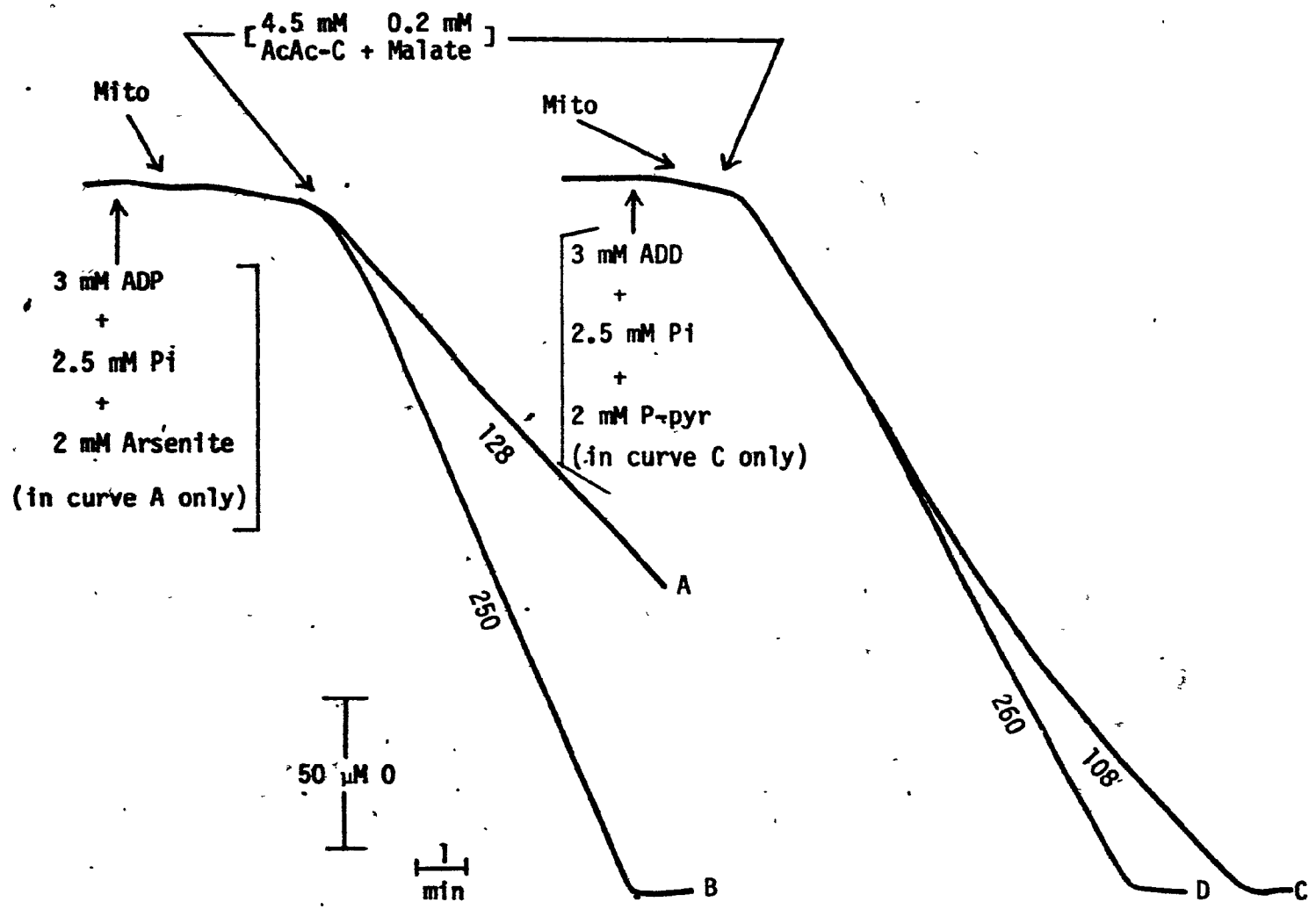

[4.5 mM AcAc-C + 0.2 mM Malate]
Mito
Mito
3 mM ADP
+
2.5 mM Pi
+
2 mM Arsenite
(in curve A only)
3 mM ADD
+
2.5 mM Pi
+
2 mM P-pyr
(in curve C only)
128
250
260
108
A
B
C
D
50 µM O
1
min

Fig. 7

Fig. 8 Effect of parapyruvate on the oxygen consumption of mitochondria with pyruvate and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 3 mM ADP, 2.5 mM Pi, 2 mM parapyruvate (in curve A only) and freshly isolated mitochondria (0.51 mg protein). 0.25 mM Pyruvate and 0.2 mM malate were added at 1 minute after the addition of mitochondria. Mito, mitochondria. Pyr, pyruvate. P-pyr, parapyruvate.

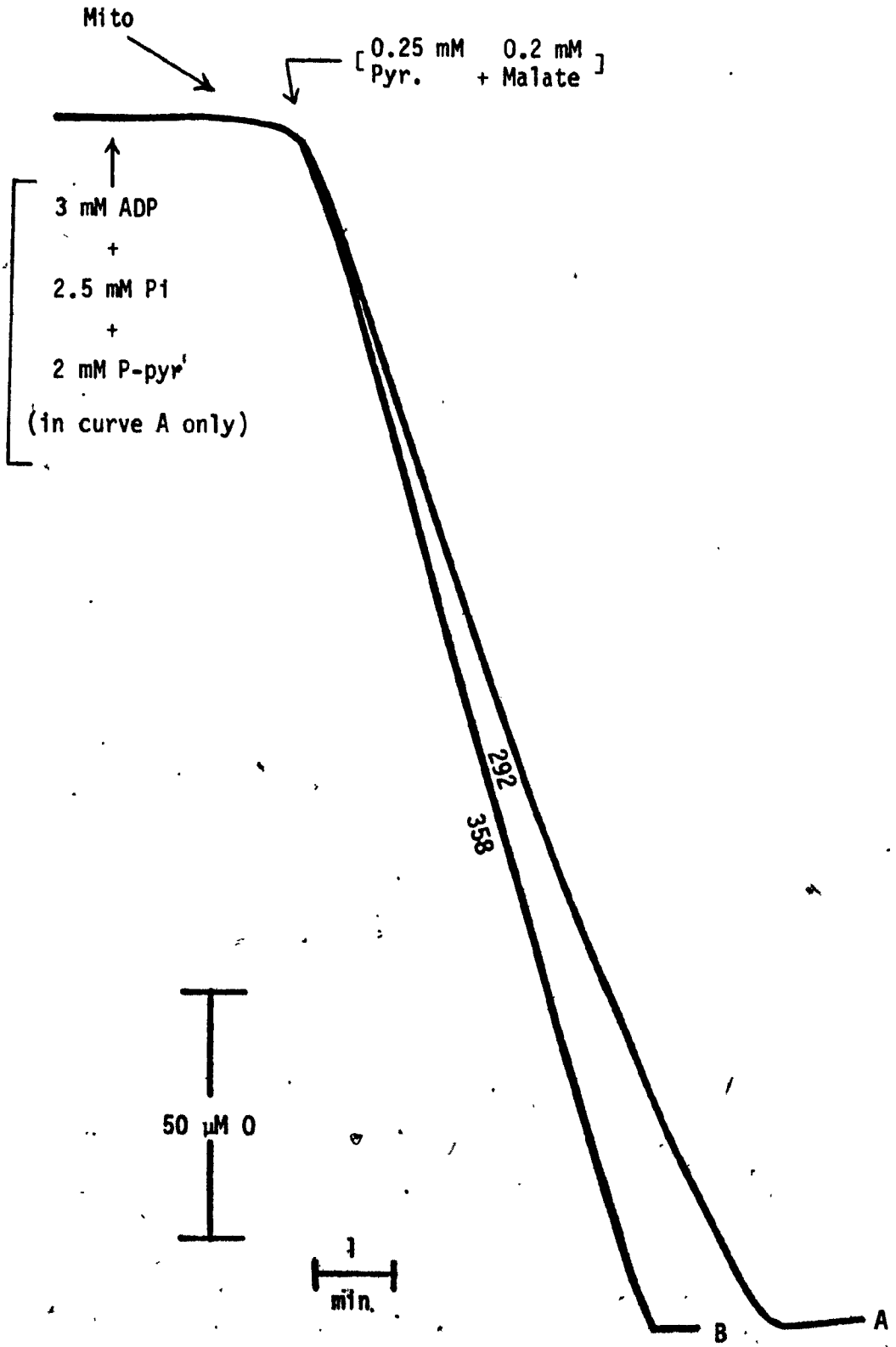

Mito
[0.25 mM Pyr. 0.2 mM + Malate]
3 mM ADP
+
2.5 mM Pi
+
2 mM P-pyr
(in curve A only)
292
358
50 µM O
1 min.
B
A

Fig. 8

Fig. 9 Effect of arsenite or of parapyruvate on the oxygen consumption of mitochondria with acetylcarnitine and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 3 mM ADP, 2.5 mM Pi, 2 mM arsenite (in curve A only), 2 mM parapyruvate (in curve C only) and freshly isolated mitochondria (0.51 mg protein). 5 mM Acetylcarnitine and 0.2 mM malate were added at 1 minute (in curves C and D) or at 3 minutes (in curves A and B) after the addition of mitochondria. Mito, mitochondria. Acetyl-c, acetylcarnitine. P-pyr, parapyruvate.

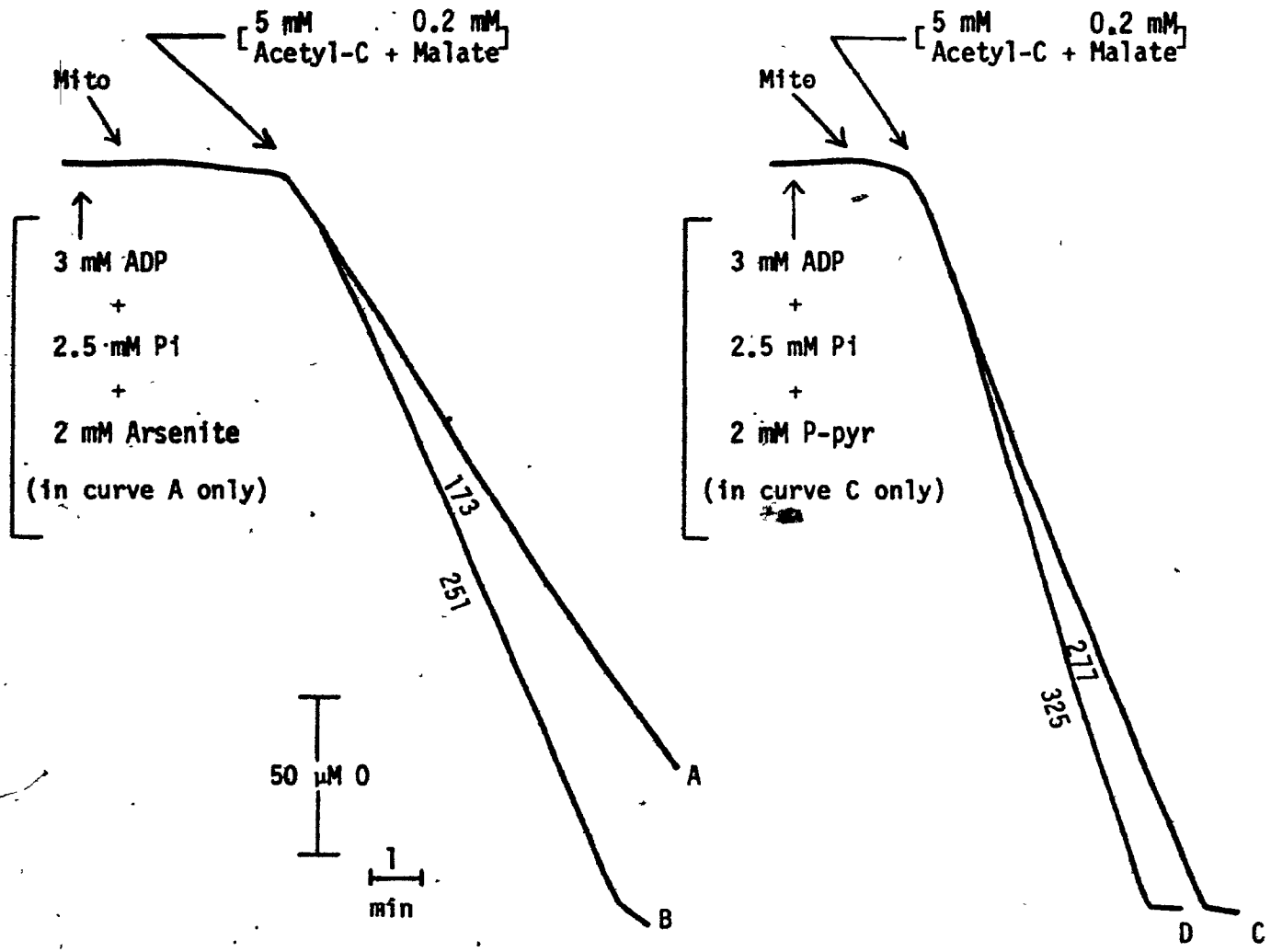

5 mM
Acetyl-C + Malate
0.2 mM
Mito
3 mM ADP
+
2.5 mM Pi
+
2 mM Arsenite
(in curve A only)
173
251
50 µM O
1
min
A
B
5 mM
Acetyl-C + Malate
0.2 mM
Mito
3 mM ADP
+
2.5 mM Pi
+
2 mM P-pyr
(in curve C only)
217
325
D
C

Fig. 9

Fig. 10 Effect of arsenite on the oxygen consumption of mitochondria with palmitylcarnitine and malate as substrates.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 3 mM ADP, 2.5 mM Pi, 2 mM arsenite (in curve A only) and freshly isolated mitochondria (0.47 mg protein). 30 μM Palmitylcarnitine and 0.2 mM malate were added at 3 minutes after the addition of mitochondria. Mito, mitochondria, Palmityl-c, palmitylcarnitine.

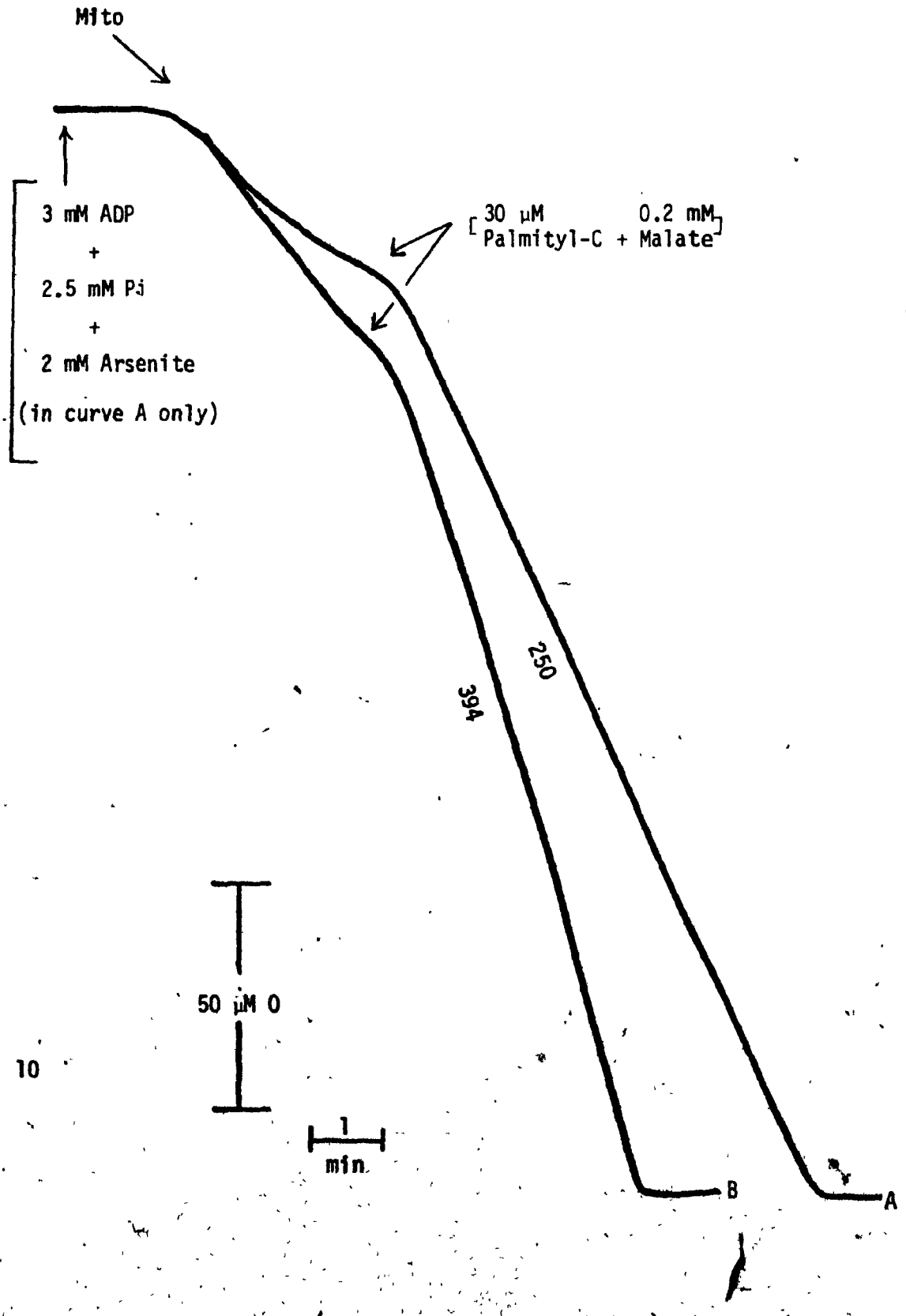

Mito
3 mM ADP
+
2.5 mM Pi
+
2 mM Arsenite
(in curve A only)
30 µM Palmityl-C + 0.2 mM Malate
394
250
50 µM O
1 min
B
A

Fig. 10

and malate. Although malate was shown to inhibit acetoacetate oxidation, it was found that apart from 2-oxoglutarate, addition of malate was also necessary for the continued oxidation of acetoacetate.

With freshly isolated mitochondria acetoacetate was oxidized even in the absence of the added sparkers of citric acid cycle provided mitochondria were uncoupled to stimulate respiration and no Pi was present (Fig. 11, curve A). Under these conditions, the respiratory rates were high to begin with but declined gradually with time presumably because of the exhaustion of endogenous sparkers, especially 2-oxoglutarate. Experiments with different limiting amounts of 2-oxoglutarate showed that heart mitochondria oxidized 2-oxoglutarate with high affinity so that it was possible to observe 2-oxoglutarate-dependent cycles of respiration (Fig. 11, curve B, C and D). The rates of oxygen consumption of 2-oxoglutarate-dependent cycles were much higher when acetoacetate was also present showing that oxidation of 2-oxoglutarate was promoting oxidation of acetoacetate (Fig. 11, curve E). The rapid phase of respiration in the presence of acetoacetate and limiting amounts of 2-oxoglutarate were followed by a slow phase of respiration (Fig. 11, curve E). The slow phase of respiration was stimulated by 2-oxoglutarate but not only by acetoacetate as acetoacetate was already present in saturating concentration.

Although when added alone, malate was inhibitory for acetoacetate oxidation, in the presence of added 2-oxoglutarate malate was no longer inhibitory and the initial high respiratory rate dependent on acetoacetate oxidation remained linear. Under these conditions, malate probably competes with 2-oxoglutarate for entry into mitochondria via the 2-oxoglutarate translocator (116). Palmieri et al (118) have reported that in mitochondria, dicarboxylates competitively inhibited the uptake of 2-oxoglutarate, and

Fig. 11 Oxidation of different limiting amounts of 2-oxoglutarate and/or of acetoacetate by rat heart mitochondria.

To 1.75 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) contained 50 μM DNP and the following substrates:

Curve A: 5 mM acetoacetate.

Curve B: 0.044 mM 2-oxoglutarate + 2.5 mM Pi.

Curve C: 0.088 mM 2-oxoglutarate + 2.5 mM Pi.

Curve D: 0.176 mM 2-oxoglutarate + 2.5 mM Pi.

Curve E: 0.044 mM 2-oxoglutarate + 2.5 mM Pi + 5 mM acetoacetate.

Reaction was initiated by adding freshly isolated mitochondria (0.44 mg protein). Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria. AcAc, acetoacetate. 2-OG, 2-oxoglutarate.

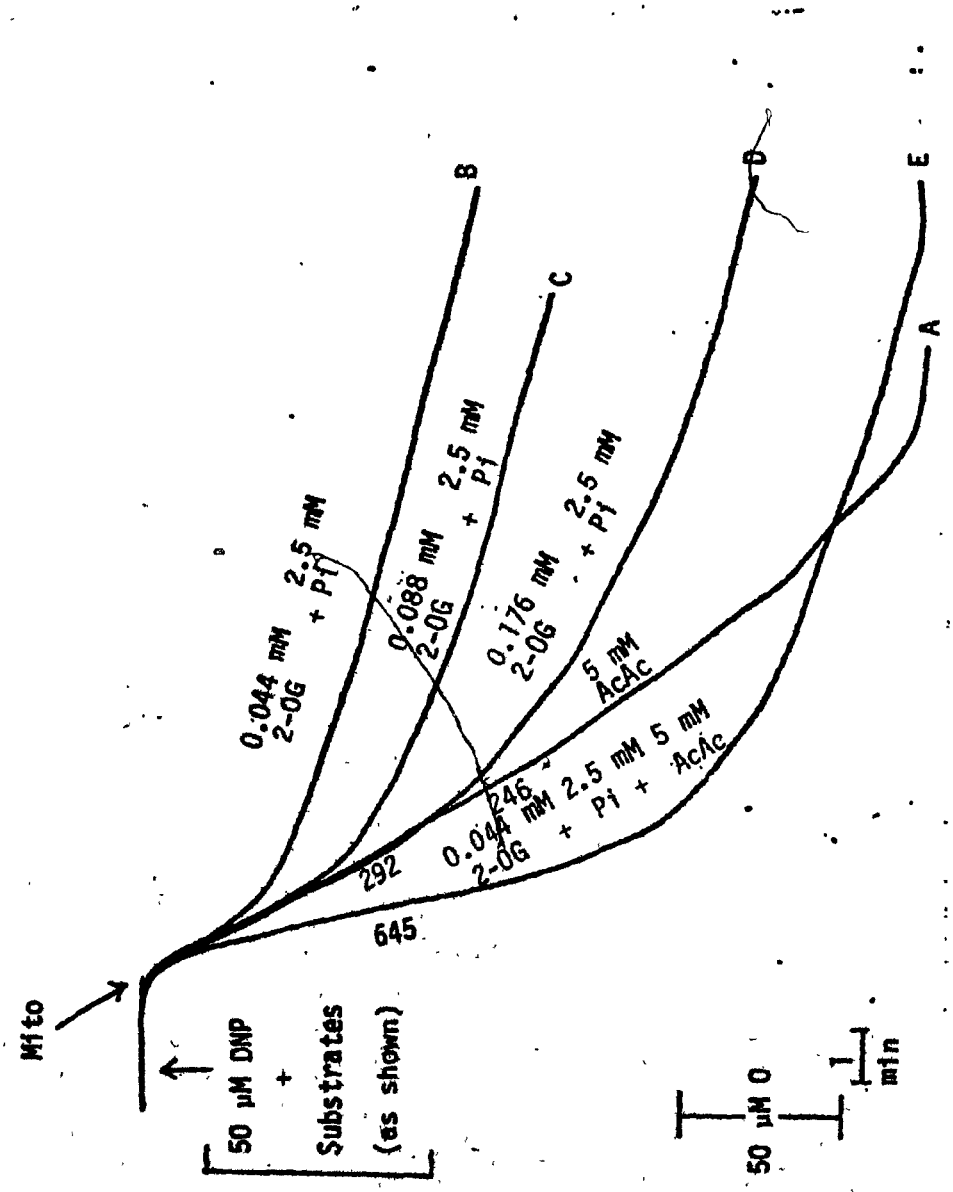

Mito
50 µM DNP
+
Substrates
(as shown)
0.044 mM 2-OG + Pi 2.5 mM
0.088 mM 2-OG + Pi 2.5 mM
0.176 mM 2-OG + Pi 2.5 mM
5 mM AcAc
0.044 mM 2-OG + Pi 2.5 mM + AcAc 5 mM
246
292
645
A
B
C
D
E
50 µM O
1 min

Fig. 11

Fig. 12 Effect of various concentrations of 2-oxoglutarate and malate on the oxygen consumption of mitochondria with acetoacetate as a substrate.

1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) contained 50 μM DNP, 10 mM Pi and the following substrates:

Curve A: 5 mM acetoacetate + 0.14 mM 2-oxoglutarate + 0.2 mM malate.

Curve B: 0.14 mM 2-oxoglutarate + 0.2 mM malate.

Curve C: 5 mM acetoacetate + 0.07 mM 2-oxoglutarate + 0.1 mM malate.

Curve D: 0.07 mM 2-oxoglutarate + 0.1 mM malate.

Curve E: 5 mM acetoacetate + 0.035 mM 2-oxoglutarate + 0.05 mM malate.

Curve F: 0.035 mM 2-oxoglutarate + 0.05 mM malate.

Reaction was initiated by adding freshly isolated mitochondria (0.75 mg protein). Numerals by the side of the tracing, the rate of the oxygen consumption in natoms per min per mg of protein. Mito, mitochondria. 2-OG, 2-oxoglutarate.

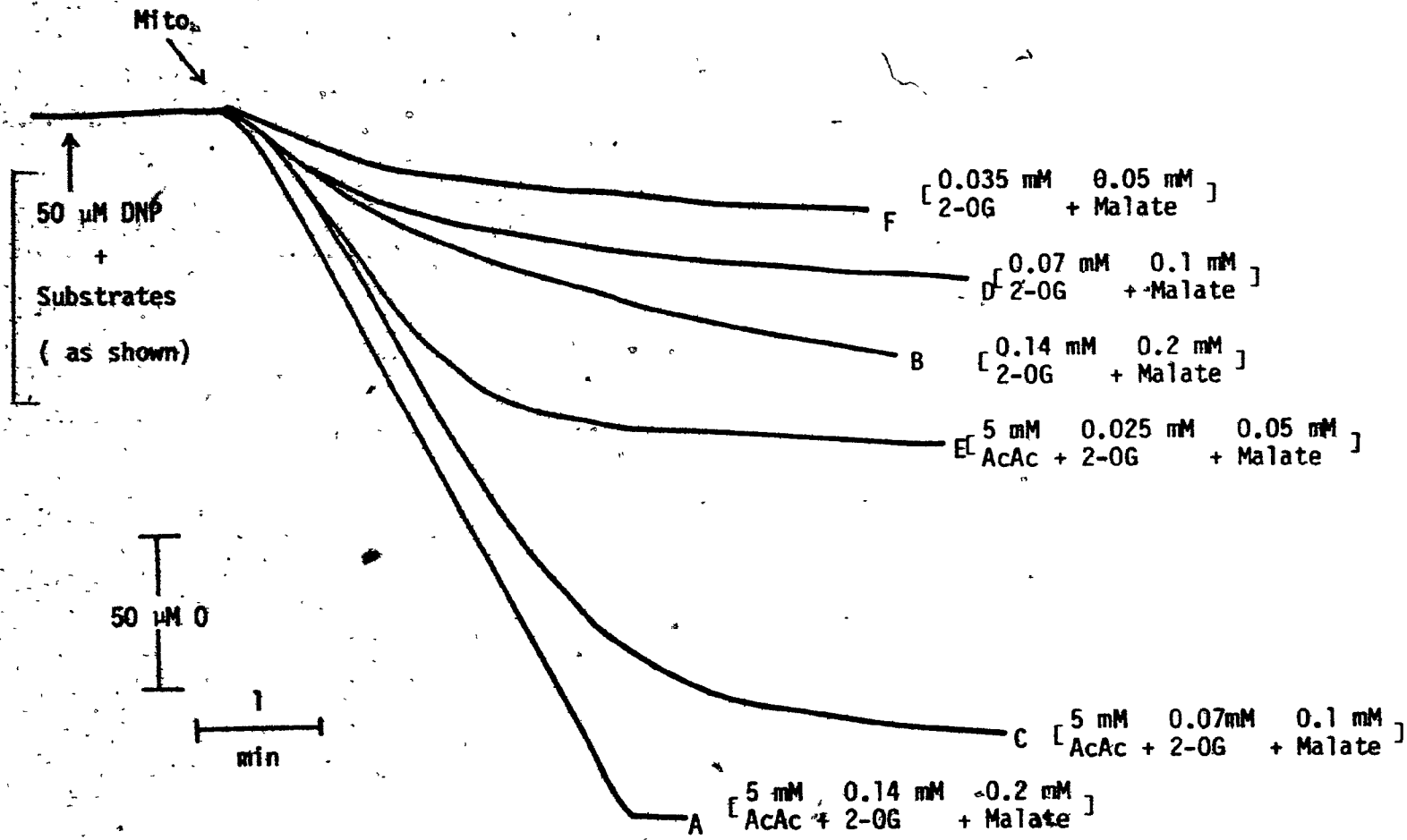

Mito.
50 µM DNP
+
Substrates
(as shown)
50 µM O
1
min
F [0.035 mM 2-OG 0.05 mM + Malate]
D [0.07 mM 2-OG 0.1 mM + Malate]
B [0.14 mM 2-OG 0.2 mM + Malate]
E [5 mM AcAc + 0.025 mM 2-OG 0.05 mM + Malate]
C [5 mM AcAc + 0.07mM 2-OG 0.1 mM + Malate]
A [5 mM AcAc + 0.14 mM 2-OG 0.2 mM + Malate]

Fig. 12

malate was found to be very effective in this regard. We also observed that the mitochondrial oxidation of 2-oxoglutarate (either in the presence of DNP plus Pi or ADP plus Pi) was inhibited by added malate. Thus when mitochondrial oxidation of acetoacetate is followed in the presence of 2-oxoglutarate and malate, the rapid oxidation of 2-oxoglutarate is prevented by malate and 2-oxoglutarate is available for longer period to support the oxidation of acetoacetate by generating succinyl-CoA. 0.14 mM 2-Oxoglutarate and 0.2 mM malate were found to be optimal to support acetoacetate dependent respiration maximally (Fig. 12, curve A).

3.2 Inhibition by Pi and by arsenate of oxidation of acetoacetate and of other substrates when respiration was followed without added sparkers of citric acid cycle.

It has been reported earlier that arsenate and Pi inhibit the oxidation of acetoacetate and this has been interpreted to be due to the diversion of succinyl-CoA from acetoacetate activation to substrate-level phosphorylation (97,98). We confirmed inhibition of acetoacetate oxidation by Pi and arsenate (Fig. 13) but found that this effect was not specific to acetoacetate oxidation alone as an inhibition of mitochondrial respiration by Pi and arsenate was also seen with other substrates such as acetoacetylcarnitine (Fig. 14) and acetylcarnitine (Fig. 15). Thus the inhibitory effect of Pi or arsenate was not specific to any one particular substrate such as acetoacetate. Moreover as described below, the effect of Pi or arsenate on acetoacetate oxidation varied according to the

Fig. 13 Inhibition of mitochondrial oxidation of acetoacetate by Pi and by arsenate in the absence of added malate and 2-oxoglutarate.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP, 5 mM acetoacetate and freshly isolated mitochondria (1.46 mg protein), a minute later, Pi (2 mM in curve B and 20 mM in curve C) and arsenate (2 mM in curve D and 20 mM in curve E) were added. Curve A lacked Pi and arsenate and served as control. Mito, mitochondria. AcAc, acetoacetate.

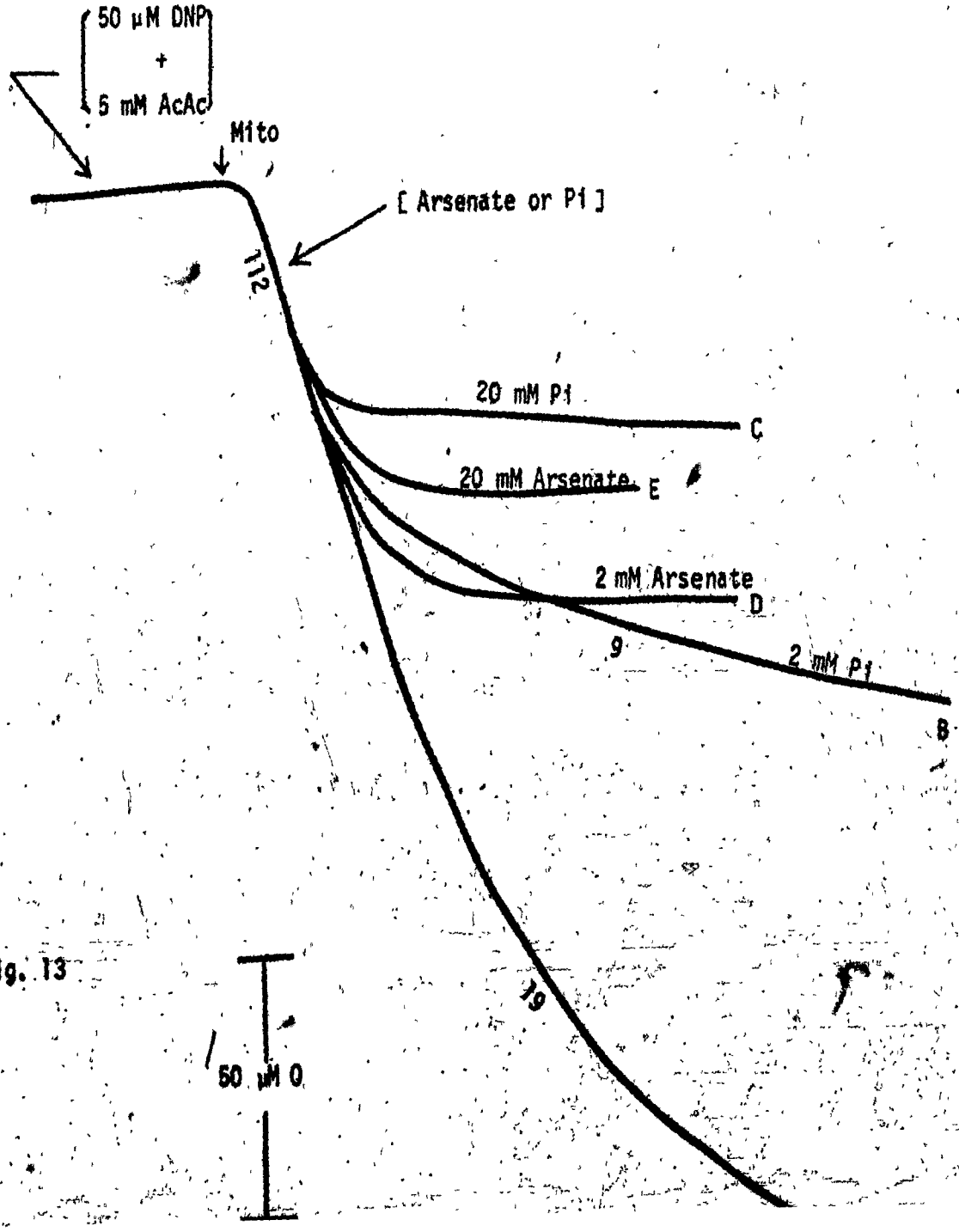

50 µM DNP
+
5 mM AcAc
Mito
[Arsenate or Pi]
112
20 mM Pi
C
20 mM Arsenate
E
2 mM Arsenate
D
9
2 mM Pi
B
19
50 µM O

Fig. 13

Fig. 14 Inhibition of mitochondrial oxidation of acetoacetylcarnitine by Pi and by arsenate in the absence of added malate.

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP, 4.5 mM acetoacetylcarnitine and freshly isolated mitochondria (1.46 mg protein), a minute later, Pi (2 mM in curve B and 20 mM in curve C) and arsenate (2 mM in curve D and 20 mM in curve E) was added. Curve A lacked Pi and arsenate and served as control. Mito, mitochondria. AcAc-C, acetoacetylcarnitine.

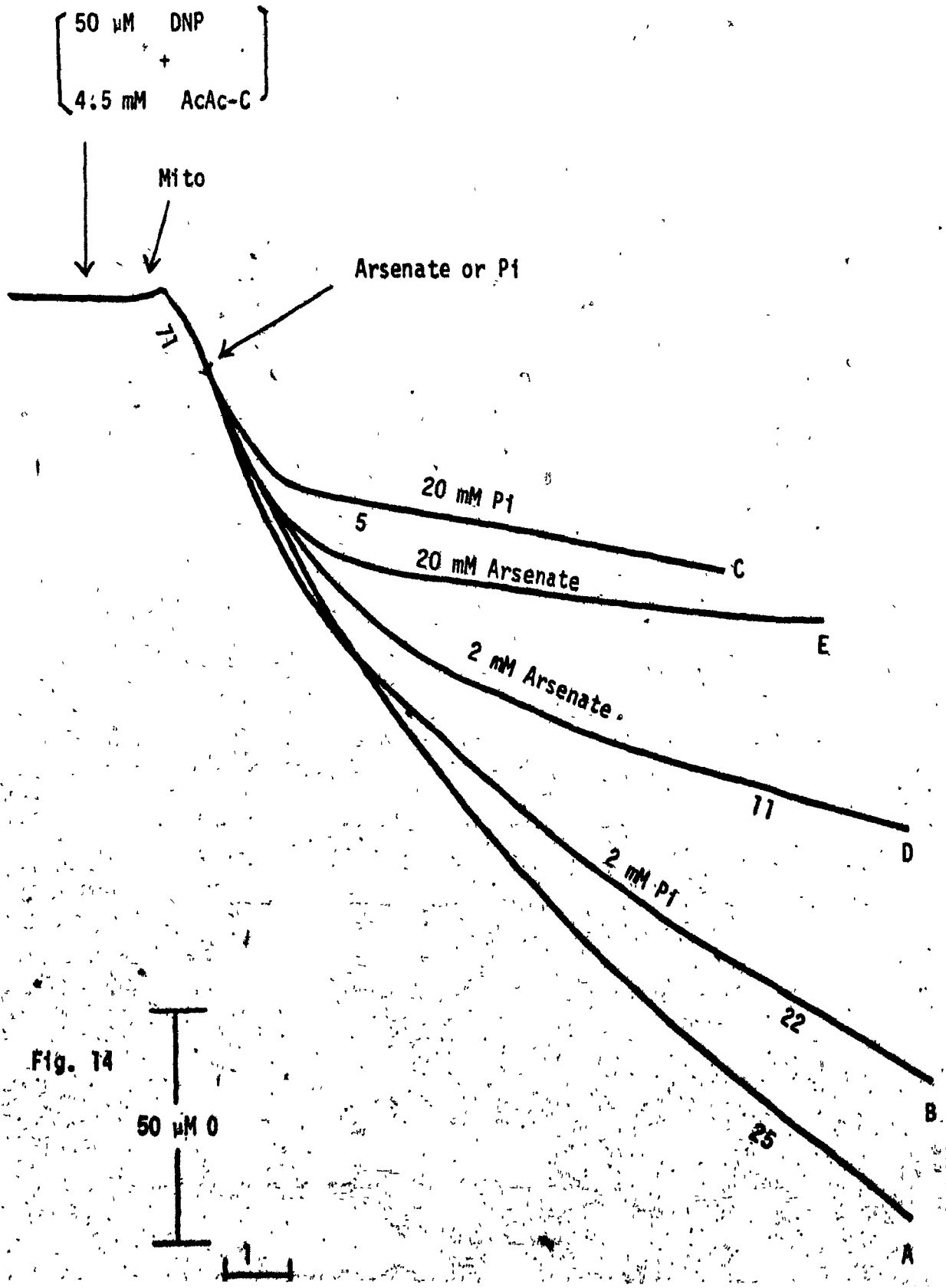

50 µM DNP
+
4.5 mM AcAc-C
Mito
Arsenate or Pi
11
20 mM Pi
5
C
20 mM Arsenate
E
2 mM Arsenate.
11
D
2 mM Pi
22
B
25
A
50 µM O
1

Fig. 14

Fig. 15 Inhibition of mitochondrial oxidation of acetylcarnitine by Pi and by arsenate in the absence of added malate.

To 1.65 ml air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP, 5 mM acetylcarnitine and freshly isolated mitochondria (1.46 mg protein), one minute later, Pi (2 mM in curve B and 20 mM in curve C) and arsenate (2 mM in curve D and 20 mM in curve E) were added. Curve A lacked Pi and arsenate and served as control. Mito, mitochondria. Acetyl-C, acetylcarnitine.

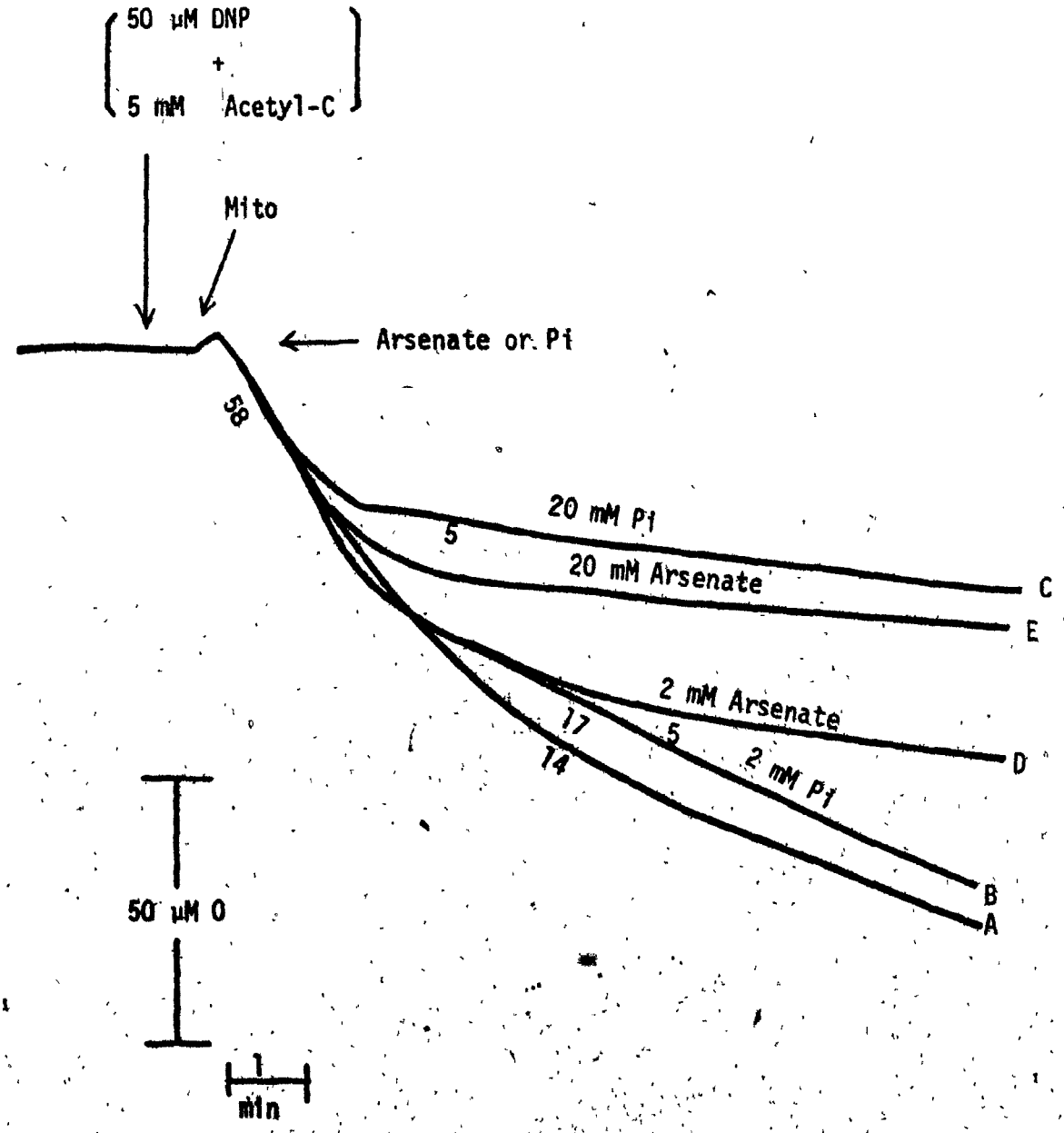

50 µM DNP
+
5 mM Acetyl-C
Mito
Arsenate or Pi
58
20 mM Pi
5
20 mM Arsenate
C
E
2 mM Arsenate
17
5
2 mM Pi
14
D
B
A
50 µM O
1
min

Fig. 15

experimental conditions. When malate and 2-oxoglutarate were not added oxidation of acetoacetate was found to be stimulated as well as inhibited by Pi. Where stimulatory effect was evident it immediately followed Pi-addition and preceded the inhibitory effect. A reciprocal relationship existed between the conditions that stimulated or inhibited the respiration. Thus, within limits, the stimulation of respiration, in magnitude and in duration was more marked (i) at lower concentrations of Pi (Fig. 16, Expt. I), (ii) at higher concentration of mitochondria (data not elaborated) and (iii) when Pi addition was made earlier in the Oxygraph experiment than added later (compare curve B and C of Expt. II of Fig. 16). Conversely, the inhibitory effect of Pi become more marked as Pi concentration was increased (Fig. 16, Expt. I), the amount of mitochondria was decreased (not shown) and the time of incubation of mitochondria at 28°C in the incubation medium (with or without DNP), preceding Pi addition was increased (Fig. 16, Expt. II).

In general, arsenate showed effects similar to those of Pi. The differences between the effect of Pi and arsenate may be summerized as follows. Whereas 5 mM Pi added to respiring mitochondria was quite inhibitory (Fig. 16, Expt. I), under similar conditions arsenate did not show any inhibitory effect (Fig. 17, Expt. I). However, when mitochondria were prior incubated for a brief period at 28°C (with or without DNP) before the addition of acetoacetate, subsequently added arsenate was more inhibitory for respiration than was Pi. (Compare curves B and C, Fig. 17). Further, under these conditions the inhibitory effectiveness of arsenate was minimized, but not reversed, by the simultaneous inclusion of Pi (Fig. 17, Expt. II). This is in contrast to an earlier report (98). With arsenate, unlike that with Pi, the inhibitory effect was found to also depend on the order of addition of components. When

Fig. 16 The effect of the order of addition of Pi on the mitochondrial oxidation of acetoacetate when followed in the absence of added 2-oxoglutarate and malate.

To 1.62 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added freshly isolated mitochondria (0.74 and 0.38 mg protein in Expt. I and II respectively). Other additions were made as shown. The two experiments were performed on different days. Numerals by the side of the tracing, oxygen consumption in natoms per min per mg of protein. Mito, mitochondria.

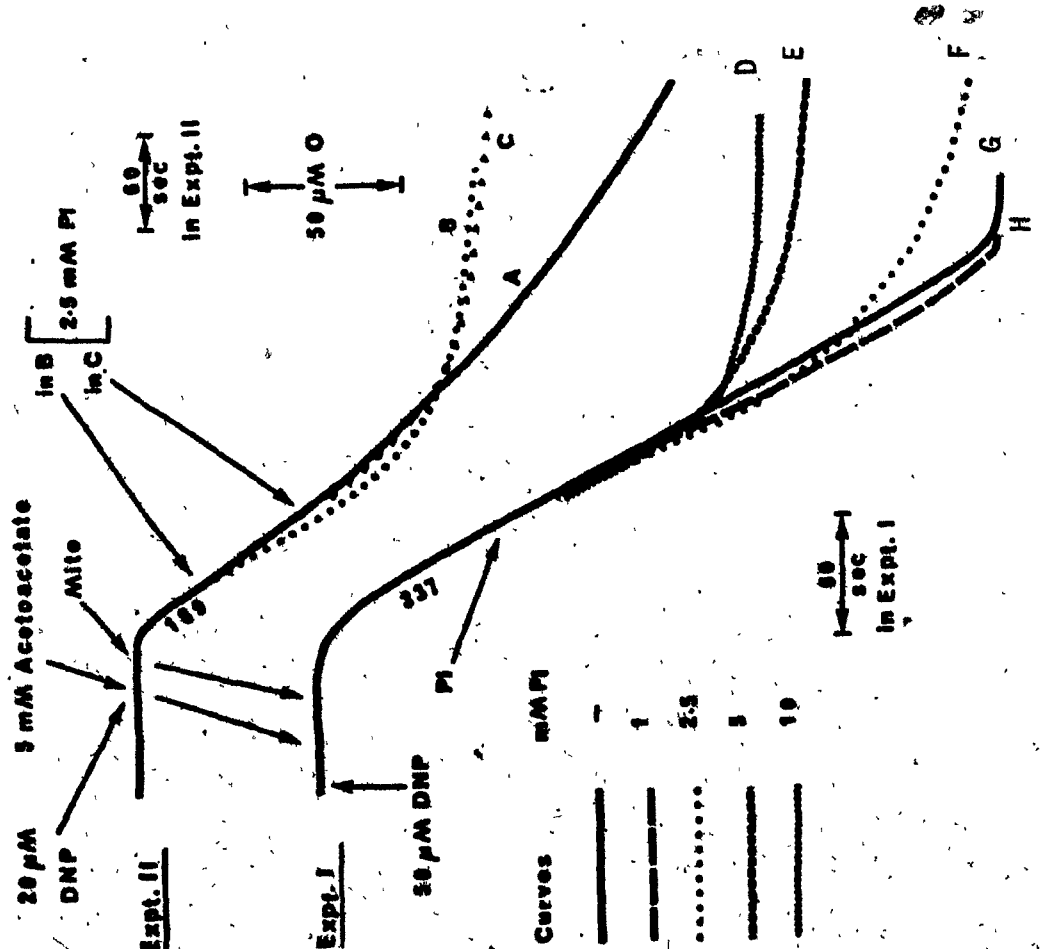

20 μM DNP
5 mM Acetoacetate
Mito
Expt. II
Expt. I
30 μM DNP
Pi
Curves
mM Pi
1
2.5
5
10
In B
2.5 mM Pi
In C
60 sec In Expt. II
50 μM O
60 sec In Expt. I
A
B
C
D
E
F
G
H

Fig. 16

Fig. 17 The effect of the order of addition of arsenate and its modification by Pi on the mitochondrial oxidation of acetoacetate when followed in the absence of added 2-oxoglutarate and malate.

To 1.62 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added freshly isolated mitochondria (0.74 mg protein). Other additions were as shown. Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria.

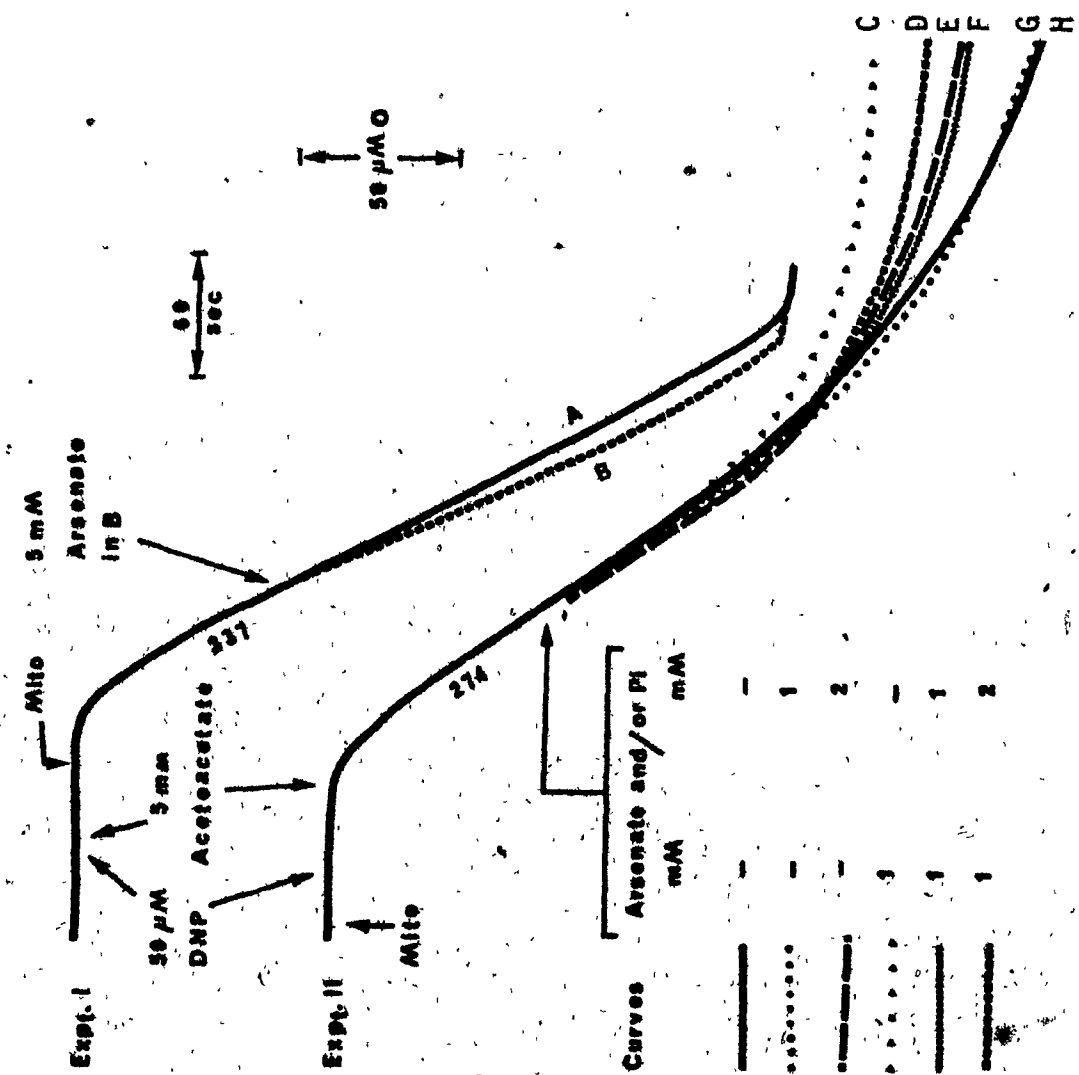

50 µM O
50 Sec
A
B
C
D
E
F
G
H
5 mM Arsenate in B
Mito
5 mM Acetoacetate
50 µM DNP
Expt. I
Expt. II
Mito
237
274
Curves
Arsenate and/or Pi mM
– – 1 2 – 1 2
– – – 1 1 1

Fig. 17

arsenate was added to mitochondria respiring with acetoacetate, only a partial inhibition of oxygen-consumption was seen. But when arseanate was added prior to the addition of mitochondria and acetoacetate, respiration was found to be fully inhibited. It could be that in the former experiment the respiration seen after the addition of arsenate resulted from the oxidation of that acetoacetyl-CoA which was formed prior to the addition of arseante; in the latter experiment, however, where a prior incubation of mitochondria with arsenate proceded aceto-acetate addition, little succinyl-CoA would be expected to remain available for acetoacetyl-CoA formation and it was perhaps because of this that the oxidation of acetoacetate was severely inhibited.

3.3 Stimulation by Pi and by arsenate of oxidation of acetoacetate and of other substrates when respiration was followed in the presence of added sparkers of the citric acid cyle.

In agreement with the earlier reports (97,98) we found that arsenate and Pi inhibit acetoacetate oxidation (Fig. 13,16 and 17). The inhibitory effect of Pi or arsenate was evident only when sparkers of the citric cycle malate and 2-oxoglutarate were not added, and was not specific for acetoacetate oxidation as oxidation of other substrates was also inhibited. On the other hand, when sparkers of the citric acid cycle were present, Pi or arsenate stimulated rather than inhbiting the oxidation of acetoacetate as well as of other substrates (as shown below).

The rate of DNP-stimulated respiration with acetoacetate in the presence of 2-oxoglutarate and malate was markedly stimulated by up to 20 mM of either Pi or of arsenate and no inhibition was observed. Under these conditions, the same concentration of Pi or arsenate which inhibited the oxidation of acetoacetate in the absence of sparkers, stimulated the acetoacetate dependent respiration in the presence of sparkers of citric acid cycle (Table 1). The optimal concentration of Pi for stimulation varied between 1 to 2.5 mM but no inhibition was noticeable even at 20 mM Pi.

The stimulatory effect of Pi and of arsenate was not restricted to the oxidation of acetoacetate as a similar effect was seen on the DNP-stimulated

TABLE 1

To 1.65 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP, 0.14 mM 2-oxoglutarate and 0.2 mM malate. 5 mM acetoacetate was added in experiments (i) to (v) only. Reaction was initiated by the addition of freshly isolated mitochondria (0.47 mg protein), Pi or arsenate was added a minute later and the respiration was followed for more than four minutes, during which period, the respiratory rates remained linear. Experiments (i) and (vi) lacked Pi and arsenate and served as controls. The acetoacetate-stimulated oxygen consumption, i.e. the natoms of oxygen consumed due to the mitochondrial oxidation of acetoacetate alone, has been calculated by subtracting the natoms of oxygen consumed due to the oxidation of 2-OG + malate from the natoms of oxygen consumed due to the oxidation of 2-OG + malate + AcAc.

2-OG, 2-oxoglutarate, AcAc, acetoacetate.

* % increase compared to the rates observed with 2-oxoglutarate, malate and acetoacetate alone [Expt.](i) .

** % increase compared to the rates observed with 2-oxoglutarate, malate alone [Expt.](vi) .

arsenate was added to mitochondria respiring with acetoacetate, only a partial inhibition of oxygen-consumption was seen. But when arseanate was added prior to the addition of mitochondria and acetoacetate, respiration was found to be fully inhibited. It could be that in the former experiment the respiration seen after the addition of arsenate resulted from the oxidation of that acetoacetyl-CoA which was formed prior to the addition of arseante; in the latter experiment, however, where a prior incubation of mitochondria with arsenate proceded aceto-acetate addition, little succinyl-CoA would be expected to remain available for acetoacetyl-CoA formation and it was perhaps because of this that the oxidation of acetoacetate was severely inhibited.

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The rate of DNP-stimulated respiration with acetoacetate in the presence of 2-oxoglutarate and malate was markedly stimulated by up to 20 mM of either Pi or of arsenate and no inhibition was observed. Under these conditions, the same concentration of Pi or arsenate which inhibited the oxidation of acetoacetate in the absence of sparkers, stimulated the acetoacetate dependent respiration in the presence of sparkers of citric acid cycle (Table 1). The optimal concentration of Pi for stimulation varied between 1 to 2.5 mM but no inhibition was noticeable even at 20 mM Pi.

The stimulatory effect of Pi and of arsenate was not restricted to the oxidation of acetoacetate as a similar effect was seen on the DNP-stimulated

TABLE 1 P_i and arsenate stimulation of the mitochondrial oxygen consumption in the presence of acetoacetate, 2-oxoglutarate, and malate

Expt.	Substrates added	P_i mM	Arsenate mM	natoms oxygen consumed in 4 mins	% increase in oxygen consumption	AcAc-stimulated oxygen consumption	
						as natoms of oxygen consumed	as % increase in oxygen consumption
(i)	2-OG + Malate + AcAc	-	-	663	-	-	-
(ii)	2-OG + Malate + AcAc	2	-	843	27*	-	-
(iii)	2-OG + Malate + AcAc	20	-	805	21	-	-
(iv)	2-OG + Malate + AcAc	-	2	744	12	-	-
(v)	2-OG + Malate + AcAc	-	20	714	8	-	-
(vi)	2-OG + Malate	-	-	93	-	-	-
(vii)	2-OG + Malate	2	-	154	65**	-	-
(viii)	2-OG + Malate	20	-	104	12	-	-
(ix)	2-OG + Malate	-	2	118	20	-	-
(x)	2-OG + Malate	-	20	95	2	-	-
	(i) - (vi)	-	-	-	-	570	-
	(ii) - (vii)	2	-	-	-	689	21
	(iii) - (viii)	20	-	-	-	701	23
	(iv) - (ix)	-	2	-	-	626	10
	(v) - (x)	-	20	-	-	619	11

oxidation of various other substrates such as pyruvate, acetylcarnitine, acetoacetylcarnitine, palmitylcarnitine (in the presence of malate) and succinate (in the presence of amytal (Table 2). In as much as effects of Pi on the oxidation of acetoacetate with rabbit heart mitochondria (data not shown) were similar to those described above for rat heart mitochondria, the observed effects of Pi are not specific to mitochondria of rat heart.

3.4 Effect of ADP on the mitochondrial oxidation of acetoacetate

Hatefi and Fakouhi (98) have reported that mitochondrial oxidation of acetoacetate was inhibited by ADP especially in the presence of Pi. In our experiments oxidation of acetoacetate in the presence of malate (0.2 mM) and 2-oxoglutarate (0.14 mM) was found to be as rapid with ADP + Pi as with DNP + Pi (Fig. 18, compare curves A and B). Moreover, the oxidation of acetoacetate was found well coupled to phosphorylation (Fig. 19); the ADP/O values with acetoacetate (2.86 ± 0.067*) as substrate were similar ($p > 0.1$) to those seen in parallel experiments with pyruvate plus malate (2.97 ± 0.063**) and the respiratory control ratios with the two substrates were also alike.

It is known that when mitochondria are uncoupled the respiration proceeds normally or may even be stimulated, but no coupled respiratory phosphorylation of ADP to ATP takes place. In line with this it was observed that the respiratory rates were higher when mitochondrial oxidation of acetoacetate was followed in the presence of an uncoupler than in the presence of ADP and Pi. (compare curve A and B, of Fig. 18 and Expt Ia and Expt IIa of Table 3). This was true not only with acetoacetate, but also with other

* Values are mean (of 11 experiments) ± SE.

** Values are mean (of 13 experiments) ± SE.

TABLE 2

To 1.7 ml (in Expt. I-III) or 1.65 ml (in Expt. IV and V) of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 50 μM DNP and various substrates as shown. Reaction was initiated by the addition of freshly isolated mitochondria (0.59 mg protein in Expt. I-III and 0.73 mg protein in Expt. IV and V); Pi or arsenate were added a minute latter and the respiration was followed for five more minutes.

TABLE 2 Stimulation by Pi and by arsenate of the mitochondrial oxidation of different substrates in the presence of 0.2 mM malate and of succinate in the presence of 1.8 mM amytal

Expt.	Substrates	Pi mM	Arsenate mM	natoms oxygen consumed in 5 mins	% increase in oxygen consumption
I.	Pyruvate	-	-	296	-
	(0.5 mM)	2	-	378	28
		20	-	366	24
		-	2	379	28
		-	20	430	45
II.	Acetylcarnitine	-	-	520	-
	(5.0 mM)	2	-	834	60
		20	-	766	47
		-	2	588	13
		-	20	636	22
III.	Acetoacetylcarnitine	-	-	484	-
	(4.5 mM)	2	-	748	55
		20	-	676	40
		-	2	558	15
		-	20	610	26

Table 2 continued

TABLE 2 (cont'd)

Expt.	Substrates	Pi mM	Arsenate mM	natoms oxygen consumed in 5 mins	% increase in oxygen consumption
IV.	Palmitylcarnitine	-	-	94	-
	(30 M)	2	-	148	57
		20	-	162	72
		-	2	134	43
		-	20	193	105
V.	Succinate + Amytal	-	-	129	-
	(5 mM) (1.8 mM)	2	-	328	154
		20	-	214	66
		-	2	286	122
		-	20	352	173

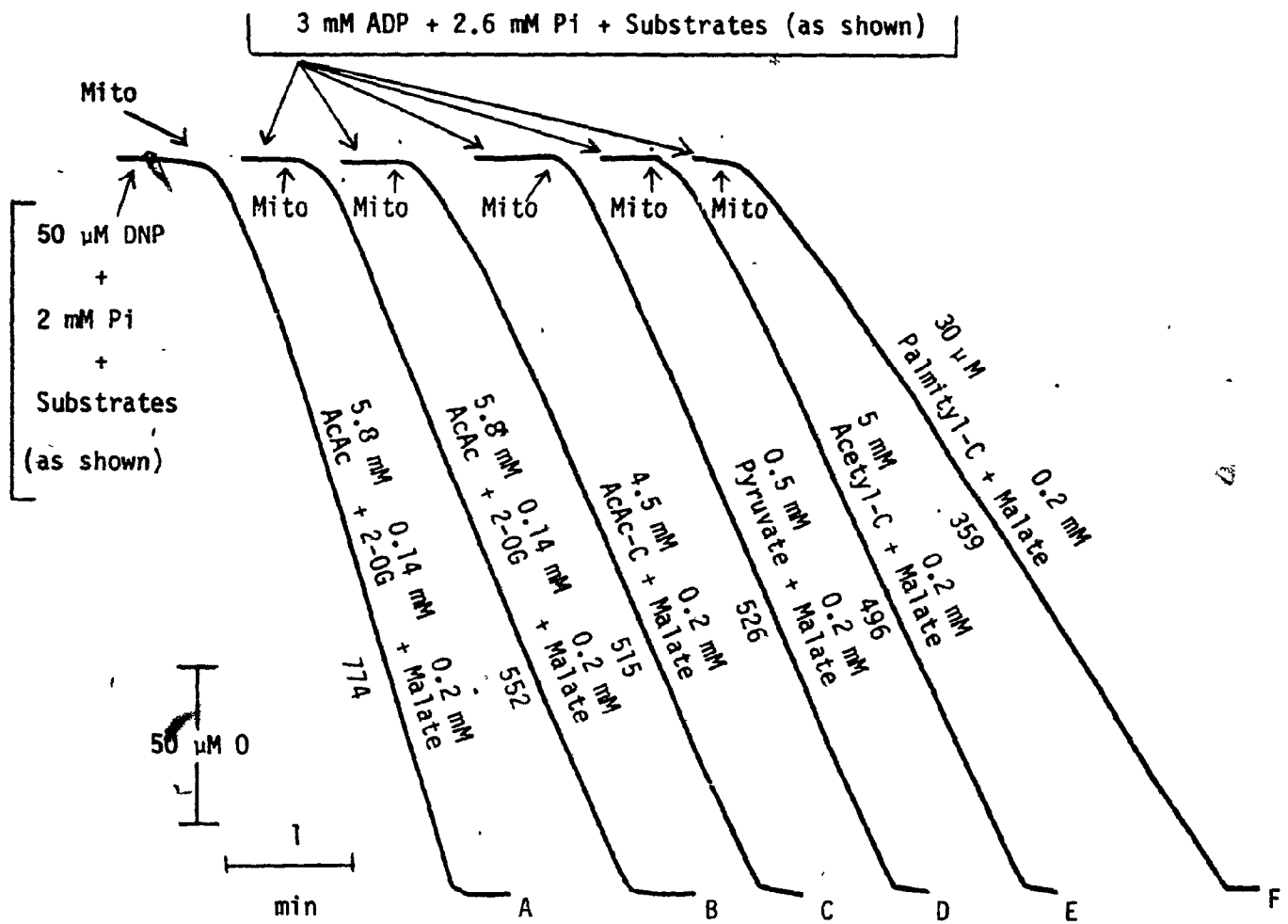

3 mM ADP + 2.6 mM Pi + Substrates (as shown)
Mito
Mito
Mito
Mito
Mito
Mito
50 µM DNP
+
2 mM Pi
+
Substrates
(as shown)
5.8 mM AcAc + 0.14 mM 2-OG + 0.2 mM Malate
774
5.8 mM AcAc + 0.14 mM 2-OG + 0.2 mM Malate
552
4.5 mM AcAc-C + 0.2 mM Malate
515
0.5 mM Pyruvate + 0.2 mM Malate
526
5 mM Acetyl-C + 0.2 mM Malate
496
30 µM Palmityl-C + 0.2 mM Malate
359
50 µM O
1
min
A
B
C
D
E
F

Fig. 18

Fig. 19 Stimulation of acetoacetate oxidation by ADP.

To 1.62 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added freshly isolated mitochondria (0.62 mg protein). Other additions were made as shown. Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria.

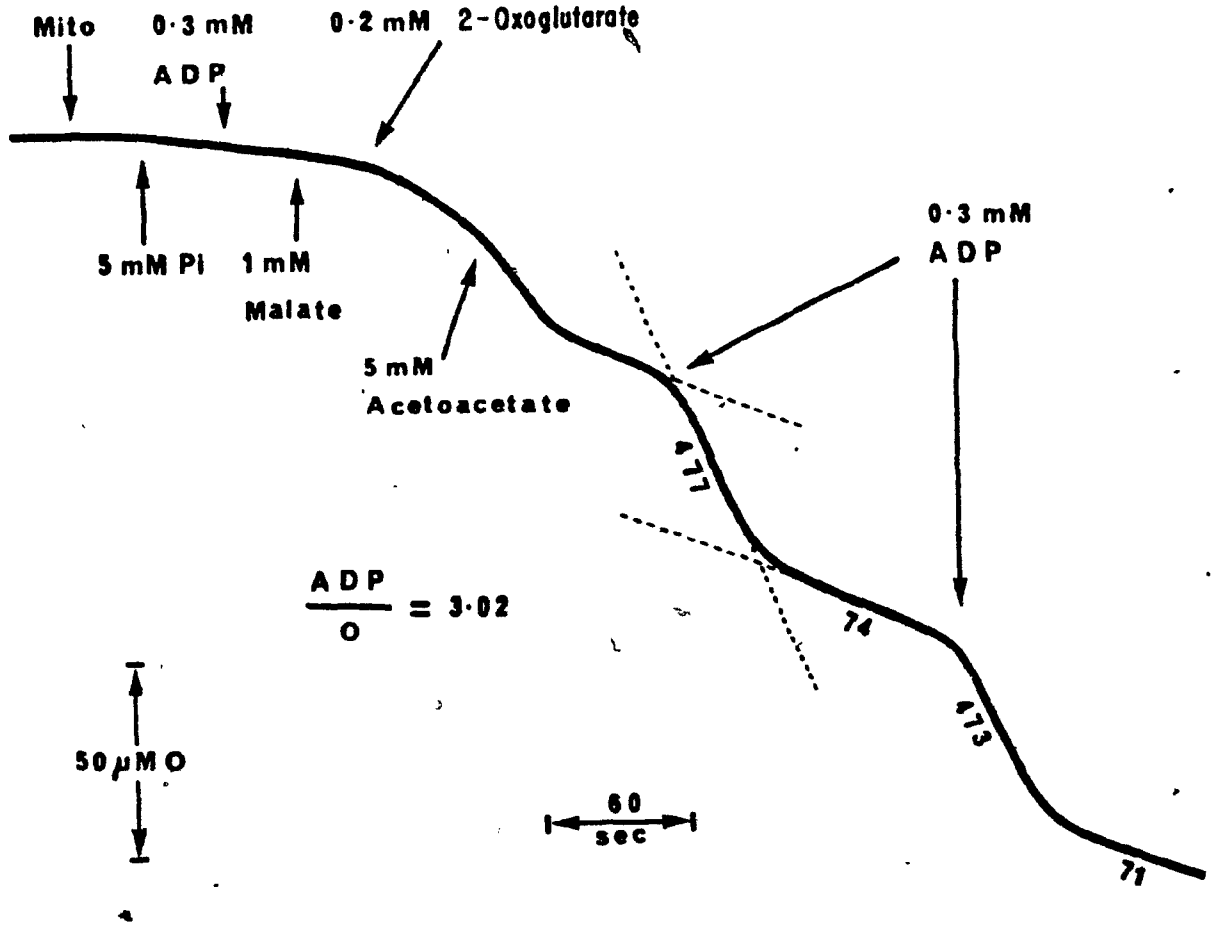

Mito
0·3 mM
ADP
0·2 mM 2-Oxoglutarate
5 mM Pi
1 mM
Malate
5 mM
Acetoacetate
0·3 mM
ADP
ADP / O = 3·02
77
74
73
71
50 µM O
60
sec

Fig. 19

substrates like pyruvate when FCCP was the uncoupler. For some unknown reason(s) it was not true with the uncoupler DNP. With FCCP and Pi, the rate of pyruvate oxidation was always found to be higher than with ADP plus Pi (Table 3, Expt Ib and Expt. IIb). It is believed that ADP in the presence of Pi inhibits acetoacetate oxidation because succinyl-CoA is directed towards substrate-level phosphorylation and hence the succinyl-CoA linked activation of acetoacetate is impaired (98). If this view is correct then in the presence of ADP and Pi mitochondrial oxidation of acetoacetylcarnitine should give higher respiratory rate than that of acetoacetate, but as can be seen from Fig. 18, it is not so. In the presence of ADP and Pi, mitochondria respired as rapidly with acetoacetate as with acetoacetylcarnitine or pyruvate (Fig. 18, curves B, C, and D). This was true not only with rat heart mitochondria but also with rabbit heart mitochondria (data not shown). Thus under these conditions the results suggest that ADP in the presence of Pi does not inhibit acetoacetate oxidation.

Based on the measurement of oxygen consumption the results described in Table 1 show that in the presence of sparkers of citric acid cycle Pi stimulated the mitochondrial oxygen consumption dependent on acetoacetate oxidation. These results, however, do not unequivocally show that in the presence of sparkers, Pi stimulates the mitochondrial oxidation of acetoacetate as oxidation of sparkers is also stimulated by Pi. This can only be shown conclusively by measurement of the disappearance of acetoacetate under appropriate conditons. Thus when Pi stimulated acetoacetate-dependent respiration more acetoacetate should disappear from the incubation medium. Therefore attempts were made to measure the disappearance of acetoacetate

TABLE 3

To 1.65 ml air saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added various substrates as shown, 2.5 mM Pi and either ADP (2 mM), DNP (50 μM) or FCCP (1 μM). Reaction was initiated by the addition of freshly isolated mitochondria (0.72 mg protein from rat heart in Expt. I or 0.87 mg protein from rabbit heart in Expt. II).

TABLE 3 Comparison of respiratory rates of mitochondria with different substrates in the presence of Pi and either ADP, DNP or FCCP

	SUBSTRATES	Respiratory rates natoms oxygen/min per mg		
		ADP (2 mM)	DNP (50 μM)	FCCP (1 μM)
Expt. I	(Rat heart mitochondria)			
(a)	Acetoacetate + 2-oxoglutarate + Malate (5 mM) (0.14 mM) (0.2 mM)	486	574	593
(b)	Pyruvate (5 mM) + Malate (0.2 mM)	500	214	666
Expt. II	(Rabbit heart mitochondria)			
(a)	Acetoacetate + 2-oxoglutarate + Malate (5 mM) (0.14 mM) (0.2 mM)	419	440	-
(b)	Pyruvate (5 mM) + Malate (0.2 mM)	406	264	-

under the conditions as described in Table 1. But the results obtained were variable. In order to get significant data, the assay volume was reduced to 40 µl and the incubation time was increased to 30 minutes. The Walker's method (106,107) was replaced by a more sensitive enzymatic method (105) to estimate acetoacetate. But inspite of these changes, the results obtained were not conclusive. It was then realized that measurement of the disappearance of substrate could not be a sensitive and accurate method because of the following considerations.

The oxygen content at 28°C is 472 natoms oxygen/ml (103) or 850 natoms O_2/1.8 ml incubation medium (the maximum capacity of the oxygraph chamber used is 1.8 ml). Since eight moles of oxygen are consumed per mole of acetoacetate oxidized, therefore 106 (850 ÷ 8) nmoles of acetoacetate can be maximally oxidized. The optimum concentration of acetoacetate is 5 mM and hence the amount of acetoacetate present is 9,000 nmoles. Therefore only 1.17% acetoacetate can disappear. If Pi causes 50% stimulation of acetoacetate oxidation, then only 53 nmoles acetoacetate (0.58%) would be oxidized in the absence of Pi. Hence measurement of the disappearance of a few nmoles out of thousands i.e. a difference of only 0.6%, proved to be an inaccurate and insensitive method.

It was therefore considered to use radioactive acetoacetate so that the formation of the product $^{14}CO_2$, instead of the disappearance of substrate, could be accurately measured. It was expected that, under the conditions as described in Table 1, more $^{14}CO_2$ should evolve due to the mitochondrial oxidation of [^{14}C] acetoacetate in the presence of Pi than in

its absence. But only negligible amount of $^{14}CO_2$ was evolved due to the oxidation of [$^{14}C_3$] acetoacetate, despite the fact that under identical conditions acetoacetate greatly stimulated mitochondrial oxygen consumption. That the above observation was not specific with this particular batch of [$^{14}C_3$] acetoacetate was evident from the fact that similar results were obtained with [$^{14}C_4$] acetoacetate obtained from another supplier. In order to check the methodology and conditions used to collect $^{14}CO_2$ from the oxidation of [$^{14}C_3$]-or-[$^{14}C_4$] acetoacetate, [$^{14}C_2$] pyruvate was used as the substrate. Experiments with pyruvate also showed that while pyruvate addition to a mitochondrial incubation system containing malate resulted in a large increase in oxygen consumption, the conversion of [$^{14}C_2$] pyruvate to $^{14}CO_2$ was only a small fraction of that expected from the stimulation of oxygen consumption. Therefore a systematic study of mitochondrial oxidation of differentially labeled pyruvate was undertaken as shown below.

3.5 Effect of malate on the conversion of differentially labeled [^{14}C] pyruvate to $^{14}CO_2$ and on the stoichiometry of pyruvate-dependent oxygen consumption.

Table 4 shows the results of an experiment in which oxidation of differentially labeled pyruvate to $^{14}CO_2$ was followed without (Expt. A) or with added malate (Expt. B). Pyruvate oxidation was also followed (Expt. C) in the presence of malonate and carnitine. Malonate was added to block the citric acid cycle and carnitine was added to allow regeneration of CoASH from acetyl-CoA so that the oxidation of pyruvate to the level of acetyl group could proceed (119). Results of Expt. B showed that when pyruvate alone

TABLE 4

The incubation system in a final volume of 180 µl contained: 0.23 M mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 µM EDTA, 2.0 mM ADP, 2.5 mM potassium phosphate, 14.3 nmoles of [^{14}C]pyruvate (115,000 to 193,000 cpm) and freshly isolated mitochondria, 52 µg protein, to start the reactions. In addition, where shown, 1 mM L-malate or 1 mM malonate plus 13 mM (-) carnitine were present. Final pH was 7.2, temperature 28°C. After 7 minutes, reactions were stopped by the addition of methanol to a final concentration of 50% (v/v). The radioactivity recovered in CO_2 was measured as described under methods.

TABLE 4 Effect of malate or malonate on the oxidation of differentially labeled [^{14}C] pyruvate to $^{14}CO_2$

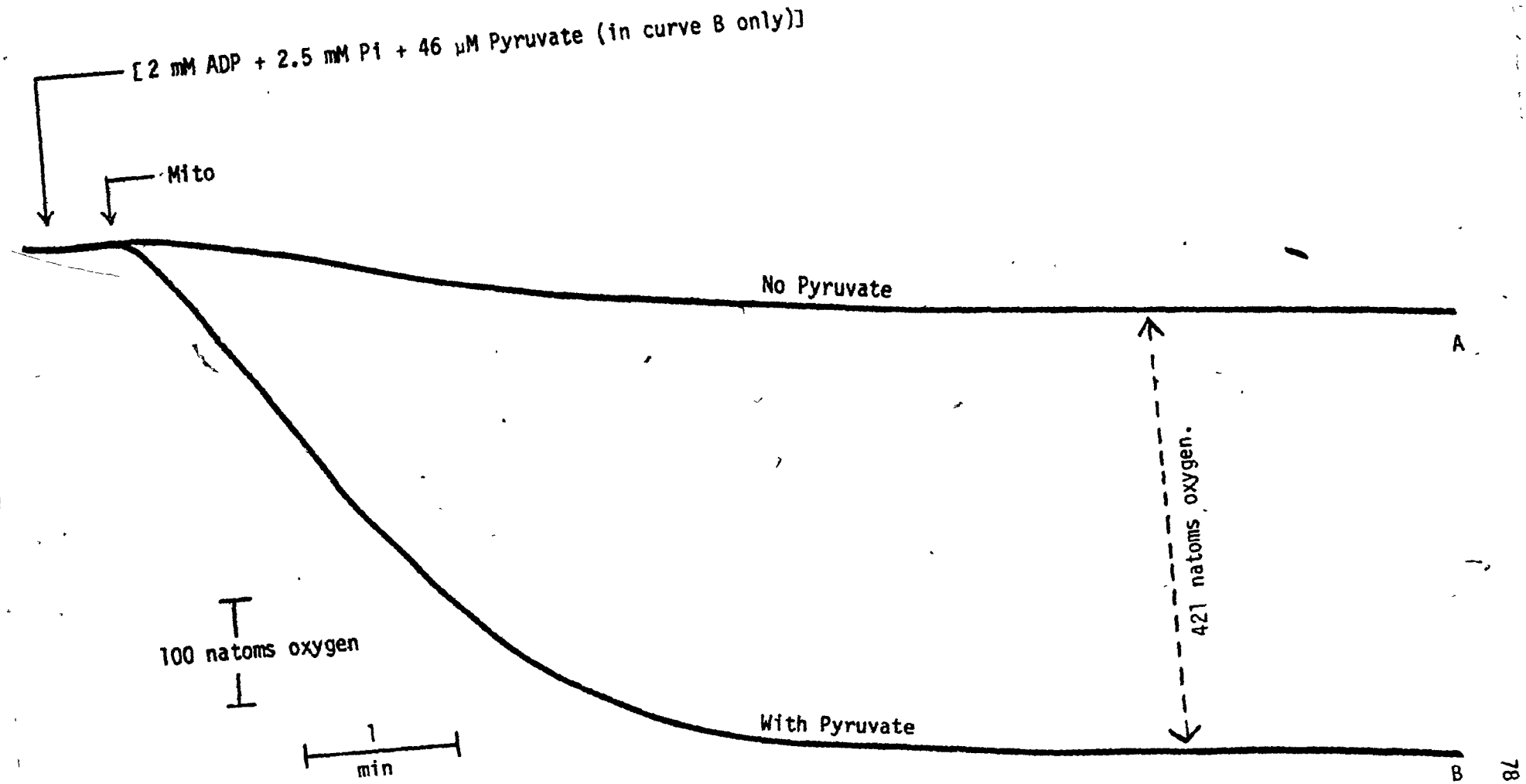
Line no.	Expt. no.	Radioactive pyruvate	Other additions	Radioactivity recovered in Co_2 (as % of added counts)
1.		[$^{14}C_1$] pyruvate	malate	75.3
2.	A	[$^{14}C_2$] pyruvate	malate	2.9
3.		[U-^{14}C] pyruvate	malate	23.2
4.		[$^{14}C_1$] pyruvate	-	68.7
5.	B	[$^{14}C_2$] pyruvate	-	72.1
6.		[U-^{14}C] pyruvate	-	69.2
7.		[$^{14}C_1$] pyruvate	malonate + carnitine	75.7
8.	C	[$^{14}C_2$] pyruvate	malonate + carnitine	2.1
9.		[U-^{14}C] pyruvate	malonate + carnitine	22.8

was incubated with mitochondria, the conversion of carbon atoms 1, 2 and 3 of pyruvate to CO_2 proceeded to about equal extent. This is to be expected if acetyl group derived from pyruvate dehydrogenase reaction were further being completely oxidized to CO_2. Results of Expt. C showed that as expected malonate had little effect on the oxidation of pyruvate to acetyl-CoA as shown by the lack of malonate effect on the conversion of [$^{14}C_1$] pyruvate to $^{14}CO_2$ (Table 4 compare lines 7 and 4) but the oxidation of carbon atom 2 of [$^{14}C_2$] pyruvate was greatly inhibited (compare lines 8 and 5). If malonate inhibits the oxidation of acetyl-CoA to CO_2 without affecting the conversion of pyruvate to acetyl-CoA, then in the presence of malonate (and carnitine) 1/3rd as much $^{14}CO_2$ should be produced from the oxidation of [U-^{14}C] pyruvate as that seen in the absence of malonate. Results of Expt. C showed that this was indeed so (compare lines 9 and 6). Hence, as expected malonate completely inhibited the operation of citric acid cycle. Results of Expt. A showed that while malate did not affect the oxidation of pyruvate to acetyl-CoA (compare lines 1 and 4) so far as the subsequent oxidation of acetyl-CoA derived from pyruvate (i.e. of carbon atoms 2 and 3 of pyruvate) was concerned, malate was as strongly inhibitory as was malonate (compare results of Expt. A, B and C). These results were unexpected because malate, a "sparker" of citric acid cycle, is known to enhance the oxidation of substances requiring the operation of the citric acid cycle (120-122).

An understanding of this anamolous behaviour of malate was obtained in the subsequent experiments in which it was realized, in agreement with the findings of Van Korff (123), that heart mitochondria exhibited a high affinity for pyruvate so that by offering a limiting amount of pyruvate, pyruvate-dependent cycle of respiration could be followed which permitted the determination of ΔO (atom)/Δ pyruvate (mole)as shown in Figs. 20-22. The ΔO/Δ pyruvate ratios were determined by measuring the mitochondrial oxygen consumption dependent on pyruvate oxidation either alone (Fig. 20) in the presence of malonate (Fig. 21) or malate (Fig. 22). These experiments showed that ΔO/Δ pyruvate approached 5 (4.84 ± 0.198, mean S.E.M. of 5 experiments with different preparation of mitochondria) when pyruvate alone was offered to mitochondria (Fig. 20). Because ΔO/Δ pyruvate of 5 is to be expected in theory for the oxidation of pyruvate to CO_2, under these conditions of Fig. 20 the acetyl-CoA groups derived from pyruvate were being nearly completely oxidized to CO_2. This is essentially in agreement with the results of radioactive experiments (Expt. B) of Table 4. Fig. 21 shows that when malonate was present the ratio ΔO/Δ pyruvate approached 1 (0.97 ± 0.038 mean ± S.E.M. of 3 separate experiments with different preparation of mitochondria). This is to be expected if malonate blocked the operation of citric acid cycle completely so that under these conditions, pyruvate oxidation could only proceed as far as acetyl-CoA. Fig. 22 shows that when malate was present, the ΔO/Δ pyruvate was 3.6 ± 0.18 (Mean ± S.E.M. of separate experiments with different preparation of mitochondria). The value 3.6 ± 0.18 is significantly different ($p < 0.01$) from the value of 4.84 observed without malate. ΔO/Δ pyruvate ratios in the above experiment were similar irrespective of whether coupled mitochondria with

Fig. 20 $\Delta O/\Delta$ pyruvate ratio during mitochondrial oxidation of pyruvate.

To 1.8 ml air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 2 mM ADP, 2.5 mM Pi and 46 μM pyruvate. Reaction was initiated by the addition of freshly isolated mitochondria (0.52 mg protein). Mito, mitochondria.



78.

ΔO/Δ pyruvate = 5.06

Fig. 20

Fig. 21 Effect of malonate plus carnitine on ΔO/Δ pyruvate ratio

To 1.8 ml air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 2 mM ADP, 2.5 mM Pi, 1 mM malonate, 13 mM L(-) carnitine and 0.185 mM pyruvate. Reaction was initiated by the addition of freshly isolated mitochondria (0.48 mg protein). Mito, mitochondria.

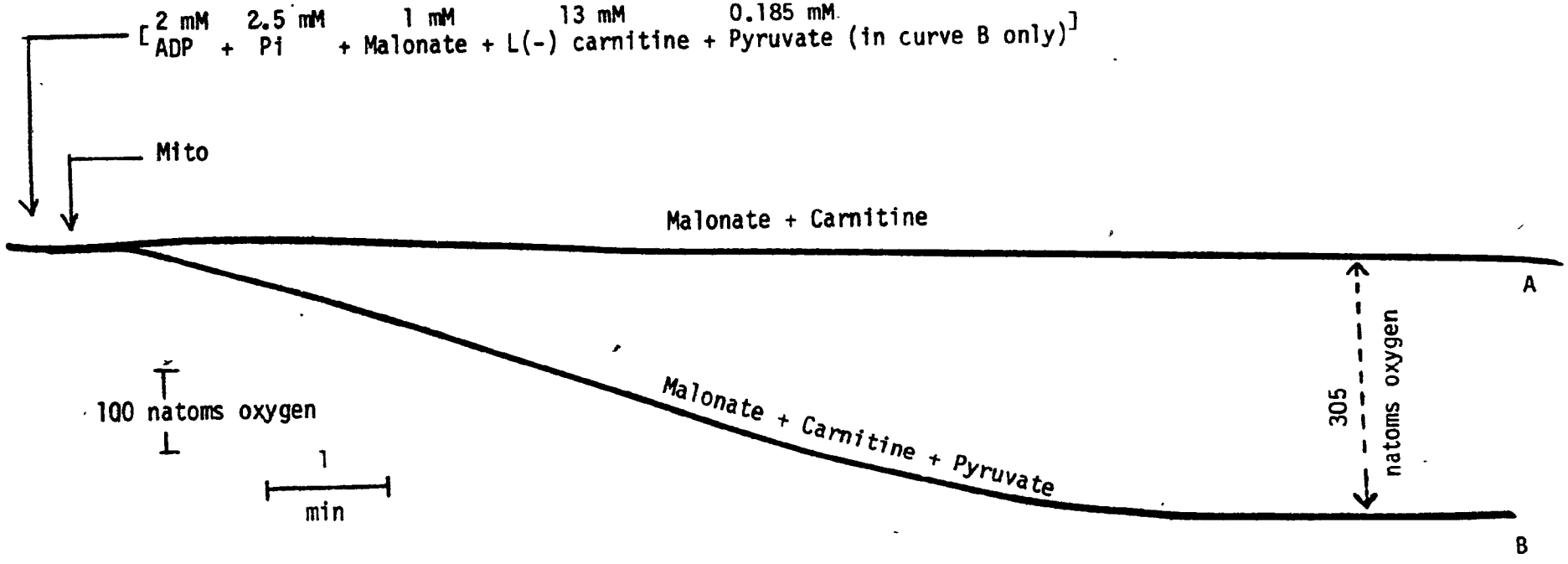

[2 mM ADP + 2.5 mM Pi + 1 mM Malonate + 13 mM L(-) carnitine + 0.185 mM Pyruvate (in curve B only)]
Mito
Malonate + Carnitine
A
Malonate + Carnitine + Pyruvate
B
305 natoms oxygen
100 natoms oxygen
1 min
ΔO/Δ pyruvate = 1.09

Fig. 21

Fig. 22 Effect of malate on $\Delta O/\Delta$ pyruvate ratio.

To 1.8 ml air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added 2 mM ADP, 2.5 mM Pi, 1 mM malate and 46 μM pyruvate. Reaction was initiated by the addition of freshly isolated mitochondria (0.48 mg protein). Mito, mitochondria.

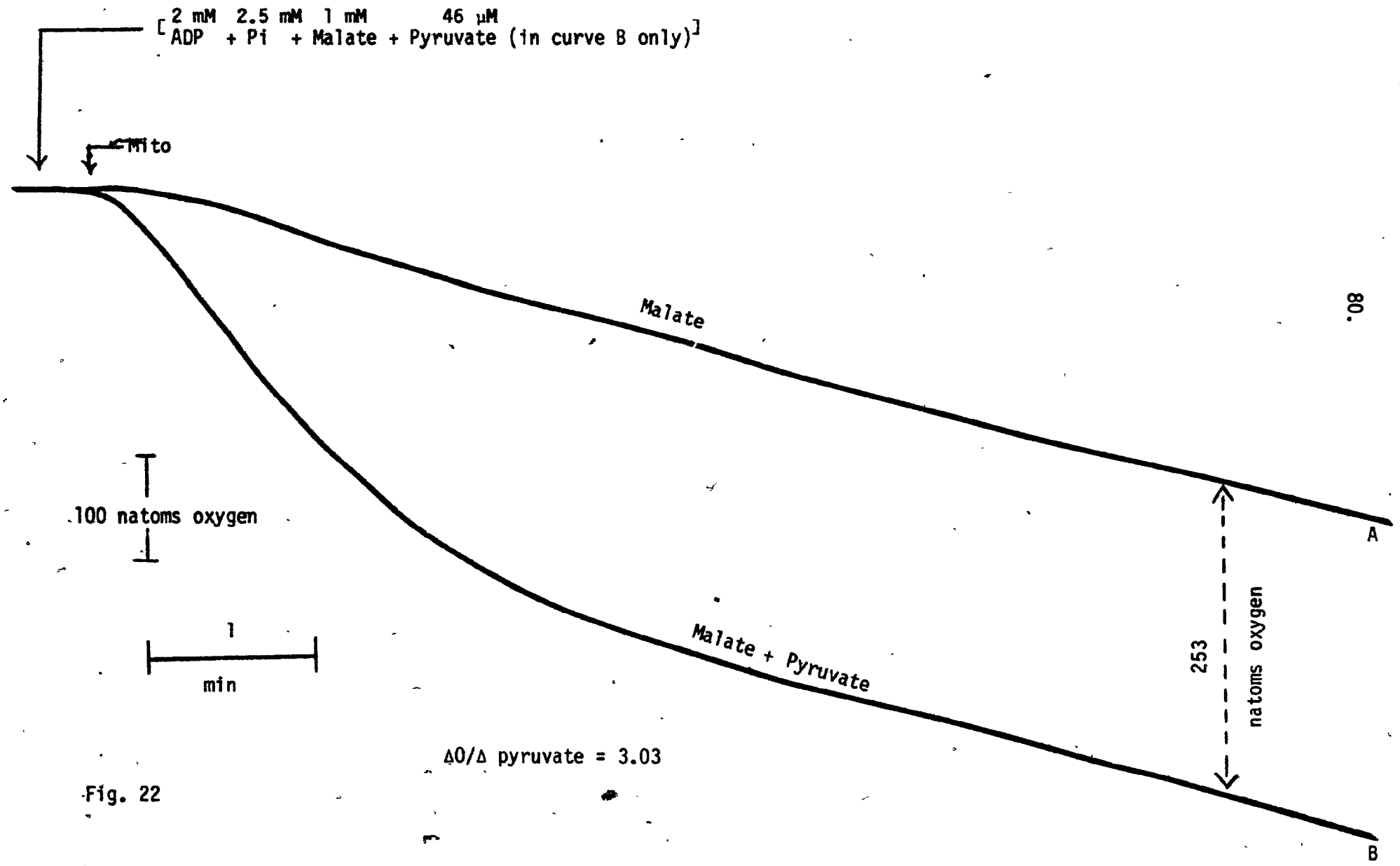

2 mM ADP + 2.5 mM Pi + 1 mM Malate + 46 µM Pyruvate (in curve B only)
Mito
Malate
Malate + Pyruvate
A
B
100 natoms oxygen
1
min
253
natoms oxygen
ΔO/Δ pyruvate = 3.03

Fig. 22

ADP ± Pi or mitochondria uncoupled with DNP ± Pi were used. Thus results of Fig. 22 where malate was present, showed that oxidation of at least part of the pyruvate was proceeding well beyond the formation of acetyl-CoA. A ration of ΔO/Δ pyruvate of greater than 1 but less than 5 could result if part of the pyruvate was being oxidized completely to CO_2 while the remainder was being oxidized only partially. However, this was not the case in the present experiments because as experiments of Table 4 showed conversion of the carbon atoms 2 and 3 of pyruvate to CO_2 was no greater with malate as compared to that seen with malonate.

The above results could be explained if there was an exchange of added malate with the intramitochondrial anions of the intermediates of citric acid cycle. Existence of such anion exchanger in heart mitochondria is known in which an entry of malate into mitochondria simultaneously leads to the exit of another permeant intramitochondrial anion such as succinate, 2-oxoglutarate or citrate (124). If the entry of malate were coupled to the exit of succinate, for example, the ΔO/Δ pyruvate would be 4.0. However, the experimentally determined values of ΔO/Δ pyruvate with malate present averaged 3.6 suggesting that besides succinate other anions like 2-oxoglutarate and citrate were also exchanging with malate. Direct measurements in one such experiment showed that while some accumulation of 2-oxoglutarate occured during the oxidation of malate alone, its amount was increased by the additional presence of pyruvate. This extra formation of 2-oxoglutarate, however, corresponded to only 14.4% of that expected from the amount of pyruvate that disappeared. Thus these results suggest that malate interrups the cyclic operation of the citric acid cycle because of the efflux of mitochondrially generated acids of the citric acid cycle in exchange for the entry of added malate.

3.6 Acetoacetyl-CoA synthetase activity in rat heart preparation

Although ATP-dependent activation of acetoacetate has been implicated in the oxidation of acetoacetate, as described earlier, there was no evidence in our experiments that such was the case. Whether an ATP-dependent activating enzyme for acetoacetate existed at all has been a subject of controversy till recently (20,77). Recently Stern (73) and Buckley and Williamson (75) using indirect assay procedure were able to demonstrate acetoacetyl-CoA synthetase activity in rat liver and brain cytosol respectively.

Having failed to find any evidence for the involvement of an ATP-dependent activatory mechanism in the oxidative utilization of acetoacetate, we wished to ascertain whether a direct ATP-linked activation of acetoacetate at all proceeded in rat heart. Therefore attempts were made to measure acetoacetyl-CoA synthetase activity in heart homogenates by direct assay procedure.

$$\text{Acetoacetate} + \text{ATP} + \text{CoASH} \xleftrightarrow[\text{Synthetase}]{\text{Acetoacetyl-CoA}} \text{Acetoacetyl-CoA} + \text{AMP} + \text{PPi}$$

This direct assay was based on the measurement of the pH-dependent ultraviolet absorption of acetoacetyl-CoA at 303 nm (~~124~~, 125). But under the conditions employed, no acetoacetyl-CoA could be estimated. Estimation of acetoacetyl-CoA formed by coupling to L-3-hydroxyacyl-CoA dehydrogenase catalyzed oxidation of NADH were also unsuccessful because despite 1 mM potassium cyanide and amytal present to inhibit the electron transport chain, the rate of NADH

oxidation in the absence of acetoacetate was so high that the condition were not appropriate for detecting relatively much slower activation of acetoacetate. The high and variable control absorbance associated with the use of homogenates further limited the amount of tissue preparation that could be used in such a procedure. Assay procedures, based on hydroxamate formation are quite sucessful for following activation of short and long chain fatty acids (126) but such a procedure was found not to be applicable for assay of acetoacetyl-CoA synthetase. This was because when acetoacetyl-CoA reacts with neutral hydroxylamine the reaction product is 3-methyl-5-isoxazolone and not a hydroxamate (127). Assays based on the formation of AMP and PPi (assuming these to be the products of acetoacetyl-CoA synthetase reactions) were unsuitable because of the presence of active enzyme in rat heart that rapidly metabolize AMP and PPi. Assays based on the disappearance of acetoacetate, ATP, and CoASH were found impractical because the activity of acetoacetyl-CoA synthetase in preparation of rat heart was not high enough to cause any measurable change in the saturating concentrations of acetoacetate, ATP or CoASH necessary during assay. Besides with ATP and CoASH presence of very active endogenous enzymes metabolizing ATP and CoASH rendered the value of such approach questionable. Measurement of acetoacetate-dependent CoASH disappearance could not have been perfect anyway because under the conditions of the assay with CoASH present a variable utilization of CoASH for acetoacetyl-CoA thiolase reaction would also have preceeded.

Both Stern (73) and Buckley and Williamson (75) have followed acetoacetyl-CoA formation by coupling acetoacetate activation to citrate formation. We found that using such an approach it was possible to qualitatively pick up ATP and CoASH dependent activation of acetoacetate.

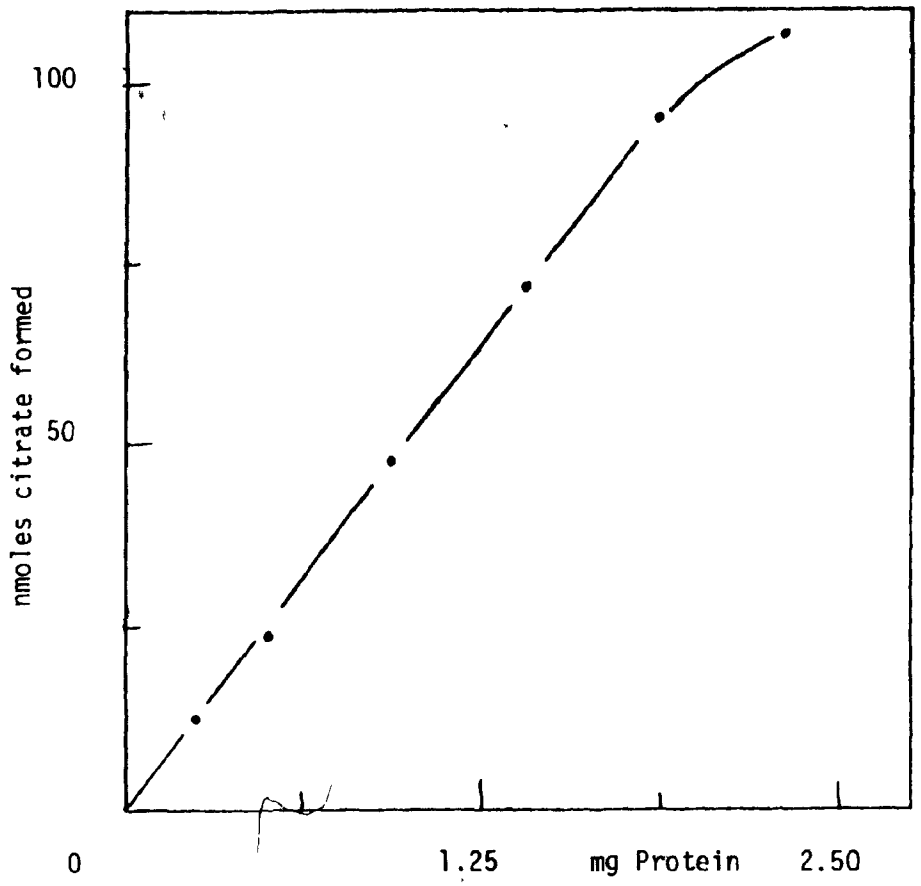
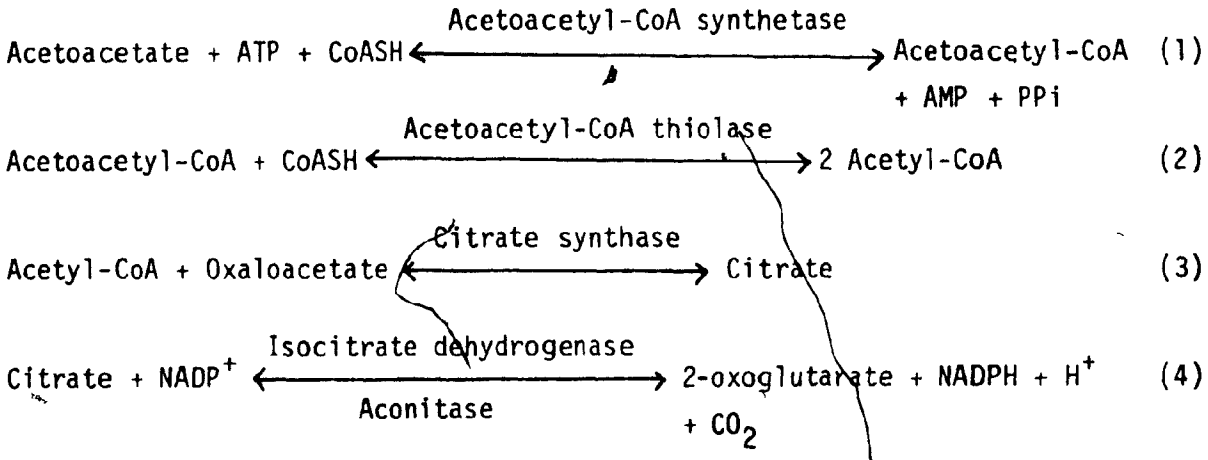


Fig. 22 A. Linearity of acetoacetyl-CoA synthetase activity of rat-heart homogenate as a function of amount of protein. The reaction medium consisted of 60 mM Tris-Cl (pH 7.4), 4 mM $MgCl_2$, 5 mM DTT, 1 mM oxaloacetate, 0.25 mM CoASH, 15 mM ATP, 2 units of citrate synthetase, 10 µg rotenone, 10 µg oligomycin and 30 mM acetoacetate. The reaction was started by the addition of freshly prepared heart homogenate which was first dialyzed and then sonicated as described under Methods. Total assay volume was 500 µl. Following incubation for 30 minutes at 37°C the reaction was terminated by adding 75 µl of 70% perchloric acid. The tubes were chilled, the contents neutralized to pH 7.4 with NaOH and then centrifuged. Citrate was determined on the supernatant as described under Methods. The values shown are the mean of triplicate analyses, correction for controls lacking ATP and CoASH was applied.

Following reaction sequences were involved in the assay procedure employed.



The citrate formed was estimated by following an increase in absorbance at 340 nm. Under the conditions employed. The rate of citrate formation increased linearly with the amount of homogenate (Fig. 22A).

Under optimally determined conditions, the highest activity of acetoacetyl-CoA synthetase was found to be present in mitochondria, whereas very little activity was found to be present in supernatant (post mitochondrial fraction). (Table 5). It was not certain whether the low cytoplasmic activity was due to the leakage of the mitochondrial enzyme or because of a possible limitation of acetoacetyl-CoA thiolase activity in the supernatant. The activity of the acetoacetyl-CoA synthetase in heart homogenates was found to decrease on storage and retained about half of its activity after one week and one-fourth after two weeks when stored at -20^{o}C.

An effect of acetoacetate concentration on activation showed that the enzyme activity rose as the concentration of acetoacetate was increased up to 240 mM (Table 5). This lack of saturation of enzyme activity by up to 240 mM acetoacetate was also seen with rat kidney homogenates (Table 6).

TABLE 5

Acetoacetyl-CoA synthetase activity was measured by coupling acetoacetate activation to citrate formation (73). The reaction medium consisted of 60 mM Tris-Cl (pH 7.4), 4 mM $MgCl_2$, 5 mM DTT, 1 mM oxaloacetate, 0.25 mM CoASH, 15 mM ATP, 2 units of citrate synthase, 10 µg rotenone, 10 µg oligomycin and various concentrations of acetoacetate. The reactions was started by the addition of heart preparation which were first dialyzed and then sonicated (details under methods). Total assay volume was 500 µl. The reaction was terminated by adding 75 µl 70% perchloric acid following incubation for 30 minutes at 37°C. The tubes were chilled and then centrifuged. Citrate was determined on the supernatant as described under methods. The values are the mean of three readings corrected for controls lacking ATP and CoASH.

TABLE 5 Acetoacetyl-CoA synthetase activity in rat heart preparation

Preparation	AcAc mM	Specific activity
		nmoles citrate formed/mg per hr
Expt. I (with fresh preparation)		
Homogenate	30	117
Mitochondria	30	108
Supernatant (post mitochondrial fraction)	30	12
Expt. II (with 1 week old preparation)		
Homogenate	.0.50	6
	1.28	8
	2.50	12
	5.0	18
	15.0	37
	30.0	51
Expt. III (with 2 week old preparation)		
Homogenate	15	12
	30	26
	60	36
	90	42
	120	50
	240	59

Thus based on indirect assay procedures, it was found that acetoacetyl-CoA synthetase is present in rat heart and kidney and that the saturation concentration of acetoacetate seems to be several fold higher than the physiological concentration present normally in body fluid.

3.7 Activation of D(-) and L(+)-3-hydroxybutyrate by rat heart.

It has been reported that both D and L-3-hydroxybutyrate are activated by rat liver and kidney (30). But there is no information available about the ability of rat heart to activate DL-3-hydroxybutyrate. Therefore it was of interest to know if the same could be true in rat heart. Using assay procedure based on hydroxamate formation (126) it was possible to detect activation of DL-3-hydroxybutyrate in rat heart homogenates. Optimum assay conditions with respect to the concentration of NH_2OH, ATP, DTT and CoASH were determined as shown in Table 6 and 7. Under the optimal assay conditions, inclusion of potassium cyanide and potasium fluoride to inhibit the electron transport chain and pyrophosphatase activity respectively, were found unnecessary. However, it was observed that when DTT was replaced by cysteine neutralized with Tris, the final color yield due to the formation of ferric hydroxamate was markedly decreased in the presence of KF (5 mM and above).

Under optimally determined conditions it was observed that DL-3-hydroxybutyrate was poorly activated by heart homogenate. An effect of DL-3-hydroxybutyrate concentration showed that the enzyme activity kept on increasing as the concentration of DL-3-hydroxybutyrate was increased up to 256 mM. (Table 7, Expt. I). Because extremely high concentration of

TABLE 6

Acetoacetyl-CoA synthetase activity was measured by coupling acetoacetate activation to citrate formation (73). The reaction medium consisted of 60 mM Tris-Cl (pH 7.4), 4 mM $MgCl_2$, 5 mM DTT, 1 mM oxaloacetate, 0.25 mM CoASH, 15 mM ATP, 2 units of citrate synthase, 10 µg rotenone, 10 µg oligomycin and various concentrations of acetoacetate. The reaction was started by the addition of kidney homogenate. The homogenate was first dialyzed and then sonicated (details as under methods). Total assay volume was 500 µl. The reaction was terminated by adding 75 µl 70% perchloric acid following incubation for 30 minutes at $37^{o}C$. The tubes were chilled and then centrifuged. Citrate was determined on the supernatant as described under methods. The values are the mean of three readings corrected for controls lacking ATP and CoASH.

TABLE 6

Acetoacetyl-CoA synthetase activity in rat kidney homogenate

(prepared fresh)

Acetoacetate	Specific activity
mM	nmoles citrate formed/mg per hr
0.25	17
2.50	23
15.00	27
30.00	33
60.00	40
120.00	42
240.00	44

TABLE 7

The reaction mixture consisted of 500 mM NH_2OH (pH 7.0), 100 mM Tris-Cl (pH 7.0), 4 mg $MgCl_2$, 7.5 mM ATP, 0.5 mM CoASH, 5 mM DTT, 0.1% Triton X-100 (to dissociate the structurally bound enzyme), 16 mM butyrate or/and various concentration of DL-3-hydroxybutyrate. The reaction was started by the addition of dialyzed heart homogenate (0.45 mg protein in Expt. I and 0.225 mg protein in Expt. II). Total assay volume was 250 µl. Following incubation for 60 minutes at 37°C, the reaction was terminated by adding 20 µl of 32% $FeCl_3$ in 60% perchloric acid (final pH ≤ 1.0). The tubes were chilled and centrifuged. The hydroxamate formed was estimated in the supernatant colorimetrically by measuring the absorbance at 540 nm (126). The values are the mean of three readings corrected for controls lacking ATP and CoASH.

TABLE 7

Activation of butyrate and DL-3-hydroxybutyrate by homogenates of rat heart

Expt.	Butyrate mM	DL-3-hydroxybutyrate mM	Hydroxamate formed nmoles/mg per hr
I	-	8	65
	-	16	92
	-	32	203
	-	64	272
	-	128	408
	-	256	511
	-	512	133
II	16	-	799
	-	16	104
	-	32	149
	-	64	239
	16	16	743
	16	32	716
	16	64	686

DL-3-hydroxybutyrate saturated the activating enzyme, we considered the possibility that this substrate was being activated non-specifically by the medium chain fatty acid activating enzyme-butyryl-CoA synthetase. If DL-3-hydroxybutyrate is activated by the same enzyme which activates butyrate then it is to be expected that the activation of butyrate would be inhibited by the simultaneous presence of DL-3-hydroxybutyrate. The results indicated (Expt. II of Table 7) that the activation of the butyrate was progressively inhibited with increasing concentration of DL-3-hydroxybutyrate.

In order to check if both D(-) and L(+)-3-hydroxybutyrate are activated, determination of D(-) and L(+)-3-hydroxybutyryl-CoA are required. Assay procedure based on the formation of hydroxamate was used to estimate both isomers of 3-hydroxybutyrate (30,126). L-3-hydroxybutyryl-CoA was estimated enzymatically by coupling to the formation of acetoacetyl-CoA according to the following equation (128).

$$\text{L-3-hydroxybutyryl-CoA} + \text{NAD}^+ \xleftrightarrow[\text{-dehydrogenase (EC 1.1.1.35)}]{\text{L-3-hydroxyacyl-CoA}} \text{Acetoacetyl-CoA} + \text{NADH} + \text{H}^+$$

The data of Table 8 show that the L-3-hydroxybutyryl-CoA formed was exactly half of the total amount of hydroxamate formed showing that both D(-) and L(+) isomers of 3-hydroxybutyrate are activated by homogenates of rat heart.

TABLE 8

The incubation system contained 100 mM Tris-Cl pH 7.4, 4 mM $MgCl_2$, 0.1% Triton X-100, 7.5 mM ATP, 5 mM DTT, 0.5 mM CoASH, 256 mM DL-3-hydroxybutyrate, and various amounts of dialyzed heart homogenate. Total assay volume was 250 µl. Time of incubation was 30 mins at 37°C. The reaction was stopped by adding 60 µl of 60% perchloric acid. The tubes were chilled, neutralized to pH 7.0 and then centrifuged.

Estimation of DL-3-hydroxybutyryl-CoA:

To 125 µl of the neutral supernatant obtained above was added 45 µl of 2 M NH_2OH (pH 7.0). Following incubation for ≥ 10 minutes at room temperature, 20 µl of 32% of $FeCl_3$ in 60% perchloric acid was added (final pH ≤ 1.0). The hydroxamate formed was estimated colorimetrically at 540 nm (126).

Estimation of L(+)-3-hydroxybutyryl-CoA:

To a cuvett with 10 mm light path was added 90 µl 0.5 M Tris-Cl pH 9.5, 5 µl 0.1 M EDTA, 10 µl 0.01 M NAD^+, 40 µl H_2O and 5 µl of L(+)-3-hydroxyacyl-CoA dehydrogenase. The reaction was initiated by the addition of 100 µl of the neutral supernatant. (Total volume 250 µl) and an increase in absorbance at 340 nm due to the formation of acetoacetyl-CoA was recorded using Gilson spectrophotometer equipped with recorder (128). The values are the mean of three readings corrected for controls lacking ATP and CoASH.

TABLE 8

Activation of both D (-) and L(+)-3-hydroxybutyrate by homogenate of rat heart

Homogenate mg protein	DL-3-hydroxybutyryl-CoA formed based on hydroxamate method n moles	L(+)-3-hydroxybutyryl-CoA formed based on enzymatic method n moles
0.14	73	36
0.29	95	47
0.57	95	47
1.14	88	43
2.15	93	46

4. DISCUSSION

It is known that addition of small amounts of acids of the citric acid cycle (such as malate, succinate, etc) greatly stimulates the mitochondrial oxidation of those substrates that require the operation of citric acid cycle (112). In line with this we observed that the oxidation of pyruvate, acetylcarnitine and acetoacetylcarnitine was stimulated by malate (Fig. 2). However, oxidation of acetoacetate was found to be inhibited by malate (Fig. 2, curve D). The reasons for this unexpected effect of malate on acetoacetate oxidation can be explained by the following considerations.

Recent studies have shown that the inner mitochondrial membrane is not freely permeable to the acids of citric acid cycle and have demonstrated the presence of several transport systems responsible for the movement of these acids across the inner membrane (8,129). Thus transport systems are known which would allow entry of malate in exchange for the exit of either intramitochondrial succinate, Pi or of 2-oxoglutarate.
If an entry of malate led to an efflux of 2-oxoglutarate then the mitochondrial oxidation of acetoacetate would be inhibited by the addition of malate. It appears that the malate inhibition of acetoacetate oxidation resulted from malate-induced loss of intramitochondrial 2-oxoglutarate. It is known that 2-oxoglutarate oxidation leads to the formation of succinyl-CoA and that succinyl-CoA can be utilized for activation of acetoacetate. The observation that malate inhibition of acetoacetate oxidation was restored by the addition of small amounts of 2-oxoglutarate lends support to the above belief (Fig. 2, curve D and Fig. 2, curve B). It may be noted that oxidation of other

substrates, which do not require succinyl-CoA for initiation of their oxidative utilization, was not as much stimulated by 2-oxoglutarate, (Fig. 2, curves A, B and C). Similarly, inhibitors of 2-oxoglutarate oxidation, arsenite and parapyruvate inhibited the oxidation of acetoacetate to a much greater extent than the inhibition of the oxidation of other substrates such as pyruvate, acetylcarnitine, acetoacetylcarnitine and palmitylcarnitine, which do not require succinyl-CoA for initiation of their oxidative utilization (Fig. 4-10).

Other workers believe that acetoacetate can be activated by an ATP-dependent activating enzyme also (71-73, 97). If ATP is able to activate acetoacetate for oxidation then the stimulatory effect of 2-oxoglutarate on acetoacetate oxidation should be less or absent when ADP plus Pi instead of DNP support the respiration. This being because in presence of ADP plus Pi ATP becomes available both due to the action of adenylate kinase and from the oxidative phosphorylation that accompanies mitochondrial respiration. However, the stimulation of respiration by 2-oxoglutarate was found to be as marked with ADP plus Pi as with DNP plus Pi (Fig. 23). Furthermore, when acetoacetate-dependent respiration was inhibited by either malate (Fig. 2), arsenite (Fig. 4) or parapyruvate (Fig. 5) addition of ATP (in the presence of oligomycin) was unable to stimulate respiration. These results, based on the measurement of oxygen consumption, failed to provide any evidence for the involvement of an ATP-dependent activatory enzyme in the oxidative utilization of acetoacetate.

Fig. 23 Stimulation of acetoacetate oxidation by 2-oxoglutarate in presence of ADP or DNP.

To 1.62 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added freshly isolated mitochondria (0.54 mg protein). Other additions were made as shown. Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria.

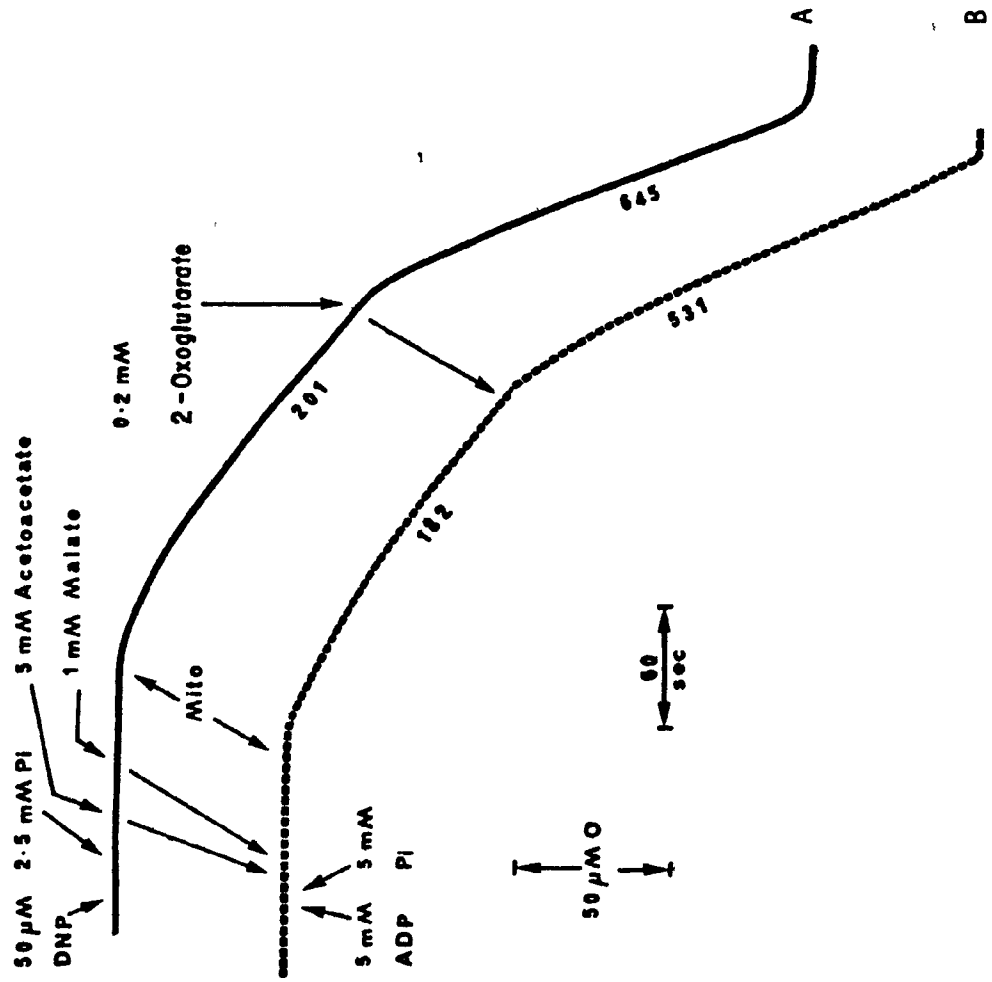

A
B
645
531
201
182
0·2 mM
2-Oxoglutarate
3 mM Acetoacetate
1 mM Malate
2·5 mM Pi
50 µM
DNP
Mito
5 mM
ADP
5 mM
Pi
50 µM O
60
sec

Fig. 23

It has been reported that Pi and arsenate inhibit the mitochondrial oxidation of acetoacetate (97,98) by directing succinyl-CoA towards substrate-level phosphorylation and towards arsenolysis, respectively, whereby the succinyl-CoA-linked activation of acetoacetate is impaired (98). Accordingly arsenate-insensitive oxidation of acetoacetate has been considered to provide evidence for succinyl-CoA-independent route (s) of acetoacetate activation (97). From the results obtained in the present study it is clear that the effects of Pi and arsenate on acetoacetate oxidation fail to indicate whether acetoacetate activation has involved a succinyl-CoA-dependent or -independent route. This being because the effect of Pi and arsenate on acetoacetate oxidation varied with the conditions and because similar effects were also seen on the oxidation of other substrates. Thus in the absence of added sparkers of citric acid cycle, Pi and arsenate inhibited the oxidation not only of acetoacetate but of other substrates as well (Fig. 14 and 15). In contrast, when added sparkers of citric acid cycle were present, Pi and arsenate stimulated rather than inhibited the oxidation of acetoacetate and of other substrates (Table 1 and 2). Table 1 shows that with DNP present, arsenate stimulated acetoacetate oxidation under conditions in which activation of acetoacetate involved succinyl-CoA-dependent route. Therefore the suggestion of Alexandre et al. (97) that oxidation of acetoacetate in the presence of arsenate indicates a succinyl-CoA-independent path of acetoacetate activation is no longer justified. Insofar as Pi promotes the leakage of intramitochondrial acids of citric acid cycle (130, 131), it is to be anticipated that when the concentration of intramitochondrial intermediates of citric acid cycle is sub-optimal, oxidation of substrates coupled to the operation of citric acid

cycle would be inhibited by Pi. Our findings that oxidation of acetoacetate and of other substrates was inhibited by Pi (and by arsenate) only when sparkers of the citric acid cycle (malate and 2-oxóglutarate) were not added, support this view. Further, although Hatefi and Fakouhi (98) considered a Pi inhibition of acetoacetate oxidation to be mediated by effects on succinyl-CoA metabolism alone, they also noted that depletion of bound (i.e. intramitochondrial ?) substrates sensitized mitochondria to inhibition by Pi.

The precise mechanisms by which Pi stimulated oxidation of acetoacetate and of other substrates is not clear but such effect(s) of Pi are unlikely to be restricted to any one step alone. For a respiration dependent on the operation of citric acid cycle a Pi involvement is to be expected because succinyl-CoA deacylation requires Pi. This would be true even during acetoacetate oxidation because while each molecule of acetyl-CoA entering citric acid cycle produces a molecule of succinyl-CoA the latter would generate twice as many acetyl-CoA molecules if Pi was lacking and the deacylation of succinyl-CoA were linked to the 3-oxoacid-CoA transferase reaction alone*. In such a situation for each turn of the citric acid cycle, one molecule of an acyl-CoA (as acetoacetyl-CoA or as acetyl-CoA) would appear as a surplus and the falling concentration of intramitochondrial-CoA would slow down respiration by limiting 2-oxoglutarate oxidase step of the citric acid cycle. Under these conditions, presence of Pi should permit regeneration of CoASH by directing part of the succinyl-CoA (and also acetoacetyl-CoA because of the reversibility of the 3-oxoacid-CoA transferase reaction) towards

* The activity of succinyl-CoA hydrolase is quite low in heart (132).

substrate-level phosphorylation. Our finding that Pi stimulated acetoacetate oxidation when sparkers of citric acid cycle were present is consistent with this view.

Pi also stimulated oxidation of substrates that did not require operation of entire citric acid cycle. Table 2 shows this for succinate oxidation (in the presence of amytal). Because Pi accelerates the reduction of oxaloacetate by mitochondrial malate dehydrogenase (133) part of the Pi stimulation of succinate oxidation could have occurred in this way. In addition when an added citric acid cycle intermediate serves as a substrate for oxidation (e.g. succinate in Table 2) or is required for supporting respiration of another substrate (e.g. malate with pyruvate, malate plus 2-oxoglutarate with acetoacetate) a stimulatory effect of Pi may be exerted in the translocation of polycarboxylate anions. It is known that permeation of several acids of citric acid cycle is directly or indirectly facilitated by Pi (8, 13, 129). Besides these other stimulatory effects of Pi are also possible e.g. oxidation of NADH by mitochondrial preparation is stimulated by Pi (~~94~~). Inasmuch as physiological levels of Pi accentuated respiration of uncoupled mitochondria by action at several sites it is possible that changes in Pi level in vivo contribute to the fine control of energy metabolism. Furthermore, because at physiological levels, Pi and ADP stimulated rather than inhibited the 2-oxoglutarate-supported oxidation of acetoacetate, it is clear that under conditions of high rates of succinyl-CoA generation acetoacetate competes successfully with Pi for the utilization of succinyl-CoA. Additional support for this view is offered below. The stimulation of the oxidation of 2-oxoglutarate to succinate by Pi is well known and is generally

interpreted to indicate a requirement of Pi for substrate-level phosphorylation (135). Fig. 24 shows that such Pi stimulated oxidation of 2-oxoglutarate in the presence of malonate was inhibited by acetoacetate. This is to be expected only if acetoacetate diverts succinyl-CoA from substrate-level phosphorylation to acetoacetyl-CoA formation because with malonate present to block the citric acid cycle, regeneration of CoASH from acetyl-CoA (derived from acetoacetyl-CoA) cannot proceed and consequently 2-oxoglutarate oxidation slows down due to a pausicity of CoASH. Addition of carnitine should reverse such an inhibitory effect of acetoacetate because, as a result of carnitine acetyl-transferase reaction, carnitine can regenerate free CoASH from acetyl-CoA (136) and from acetoacetyl-CoA (97). Fig. 24 shows that such an effect was indeed observed. No such effect of carnitine was seen in the absence of acetoacetate. It is evident, therefore, that contrary to the belief commonly held (97, 98, 137) even in the presence of Pi, succinyl-CoA can be well utilized for acetoacetate activation.

Among various substrates tested, mitochondria from heart show highest and similar respiratory rates with pyruvate and palmitylcarnitine (138) and our present finding show that mitochondria respire as rapidly with acetoacetate as with pyruvate. The ADP plus Pi stimulated respiratory rates with acetoacetate (492 ± 17.6) were found to be quite similar ($p > 0.1$) to those (519 ± 12.8) seen in parallel experiments with pyruvate. Inasmuch as acetoacetate oxidation was well coupled to phosphorylation, it is evident that heart can derive nearly as much energy by utilizing ketone bodies as that possible from the aerobic oxidation of carbohydrate or of fat which of course is quite appropriate for this tissue. Because oxidation of acetoacetate

Fig. 24 Inhibition of the Pi-stimulated oxidation of 2-oxoglutarate by acetoacetate and its reversal by carnitine.

To 1.6 ml of air-saturated medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl, 20 μM EDTA, pH 7.2, 28°C) was added freshly isolated mitochondria (1.2 mg protein). Other additions were made as shown. Numerals by the side of the tracing, the rate of oxygen consumption in natoms per min per mg of protein. Mito, mitochondria.

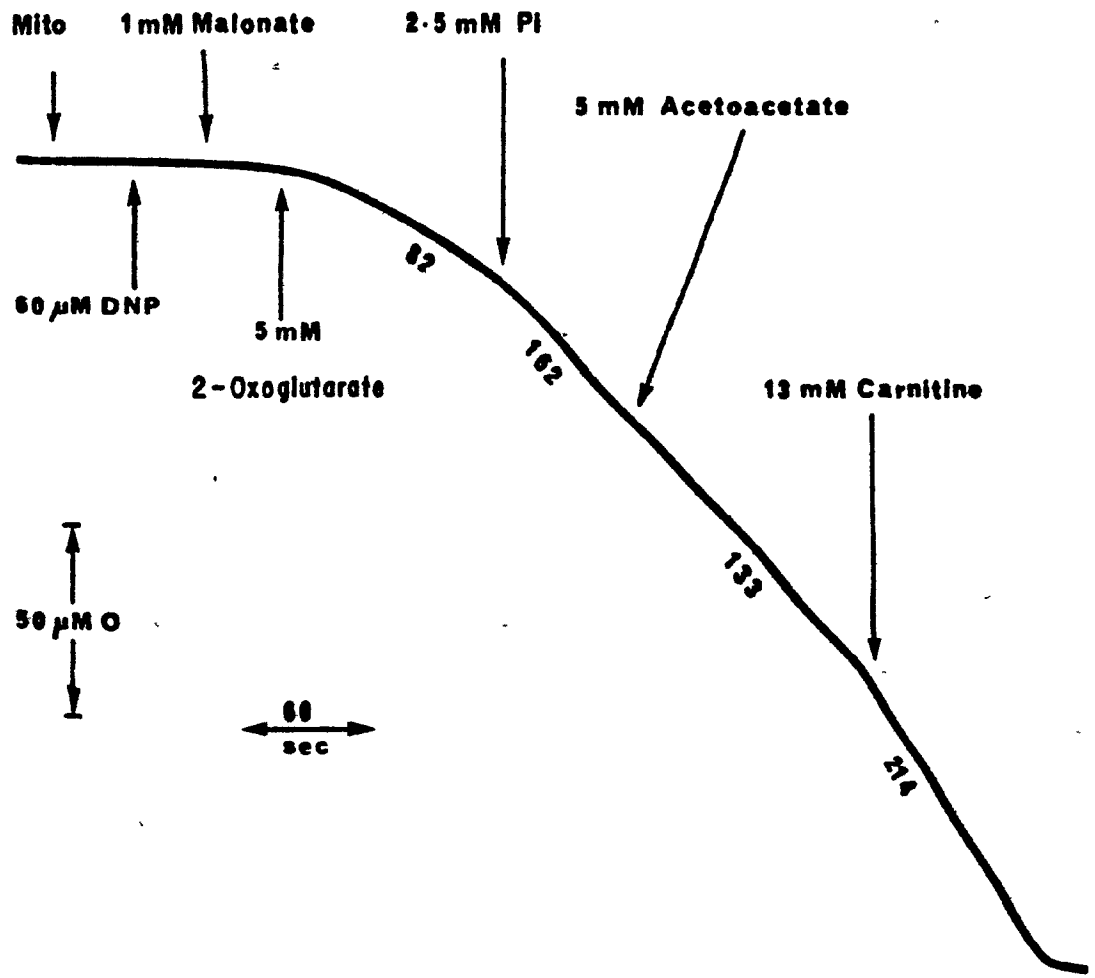

Mito
1 mM Malonate
2·5 mM Pi
5 mM Acetoacetate
60 µM DNP
5 mM
2-Oxoglutarate
13 mM Carnitine
50 µM O
60
sec
82
162
133
214

Fig. 24

was coupled to succinyl-CoA-linked activation of acetoacetate it appears that the 3-oxoacid-CoA transferase of intact heart mitochondria has adequate capacity so as not to limit the utilization of acetoacetate for energy production.

As described earlier, Pi and arsenate stimulated the acetoacetate-dependent respiration of rat heart mitochondria when malate and 2-oxoglutarate were present. However, attempts to ascertain if under these conditions Pi stimulated the oxidation of acetoacetate by following the conversion of [$^{14}C_4$]-acetoacetate or of [$^{14}C_3$] acetoacetate to $^{14}CO_2$, were unsuccessful because only negligible amount of $^{14}CO_2$ was produced in such experiments despite the fact that under identical conditions acetoacetate greatly stimulated mitochondrial oxygen consumption. Experiments with pyruvate showed that while malate - a known sparker of the citric acid cycle - stimulated pyruvate oxidation as seen by stimulation of oxygen consumption, simultaneously the conversion of carbon atoms of acetyl group (derived from pyruvate oxidation to CO_2 was inhibited by malate (Table 4). A clue to this anamolous behavior of malate was obtained by the determination of the $\Delta O(atom)/\Delta$ pyruvate (mole) ratio. When pyruvate alone was offered to mitochondria, the $\Delta O/\Delta$ pyruvate ratio was found to be close to 5 (Fig. 20). Because ratio of 5 is to be expected from the complete oxidation of a mole of pyruvate to CO_2,

$$CH_3.CO.COOH + 5O \longrightarrow 3CO_2 + 2H_2O$$

it is clear that pyruvate was nearly completely oxidized to CO_2 and water when oxidation of pyruvate depended on the presence of endogenous sparkers of the citric acid cycle. When mitochondrial oxidation of pyruvate was followed in the presence of added malate, the $\Delta O/\Delta$ pyruvate ratio averaged 3.6. This showed that pyruvate was incompletely oxidized in the presence of malate. It

is known that mitochondria have transport systems because of which entry of malate leads to the exit of another permeant intramitochondrial anion such as succinate, 2-oxoglutarate or citrate (124). Chappell and Haarkoff (139) on the basis of experiments with rat liver mitochondria have suggested that during the oxidation of succinate, entry of succinate appeared to be obligatorily linked to the exit of malate. If the reverse of above were operating in the present experiments with heart mitochondria (i.e. if the entry of malate were coupled to the exit of succinate) then the $\Delta O/\Delta$ pyruvate would have been 4.0. However, the experimentally determined values of $\Delta O/\Delta$ pyruvate with malate present averaged 3.6 suggesting that besides succinate other anions like 2-oxoglutarate and citrate may also be exchanging with malate. Direct measurement in one such experiment showed that while some accumulation of 2-oxoglutarate occurred during the oxidation of malate alone, its amount was increased by the additional presence of pyruvate. This extra formation of 2-oxoglutarate, however corresponded to only 14.4% of that expected from the amount of pyruvate disappearing. In somewhat similar experiments but with 5 mM malate, LaNoue et al.(140) have found that pyruvate oxidation led initially to the accumulation in the incubation medium of significant amounts of succinate, some of 2-oxoglutarate and much smaller amounts of citrate. Although Davis (141) has reported that commercial preparations of pyruvate have significant amounts of parapyruvate as contaminant which inhibited the oxidation of 2-oxoglutarate, the accumulation of 2-oxoglutarate in our experiments could not be due to this reason because at the low levels of pyruvate employed in the present experiments, inhibition by

contaminating parapyruvate was not noticeable*. Furthermore, this accumulation of 2-oxoglutarate due to pyruvate occurred only in the presence of malate.

Though not quite so marked, an inhibition of mitochondrial oxidation of [^{14}C]-acetyl-CoA by higher concentrations of added sparkers of citric acid cycle has been observed by other investigators before and this has been attributed to the dilution of acetyl-CoA pool by acetyl-CoA supposedly derived from the added "sparkers" (142, 143). However, as the present study shows the inhibition of the conversion of carbon atoms of acetyl group to CO_2 by added sparkers of citric acid cycle such as malate results instead from the interruption of the cyclic operation of the citric acid cycle brought about by the efflux of mitochondrially generated acids of citric acid cycle in exchange for the entry of added malate.

Evaluation of the involvement of ATP-dependent activation of acetoacetate: -

ATP-dependent activation of acetoacetate has been described for several tissues (71-76). Acetoacetyl-CoA synthetase activity in all the studies cited above was measured by coupling acetoacetate activation to citrate formation. In the present study, based on the measurement of oxygen consumption, no evidence could be obtained for the participation of an ATP-dependent activatory mechanism in the oxidative utilization of acetoacetate by rat heart mitochondria. Assay of acetoacetyl-CoA synthetase activity in preparations of rat heart based on measurement of the pH-dependent absorption of acetoacetyl-CoA at 303 nm (125) or estimation of the acetoacetyl-CoA formed by coupling to L-3-hydroxyacyl-CoA dehydrogenase were unsuccessful. However, by coupling

* We did obtain, in agreement with the finding of Davis (141) non-linear rates of respiration with commercial preparations of pyruvate being used (Sigma Chemical Co.) but this was marked only above concentrations exceeding 90 μM.

acetoacetate activation to citrate formation (73, 75), we were able to detect the presence of an ATP-dependent acetoacetyl-CoA synthetase in preparations of rat heart and kidney. But such an enzyme exhibited a very poor affinity for acetoacetate because the rate of acetoacetate activation kept on increasing with rising concentration of acetoacetate and there was no evidence that the rate became saturated at 240 mM (Table 5). DL-3-Hydroxybutyrate was also found to be poorly activated by homogenates of rat heart as extremely high concentration (256 mM) of DL-3-hydroxybutyrate were required to saturate the activity of the enzyme (Table 7).

Because of the low affinity for acetoacetate and DL-3-hydroxybutyrate it is unlikely that the ATP-dependent activating enzyme would be involved in the oxidative utilization of acetoacetate and DL-3-hydroxybutyrate in heart or kidney even under ketonemic conditions. The concentrations of acetoacetate and D-3-hydroxybutyrate in blood of normal adult persons fasted for 12-20 hours, are 0.015-0.226 mM and 0.031-0.650 mM respectively (49). Normally about two-third of the ketone bodies are found as D-3-hydroxybutyrate and one-third as acetoacetate and acetone. In severe cases of ketoacidosis, blood ketone bodies levels may be raised up to 5 mM (49). In rats starved for 48 hours, the concentrations of acetoacetate and D-3-hydroxybutyrate were found to be 0.47 mM and 1.62 mM respectively (19).

The poor activation of acetoacetate and DL-3-hydroxybutyrate by rat heart raised the question as to whether these substrates were activated non-specifically by one of the short-chain acyl-CoA synthetases such as butyryl-CoA synthetase. Some support for this was obtained in experiments where the activation of butyrate was found to be inhibited by the simultaneous

presence of DL-3-hydroxybutyrate (Table 7).

It has been reported that both D(-) and L(+) isomers of 3-hydroxybutyrate are activated by rat liver and kidney (30). We found this to be true for heart tissue as well.

5. CONCLUSION

Malate stimulated the mitochondrial oxidation of pyruvate and acylcarnitine as expected but oxidation of acetoacetate was found to be inhibited by malate. This inhibition of acetoacetate oxidation by malate was restored by the addition of 2-oxoglutarate. Oxidation of other substrates was relatively little affected by the presence of 2-oxoglutarate. This requirement of 2-oxoglutarate for acetoacetate oxidation stems from the ability of 2-oxoglutarate to provide succinyl-CoA. It appears that inhibition of acetoacetate oxidation by malate resulted from the loss of intramitochondrial 2-oxoglutarate (or its precursors) that accompanied entry of malate into mitochondria.

Unlike the oxidation of pyruvate and acylcarnitine, optimal oxidation of acetoacetate required the presence of both malate and 2-oxoglutarate. When 2-oxoglutarate was present small amounts of malate stimulated instead of inhibiting the oxidation of acetoacetate.

The 2-oxoglutarate supported oxidation of acetoacetate was markedly inhibited by arsenite and parapyruvate. These inhibitors of 2-oxoglutarate oxidation also inhibited mitochondrial oxygen consumption with pyruvate and acylcarnitines when respiration was followed in the presence of malate but the extent of such inhibition was relatively small.

Inhibition of acetoacetate oxidation by arsenite, parapyruvate (in the presence of 2-oxoglutarate and malate) or malate (in the absence of added sparkers) was not at all restored by the addition of ATP showing that ATP was unable to activate acetoacetate for oxidation. Thus there is no evidence that ATP-dependent activation of acetoacetate may be involved in the oxidative utilization of acetoacetate. The above results thus suggest that a continual generation of mitochondrial succinyl-CoA is obligatory for initiating acetoacetate oxidation.

When sparkers of citric acid cycle were not added, Pi and arsenate inhibited the mitochondrial oxidation of acetoacetate: however, under these conditions Pi and arsenate inhibited the oxidation of pyruvate and acylcarnitines as well. This was true with uncoupled mitochondria and under conditions in which production of ATP was not expected. Thus the inference of Alexandre et al.(97) that oxidation of acetoacetate in presence of arsenate indicates involvement of succinyl-CoA-independent route in the activation of acetoacetate is not true.

Acetoacetate was able to support as high rate of coupled respiration of rat heart mitochondria as seen in parallel experiments with pyruvate, showing that rat heart mitochondria can derive as much energy by oxidizing acetoacetate as that possible from the oxidation of pyruvate.

Because in our experiments activation of acetoacetate proceeded via succinyl-CoA route, the capacity of 3-oxoacid-CoA transferase of rat heart mitochondria appears to be adequate so as not to limit the utilization of acetoacetate for oxidation.

Under conditions in which oxygen consumption of mitochondria with acetoacetate as substrate was stimulated by the addition of 2-oxoglutarate and malate, conversion of [$^{14}C_3$]- or [$^{14}C_4$]-acetoacetato to $^{14}CO_2$ did not proceed. This discrepancy was resolved by following the effect of malate on the oxidation of differentially labeled pyruvate. The results showed that although malate stimulated pyruvate-dependent consumption of oxygen, production of $^{14}CO_2$ from [$^{14}C_2$]- and [$^{14}C_3$] pyruvate was inhibited. It appears that entry of malate in mitochondria accompanied an efflux of intramitochondrial intermediates of citric acid cycle resulting in interruption of the cyclic operation of citric acid cycle.

In the presence of ATP and CoASH, rat heart preparations activated acetoacetate as well as D(-) and L(+) isomers of 3-hydroxybutyrate. But the activating enzyme showed such poor affinity for acetoacetate and 3-hydroxybutyrate that its involvement in the activation of ketone bodies in vivo is questionable. It is possible that butyryl-CoA synthetase itself activates non-specifically acetoacetate and 3-hydroxybutyrate because presence of DL-3-hydroxybutyrate was found to inhibit the activation of butyrate.

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