THE CONTROL OF ENERGY METABOLISM IN YEAST

THE CONTROL OF ENERGY METABOLISM IN THE YEAST *SACCHAROMYCES CEREVISIAE*

By

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TITLE: The Control of Energy Metabolism in the Yeast *Saccharomyces cerevisiae*.

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SCOPE AND CONTENTS: The yeast *S. cerevisiae* is able to use several different metabolic pathways for the generation of metabolic energy, and is able to vary the synthesis of these multi-enzyme systems in response to changes in the external environment. The responses of the yeast to various changes in the environment have been studied and correlated with changes in the rate of synthesis of various enzymes. Two of these pathways are found in the mitochondrion, a sub-cellular organelle, the structural integrity of which is necessary for the functioning of these integrated systems. The experiments described thus provide information about the control of structure and function in cellular organelles, as well as about the general changes in energy metabolism.

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DIAGRAMS AND TABLES

Introduction

The metabolic pathways existing in plants, animals and micro-organisms can be broadly divided into two kinds; those pathways concerned with the generation of metabolic energy, and those pathways which utilize such energy. This study will be concerned primarily with pathways of the former kind. In the last decade the functioning of multi-enzyme systems has received increasing attention from chemists and biochemists. Of equal importance to the understanding of the life process are the mechanisms by which organisms control the synthesis and assembly of multi-enzyme systems. For most organisms, including many micro-organisms, the energy generating systems are "fixed" i.e. the organism uses one or two pathways continuously and the enzymes of these pathways are always present in the organism. In order to study the "on/off" control mechanisms involved in the genetic control of protein synthesis, and the subsequent assembly of multi-enzyme complexes the organism concerned must be able to synthesize more than one energy generating system. Also, the organism must be able to exist equally well by using any of the several pathways available to it without the intercession of any of the alternative pathways. Such a system would allow one to study the transition from one mode of energy generation to a second. A class of micro-organisms which fall into this category are the facultative anaerobes.

Saccharomyces cerevisiae was chosen for this study because it is a facultative anaerobic yeast. This implies that, given the

appropriate conditions, this yeast can grow with equal facility under both aerobic and anaerobic culture conditions. The metabolic needs of the cell are very different under these two contrasting atmospheric conditions, and the energy generating systems are modified to accommodate these particular environmental conditions.

Under anaerobic conditions the yeast realizes energy from the fermentation of sugars to produce alcohol and carbon dioxide. This alcoholic fermentation was first attributed to yeast by Pasteur, who also realized that this micro-organism was a facultative anaerobe. Further studies on the fermentation of glucose to alcohol gave rise to the isolation of the first enzymes (zymase) by Buchner and eventually, through the work of Embden, Meyerhoff, Parnas, Warburg and others, to the elucidation of the glycolytic or Embden, Meyerhoff, Parnas pathway as it is known today. This pathway, with slight modification of the steps prior to glucose-6-PO_4, provides a route by which yeast is able to ferment mono,-di,-tri,- and oligosaccharides to produce ethanol, carbon dioxide and energy. This then, is the major pathway by which yeast, growing on a fermentable carbon source, are able to generate the energy needed for growth, [for review, see Axelrod[1]].

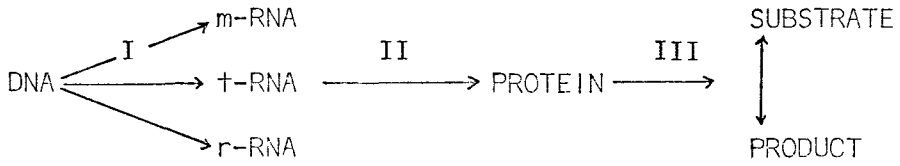
When yeast are growing aerobically on a non-fermentable carbon source, the metabolic energy is derived from respiration. Non-fermentable, or respirable carbon sources, such as ethanol or glycerol are oxidized via the tricarboxylic acid, or Kreb's cycle to produce carbon dioxide and reduced nicotinamide nucleotides (NADH, NADPH). The NADH is then oxidized via the respiratory chain to produce the

high energy intermediate adenosine triphosphate (ATP) with molecular oxygen acting as the terminal oxidizer. Thus respiration is dependent upon the production of two separate metabolic pathways and the presence of molecular oxygen in the environment. The Kreb's cycle is a multistep system by which the yeast can metabolize acetyl coenzyme A to produced reduced NAD and carbon dioxide. During respiratory growth this is a major function of the Kreb's cycle, but it is also involved in other, anapleurotic functions, such as gluconeogenesis and carbon-skeleton production for amino-acid synthesis. One must consider these other metabolic functions as well as that connected with respiration when considering the probable role of the Kreb's cycle during any particular phase of the yeast growth cycle.

In contrast, the respiratory chain is concerned solely with transfering electrons from the Kreb's cycle to molecular oxygen, and utilizing this oxidation to generate ATP. Thus the yeast will only need the respiratory chain under conditions where the energy derived from fermentation is insufficient for growth, and an alternative, respirable energy source is available.

In the growing yeast these three enzyme systems are compartmentalized (glycolysis is cytoplasmic; Kreb's cycle and the respiratory chain are mitochondrial[2]) and physically separate, but are connected by metabolic intermediates. Pyruvic acid, which is decarboxylated during fermentation to give rise to ethanol, can also be oxidized to acetyl CoA, which then enters the Kreb's cycle. During respiratory growth the mitochondrion must release nicotinamide nucleotides, ATP, and other metabolites into the cytoplasm, so that the

various compartmentalized metabolic pools will also be connected by chemical equilibrium or by membrane transport mechanisms. Although these enzyme systems are physically separate in yeast, their functioning is almost certainly integrated under the appropriate metabolic conditions. This description of the major energy producing pathways of yeast leads naturally to the questions of how these systems are produced and of how this production is controlled? These are unanswerable at the present time, but the answers must be considered in the light of the control mechanisms generally available to micro-organisms. The information which specifies enzyme and subcellular structure is encoded in the deoxyribonucleic acid (DNA). The processing of this information involves the transcription of DNA genes to produce various ribose-nucleic acids (RNA). The structural genes, which encode the amino-acid sequence for proteins produce messenger RNA (m-RNA), other genes produce transfer RNA (t-RNA), which participates in the transport and activation of amino-acids, and ribosomal RNA (r-RNA), which in the form of a ribosome, co-ordinates the actions of m-RNA, t-RNA and the relevant enzymes to produce proteins. If the protein is an enzyme it will then interact with a substrate(s) and produce a product(s). This process can be summarized:



The Roman numerals (above) denote probable control sites. Type I control [See Fig. I(i)] at the level of specific structural gene

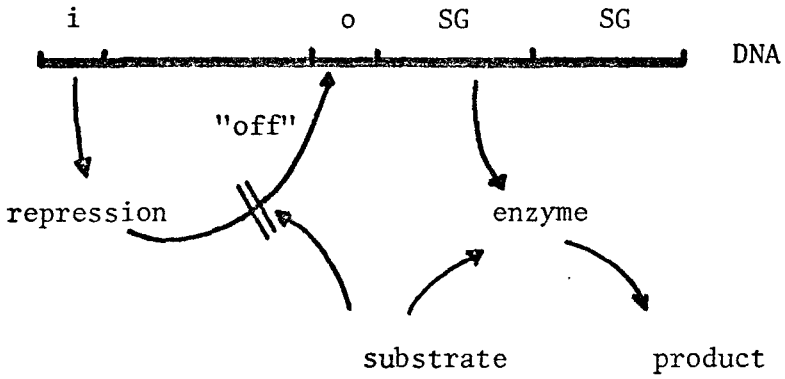


Fig 1(i) Type I Control

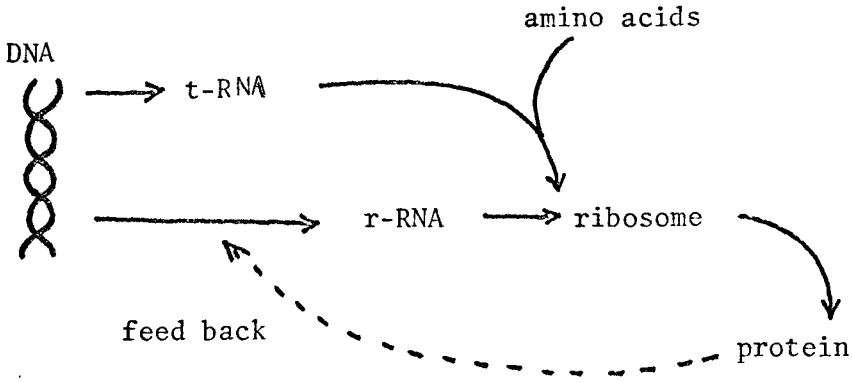


Fig 1(ii) Type II Control

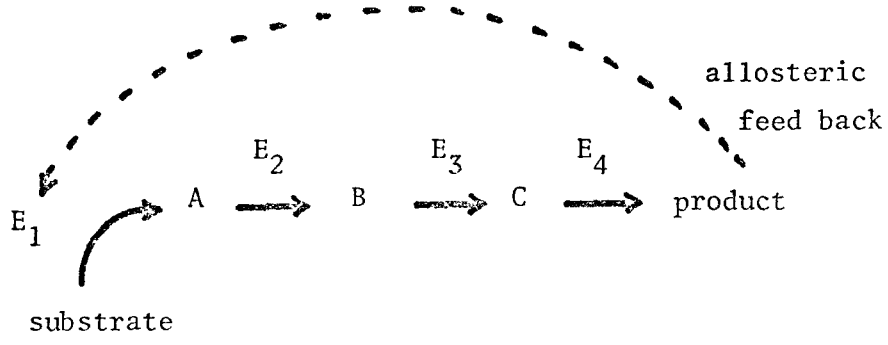


Fig 1(iii) Type III Control

translation has been demonstrated in many micro-organisms. The control of enzyme production by substrates/products specific to that enzyme as demonstrated by the β galactosidase studies of Jacob and Monod[3] provides a specific control mechanism at the level of DNA transcription. A number of variations upon this theme, involving multi-enzyme systems is discussed by Pontecorvo[4]. One operon, multiple structural gene systems have been observed in bacteria[5], fungi[6], and other micro-organisms. Cascade regulation, which is the simultaneous induction of a number of unrelated operons has not been specifically reported, but sequential induction of a number of related structural genes has been reported in *Pseudomonas*[7].

Control of Type II [See Fig. I(ii)] is more general than that of Type I, and involves an alteration in the total rate of RNA transcription in response to gross environmental changes. The co-ordinated changes between the rates of DNA, RNA and protein synthesis, and the growth rate have been described by Maaloe and Kjeldgaard[8]. At the genetic level the "relaxed-stringent" mutants of *E. coli* also exhibit this control[9]. Briefly, in the wild type coli when protein synthesis is inhibited, the overall rate of RNA synthesis becomes decreased, and conversely when the rate of protein synthesis is stimulated, the overall rate of RNA synthesis is also increased. These changes are also mimicked by the rate of DNA synthesis.

The third level of control [See Fig. I(iii)], is at the level of enzyme function, and can be considered as a metabolic "fine control". In many enzyme systems, especially multi-enzyme pathways[5] there is a

stimulatory/inhibitory relationship between the various substrates and products of the pathway and the respective enzymes. The rate of production of one substance often controls the rate of utilization of the substrate which is acted upon to give rise to that product. There are many examples of this type of control, and some have been observed which inter-relate the glycolytic pathway, Kreb's cycle and the respiratory chain[10].

The above control mechanisms are generally available to micro-organisms and the control of energy metabolism in yeast will be discussed with the above information in mind (Chapter 6). The enzyme systems of yeast have long provided information about the intermediary energy metabolism (see above). In 1950, when Ephrussi and Slonimski[11] began the characterization of the "petite" mutation of yeast, the study of the fermentation:respiration balance gained added impetus. The "petite" mutation shows cytoplasmic inheritance and its phenotype is respiratory deficiency. Such strains are characterized by an inability to grow on non-fermentable carbon sources[11]. The lack of respiratory ability correlates cytologically with degenerate mitochondrial profiles (Yotsuyanagi[12], Shatz *et al.*[13]) and biochemically by reduced amounts of various dehydrogenases and the loss of cytochromes $a+a_3$, b and c, (Slonimski[14] Slonimski and Sherman[15], Mahler *et al.*[16]). As these biochemical, cytological and genetic studies were progressing, other researchers were elaborating the effects of various environmental factors upon the wild type yeast. A number of growth conditions caused the wild type yeast to mimic the

"petite" phenotype. Anaerobic, fermentative growth causes the yeast to lose its respiratory ability, the mitochondrial profiles become degenerate and decrease in numbers, and the levels of cytochromes $a+a_3$, b and c, are much decreased (Somlo[17], Slonimski[18], Tustanoff and Bartley[19]). The same phenomena were also observed when respiratory sufficient yeast were transferred to a fermentative carbon source such as glucose (Polakis and Bartley[20], Utter *et al.*[21], Jayaramann *et al.*[22]). The above studies delineated the major factors which could produce the respiratory deficient phenotype in yeast. Later studies on the adaptation of anaerobically grown yeast to aerobic conditions demonstrated the restoration of the respiratory sufficient phenotype (Slonimski[23], Tustanoff and Bartley[24] Linnane *et al.*[25]). These studies led Slonimski to propose that the synthesis of the mitochondrial proteins was an inductive process, triggered by molecular oxygen, and Bartley and his co-workers to propose the alternative hypothesis that it was release from glucose repression, mediated by fermentation, which led to the elaboration of mitochondrial profiles.

A preliminary report by Shatz *et al.*[26] suggested that there was DNA associated with the yeast mitochondria. This report was soon followed by the isolation of DNA from the mitochondrion of a fungus (*N. crassa*) and from the mitochondria and other sub-cellular organelles of higher plants and animals. Each of these DNA's has a unique structure and a characteristic bouyant density (review, Gibor and Granick[27]). In 1966 Tewari, Votsch and Mahler[28] isolated and

characterized the DNA of yeast mitochondria, using cells grown under various conditions. This finding was very quickly substantiated by other laboratories and Monoulou *et al.*[29] demonstrated that "petite" mutations could be associated with deletions in the mitochondrial DNA.

Recently these studies have been amplified by the isolation of a DNA-DNA polymerase (replication enzyme)[30], and a DNA-RNA polymerase (transcription enzyme)[31], both specific for mitochondrial DNA, and also of ribosomal and transfer RNA specific to the mitochondrion[30]. Winterberger[30] has also been able to demonstrate base pair homology between mitochondrial DNA and mitochondrial RNA, The discovery of the "petite" cytoplasmic inheritance and the DNA associated with the mitochondrion led to a re-awakening of the self-reproducing theory for mitochondrial synthesis originally proposed by Altmann. The more recent evidence indicates that the mitochondrion is equipped with most of the apparatus essential for self-replication. Although the characteristics of this system are similar to that possessed by bacteria, the amount of DNA associated with the mitochondria of most eukaryotes is sufficient to code for only a small fraction of the proteins involved in the mature mitochondrion (Work and Ashwell[32]).

That the mitochondrion is actually synthesizing proteins coded for by mitochondrial DNA is still in doubt, but the incorporation of radioactive amino acids by isolated mitochondria has been firmly established for mammalian mitochondria (Roodyn[33]), and for isolated yeast mitochondria (Linnane *et al.*[34,35]).

Further evidence for the coding properties of mitochondrial DNA comes from the works of Winterberger[30], who showed that a "petite" mutant could no longer synthesize mitochondrial ribosomes; from Linnane *et al.*[34,35] who showed that in yeast an erythromycin-resistant mitochondrial protein synthesizing system is transmitted via cytoplasmic inheritance, and from Thomas and Wilkie[36] who have been able to demonstrate cytoplasmic-recombination under anaerobic mating conditions. Perhaps significantly, this would be the condition when the mitochondrial membrane systems would be most degenerate.

Lehninger[37] discusses the evidence for the three major alternative hypotheses proposed to explain the synthesis of mitochondria. The first is *de-novo* synthesis, the second is formation from preformed cellular structures e.g. the cytoplasmic and/or nuclear membrane, and the third is that of self proliferation - each mitochondrion "growing" and giving rise to daughter mitochondria by some form of fission process. The evidence for the first two hypotheses is at best circumstantial, and although the accumulated evidence makes the third hypothesis very attractive, it is difficult to envisage the mechanism by which this "growth" is controlled.

Previous evidence suggests that some components of the respiratory chain are coded for by the mitochondrial DNA i.e. affected by "petite" mutations, whereas the great bulk of the mitochondrial proteins are either unaccounted for, or coded for by nuclear genes. Munkres[38], working with *N. crassa* has shown that the structural genes for the mitochondrial isozymes of malic dehydrogenase (MDH) are

nuclear in location. Sherman[39] and Sherman and Slonimski[15] have isolated a large number of "petite" mutants which exhibit Mendelian inheritance. The best studied of these mutants are those of the CY locus, which is the structural gene for cytochrome-c, an integral part of the respiratory chain. Evidence from mammalian systems indicate that cytochrome-c is synthesized on the cytoplasmic ribosomes, and then integrated into the mitochondrion[40]. This has not however been demonstrated in yeast.

The evidence obtained by Luck[41] working with *N. crassa* is the least equivocal, with respect to the self-proliferation or growth hypothesis. Working with a choline requiring mutant and radioautography, Luck was able to demonstrate that in a pulse-chase experiment, the number of grains/mitochondrion is halved in one generation time. The fungus was growing synchronously and this evidence implies that the mitochondria are growing in phase with the cellular division cycle.

This is obviously untrue for yeast, as the number of mitochondria per yeast varies greatly under the various growth conditions. Cytological and biochemical studies[42] have led to the theory of the pro-mitochondrion, which is an immature mitochondrion, present in anaerobic cells, which is transformed into a mature mitochondrion when the cell is exposed to oxygen. Thus the conventional wisdom, as exemplified by the recent work of Shatz, Criddle, Plautner and Paltauf[42] is a mixture of the *de-novo* and "self-proliferatory" theories, which will be more fully discussed in the conclusion (Chapter 6).

From the evidence so far presented it seems that the yeast mitochondrion has all the characteristics of a self-replicating system,

except that the DNA is insufficient in quantity to code for more than a very few mitochondrial proteins. An explanation of the mode of mitochondriogenesis in yeast must therefore embody the synthesis and integration of nuclear-coded proteins into the mitochondrion, the mitochondrial synthesis of mitochondrially coded protein, and the integration of these two processes with general cell metabolism.

As described above, the energy generating pathways in yeast are altered in response to changes in the atmospheric conditions and to carbon-energy source changes. The object of the experiments to be described were:

(i) to further document the changes in enzymic concentration and function under the various growth conditions.

(ii) to interpret these results with respect to the way in which yeast control the fermentation:respiration balance.

(iii) from the results involving mitochondrially located enzymes, to gain a further insight into the processes involved in mitochondriogenesis.

(iv) finally, to try and draw some conclusions about the relative importance of nuclear and mitochondrial DNA in mitochondriogenesis.

CHAPTER I

Methods

Microorganism and culture procedure

Saccharomyces cerevisiae, strain no. 77 of the National Collection of Yeast Cultures (Brewing Industries Research Foundation, Nutfield, Surrey) was maintained aerobically on agar slopes containing inorganic salts (see media), 2.25% (W/V) Difco malt extract, 0.05% (W/V) Difco yeast extract and 0.5% (W/V) sucrose. The yeast was grown for 48 hrs at 30.0° prior to inoculation. The yeast was subcultured once a month, and after 48 hrs at 30° the slopes were stored at 2°.

Growth of cells

Liquid growth medium utilized in this study was the complete anaerobic medium described by Tustanoff and Bartley[19] which contained ergosterol and Tween 80. The carbon source was added as described in the text. The yeast inoculum was prepared by washing the organism from the agar slope with sterile distilled water. Sufficient inoculum (determined by nephelometry) was added to the growth medium to give 0.40 mg. dry wt. of cells for each g of carbon source compound. Initially cell growth was commenced aerobically in wide-based conical flasks which were aerated by vigorous shaking (135 oscillations/min) in a thermostatically controlled water bath (30.0°). When various

kinetic parameters were to be measured in aerobically grown yeast, these cells were grown to their stationary phase and aliquot samples were taken sequentially during this growth period. When transfer experiments were undertaken cells were grown to their mid-exponential phase (3.5 mg dry wt/ml) and immediately centrifuged (2,000 x g for 5 min) at 30°, then resuspended in sterile distilled water and added to fresh anaerobic medium so that the cell concentration was 4.0 g wet wt/l of medium. The nature of the carbon source in this latter medium varied and is given in the text. Incubation of these cells was then continued for a specific length of time in the manner described above either aerobically or anaerobically. Anaerobic conditions were attained by bubbling the medium with a continuous flow of oxygen-free nitrogen gas which was further chemically purged of the last traces of oxygen[43]. An anaerobic milieu was insured by pre-bubbling the liquid medium for 30 min prior to inoculation.

Harvesting the organisms

Cells were collected at different time periods, transfered into pre-cooled centrifuge tubes and centrifuged at 5,000 x g for 5 min (0°). The supernatant was decanted and stored at -20.0° for carbon source and ethanol analysis where indicated. The resulting pellet was washed twice by suspension in distilled water (0°) and then stored for 24 hrs at -20.0° if enzyme assays were to be carried out. For dry wt determinations aliquot samples were taken and treated as described above and the final pellet was heated at 115° for 24 hrs.

Cell free extracts

After 24 hrs, stored pellets were thawed, suspended in a volume of tris-HCl buffer (0.05 M, pH 7.4) and then passed through a chilled French pressure cell (23,000 p.s.i.) which was maintained at 4°. The cell walls and debris were removed by centrifugation (1,000 x g for 5 min at 0°). The cloudy supernatant was used for enzymic assays.

Preparation of yeast mitochondria

Mitochondria were prepared using a modification of the protoplast technique described by Duell *et al.*[44] using late exponential aerobic cells. Serum albumin (3 mg/ml) was added to the lysis-medium. Approximately 80% formation of spheroplasts were obtained (microscopic examination) using the crude snail gut extract (400 mg protein/g wet wt of cells).

Measurement of respiratory ability

Five ml aliquot samples were removed from the culture at various times during the growth period and transferred quantitatively to a chamber of the Biological Oxygen Monitor (Yellowspring Instrument Co., Yellowspring, Ohio; Model 53) and aerated for exactly 1.0 min at a constant rate. Oxygen consumption of the whole cells was monitored polarographically using a Clark type electrode for 3.0 min at 30.0°. This measurement reflected the respiratory capacity of these cells. Very active cell cultures were diluted 1:5 with supernatants obtained from samples taken for dry wt determinations.

Measurement of mitochondrial respiration

Oxygen consumption of mitochondria was also measured polarographically. The reaction medium contained 0.65 M sorbitol, 25 mM potassium phosphate buffer (pH 6.8), 10 mM KCl, 1.0 mM EDTA and 1.0 mg bovine serum albumin (in 3.0 ml). The reaction was carried out at 30°. Oxidations were initiated by the addition of 0.05 ml mitochondrial suspension (400 - 500 µg protein), followed by 0.02 ml substrate (concn. indicated in text), 0.01 ml ADP (180 µM) and 0.01 ml of either D (-) or L (+) isomers of chloramphenicol. Because of the limited solubility of this drug in water it was solubilized in 95% ethanol (600 mg/ml at 35°). P:O and respiratory control ratios were determined by graphic analysis of the polarographic tracings recorded in response to the addition of a definite amount of ADP, as described by Chance and Williams[45].

Analytical methods

Protein was determined by the biuret or Folin-Ciocolleau method[46] with crystalline bovine serum albumin as standard. Glucose, galactose and ethanol were determined in the supernatants utilizing the glucose or galactose oxidase[47] and alcohol dehydrogenase[48] procedures respectively.

Enzyme assays

All assays were performed in a temperature controlled Gilford 2000 recording spectrophotometer. The temperature was maintained at 30°C. By using 1.0 cm lightpath, 3.0 ml cuvettes in a micro-cuvette

holder it was possible to reduce the assay volumes to 1.5 - 2.0 mls without encountering the mixing and edge effect problems inherent in micro-cuvettes.

Pyruvate decarboxylase (2-oxo-acid carboxy-lyase, E.C.4.1.1.1.) was assayed according to Holzer and Goedde[49]. The decarboxylation was measured by using excess alcohol dehydrogenase to couple acetaldehyde production to the oxidation of NADH. The system contained phosphate buffer (0.05 M, pH 6.5), $MgCl_2$ (8.33 mM), cysteine HCl (20 mM), thiamine pyrophosphate (1.67 mM), NADH (0.15 mM), pyruvate (33 mM), alcohol dehydrogenase (60 µg) and water to a final volume of 2.0 mls. Absorbance was monitored at 340 mM, $E = 6.22 \times 10^{-3} M^{-1} cms^{-1}$.

Hexokinase (ATP glucose-6-phosphotransferase E.C.2.7.1.2.) was measured according to Bergmeyer[50]. The reaction was coupled to NADP using glucose-6-P. dehydrogenase and the increase in absorbance at 340 nM due to NADPH was measured. $E = 6.22 \times 10^{-3} M^{-1} cms^{-1}$. Each cuvette contained $MgCl_2$ (0.01 M), ATP (0.01 M), NADP (3×10^{-3} M), tris-HCl buffer (0.05 M, pH 7.4), glucose-6-P. dehydrogenase (1.0 µg) and glucose (20 µg) in a final volume of 1.5 mls.

Glucose-6-P. dehydrogenase (D-glucose-6-phosphate NADP oxidoreductase, E.C.1.1.1.49) was assayed by the method of Bergmeyer[51]. Each cuvette contained tris-HCl buffer (0.05 M, pH 7.4), NADP (0.05 mls of 25 mg/10.0 mls solution), glucose 6-P. (4×10^{-2} M) and water to a final volume of 1.5 mls. The reaction was started by the addition of glucose-6-P., and the increase in absorbance at 340 nM was recorded.

Malic dehydrogenase (L-malate:NAD oxidoreductase E.C.1.1.1. 37) was assayed according to Witt, Kroneau and Holzer[52]; cuvettes contained NADH (0.1 mM) oxaloacetic acid (0.1 mM) and tris-HCl buffer (0.05 M, pH 7·4) to a final volume of 1.5 mls. Absorbance was monitored at 340 nM.

Succinic dehydrogenase (Succinate:paramethosulponate oxidoreductase, E.C.3.9.9.1.) was assayed by the method of Singer, as modified by Hauber[53]. To each cuvette was added KCN (1.5 µmoles), sodium succinate (45 µM), phosphate buffer (150 µmoles, pH 7.6) enzyme extract and water to a final volume of 1.48 mls. The cuvettes were equilibrated for 3.0 mins at 30°. After 3.0 mins, 0.02 ml of a parametho-sulphonate (PMS)/dichlorophenol indolephenol (DCIP) solution was added and the absorbance at 600 nM was measured for the first minute. PMS (30 mg/ml)/DCIP (90 mµmoles/ml) E = 20 m moles^{-1} cms^{-1}. Estimates were corrected for a minus-succinate blank reaction.

Isocitric dehydrogenase (L-isocitric:NAD oxidoreductase, E. C.1.1.1.41.) was assayed according to B. D. Sanwal *et al.*[54], modified as follows: each cuvette contained $MgCl_2$ (0.05 mls, 0.1 M), NAD (0.05 mls, 10 mg/ml), AMP (0.02 mls, 0.025 M), KCN (0.05 mls, 30 mM) and tris-HCl buffer (0.05 M, pH 7.4) to a final volume of 1.48 mls. The reaction was started by the addition of 0.02 mls of isocitrate (0.25 M). The reaction was monitored at 340 nM.

Alcohol dehydrogenase (Alcohol:NAD oxidoreductase E.C.1.1.1. 1,) was assayed according to the method of Bergmeyer[55] modified as follows. To each cuvette was added NAD (0.01 mls, 0.075 M), ethanol

(0.1 mls, 2% solution) glycine-phosphate buffer (1.0 mls, pH 8.8). Absorbance was monitored at 340 nM. Glycine-phosphate buffer consisted of sodium pyrophosphate (1.66 gms), glycine (0.0833 gms), semicarbazide-HCl (0.216 gms) made up to 50 mls at pH 8.8.

NADH-cytochrome c reductase (NADH:cytochrome c oxidoreductase E.C.1.6.2.1.) was assayed by a method modified from that of Y. Halefi and J. S. Rieske[56]. Phosphate buffer (0.05 M, 10^{-3} M EDTA, pH 7.0), ferricytochrome c (0.01 mls of a 0.80% solution), KCN (0.05 mls, 0.01 M), NADH (0.1 mls, 0.01 M) and water to a final volume of 2.0 mls. Reaction rates were corrected for auto-reduction of cytochrome c and for minus-enzyme blank reaction. E = 29.0 $mmoles^{-1}$ cms^{-1} at 550 nM.

Cytochrome c oxidase (cytochrome c:oxygen oxidoreductase, E.C.1.6.2.1.) was assayed by measuring the oxidation of cytochrome c at 550 nM, according to the method of L. Smith[57]. Each cuvette contained horse heart cytochrome c (0.75 mg) in 1.5 mls of tris-HCl buffer (pH 7.4). Cytochrome c was reduced with ascorbic acid, such that the ratio $OD_{550}:OD_{565}$ was between 12:1 and 14:1. Data are expressed as 1^{st} order rate constant, or converted to mmoles/min using E = 29.0 $mmoles^{-1}$ cms^{-1}.

Separation of malic dehydrogenase (MDH) iso-enzymes

(i) *Column chromatography*

DEAE-A50 Sephadex was cycled through NaOH and HCl, neutralized, and equilibrated to phosphate buffer NaH_2PO_4 (0.02 M, pH 7.0) 20 x 1.0 cm columns were poured at room temperature, and

equilibrated to 4.0°C. after packing. Samples were layered on top of the column and eluted with 25.0 ml phosphate buffer(NaH_2PO_4, pH 7.0) followed by a further 25.0 ml of phosphate buffer(NaH_2PO_4 0.2 M NaCl, pH 7.0). Sixteen fractions of 3.0 mls were collected. Fractions were assayed spectrophotometrically for MDH as described above. This method was modified from that of Schmidt, Schmidt and Mohr[58].

(ii) *Polyacrylamide gel electrophoresis*

Anionic conditions, as described by Baruch Davis[59], were used throughout. Bands were visualized by incubating the gels at 37°C totally immersed in the following solution: paramethosulphonate (PMS), 4.0 mg, nitrotetrazolium blue (NTB) 10.0 mg, malic acid (neutralized) 1.0 gm, NAD 50 mg, dissolved in 50 ml of 0.1 M tris-HCl buffer, pH 8.5. Electrophoresis times were as described in the text.

Extraction and determination of nicotinamide nucleotides and adenosine phosphates

Duplicate samples of yeast suspensions were serially removed from the growth medium and immediately centrifuged at 2,000 x g for 1.5 minutes. After discarding the supernatant the yeast pellets were immersed in liquid N_2 and immediately frozen. These cells were then stored in liquid N_2 until they were used for subsequent analyses. Extraction of adenosine phosphates and nicotinamide nucleotides from these cells was carried out according to the procedures outlined by Polakis and Bartley[60]. Recovery studies of this method in our hands

gave results which ranged from 98.5 to 100.5%. (See Tables I(II) and I(III). Quantification of these cofactors was then carried out using the fluormetric procedure described by Maitra and Estabrook[61]. The output signal of an Eppendorf fluorimeter was fed into a compensating voltage attachment[62] which in turn was amplified and recorded by a 10" Beckman Potentiometric recorder. Pyridine nucleotide oxidation or reduction was assessed from the change in fluorescence using a 313 + 366 nM excitation light and a secondary emission filter with a band pass of 400 to 3,000 nM. Nucleotide solutions were standardized in the conventional manner by determining the change in absorbance at 340 nM spectrophotometrically using similar enzymic reactions. Glucose-6-phosphate and pyruvate concentrations were estimated as a by-product of the adenosine phosphate determinations.

Analysis for DNA

DNA was assayed according to the method of Burton[63]. DNA was extracted from whole cells with perchloric acid (0.5 N) and digested at 70°C for 15 mins. The diphenylamine reaction was used to visualize the deoxyribose produced and deoxyribose was used for the standard curve.

Expression of results

Enzyme activity and oxygen consumption data are expressed both as specific activity in the cell and total activity in the culture medium. By contrasting these different activity expressions it is

possible to obtain an insight into the state of the catalytic protein during various phases of yeast propagation. Specific activity does not necessarily reflect changes in catalytic activity or changes in the gene expression which controls that catalytic activity. By comparing and contrasting the specific activity (which is a relative rate) with that of the total activity (which is an absolute rate) it is possible to characterize the various modes by which the concentration of catalytic protein per cell can be altered. Synthesis of enzymes is reflected in a positive slope of the total activity curve, a zero slope indicates no net synthesis and a negative slope degradation. Inflection points in these curves represent "rate changes" or transitions from one functional state to another. On this basis it is possible to decide whether changes in enzyme concentration/cell mass are due to changes in enzyme concentration, altered expression of existing enzyme activity or to increased or decreased cell growth relative to a constant rate of enzyme synthesis.

Total activities are also expressed as semi-log and log-log plots. A straight line semi-log plot indicates an exponential synthetic rate and inflexions in the curve indicate rate changes. The slope of the line is a measure of the doubling time. A straight line on a log-log plot indicates a parabolic synthetic rate of the type $y = c^{-mx}$. Both types have been observed, and the possible significance of these results is discussed in Chapter 4.

Estimates of the errors involved in the analytical data

(i) *Enzyme assays*

All samples were passed through the French press twice to ensure maximum cell breakage. Each sample was then assayed three times for each activity, and the values recorded in the results is the average for the three estimates. The data in Table I (I) illustrates the range of some estimates. In general, individual estimates agreed within 5%. [cf. Table I(I)]

(ii) *Nucleotide analysis*

The data in Tables I (II) and I (III) illustrates recovery of nicotinamide and adenine phosphate nucleotides experienced with the respective extraction techniques. Duplicate samples were taken at each point in the experimental time course and then each sample was extracted separately. Each sample was assayed three times, with individual estimates agreeing within 5%. The points used in the results represent the average of six estimates (three for each sample).

(iii) *Other analysese*

Assays for glucose, galactose, alcohol, protein and DNA were all performed in duplicate on single samples. The values used in the results represent a mean of two estimates; in general estimates agreed within 5% or less.

TABLE I (I)

The Effect of French Pressing on the Recovery of Cyt. c oxid. Activity

Extract* mg.protein /ml	# of times pressed	ΔE_{550}/min/ 0.01 ml	Average Specific Activity 1^{st} order rate const./mg protein
56.0	1	0.0285 0.0270 0.0290	1.07
50.0	1	0.0290 0.0275 0.0300	1.27
54.0	2	0.0275 0.0250 0.0260	1.07

* 25.0 mls of aerobic yeast: 24.0 hrs, 2.7% glucose Y.AnO_2

TABLE I (II)

Recovery of ATP from adenosine phosphate cell extracts

Sample	% change in fluorescence	Expected % change	Average %
ATP standard solution *	16.5 16.5 17.5 18.5	17.5	98.7
ATP standard solution	17.5 17.75 16.75 15.5	17.5	98.0
ATP & 5.0 mls of cells**	22.5 23.0 21.1 21.15	22.13	100.1
2 x ATP & 10.0 mls of cells	53.0 50.25 52.25 54.0	53.0	99.0

* equivalent aliquots of ADP and AMP were also included in these samples

** yeast suspensions were aerobic, 5% glucose-grown; 54.6 mg. dry wt/10.0 mls.

TABLE I (III)

Recovery of NAD and NADH from cell extracts

NAD		NADH	
Sample	% Recovery	Sample	% Recovery
Standard NAD Solution*	94.7	Standard NADH Solution*	115.0
Standard + 5.0 mls of ** cells	83.8	Standard + 5.0 mls of cells	94.0
Standard + 10.0 mls of cells	98.7	Standard + 10.0 mls of cells	120.0

* NAD and NADH were added to each sample to check for interconversion

** yeast suspensions were aerobic, 5% glucose-grown; 54.6 mg. dry wt/10.0 mls.

Chemicals

Horse heart cytochrome c (Type III), adenosine phosphates, NAD^+ and NADH were purchased from the Sigma Chemical Co. (St. Louis, U.S.A.). Bovine serum albumin was obtained from Armour Pharmaceutical Co. (Kankakee, Ill., U.S.A.). Snail gut enzyme (Suc d'Helix pomatia) was secured from Industrie Biologique Francaise (Seine, France). Casein hydrolysate (acid) originated from Oxo Ltd., (London, England), yeast extract from Difco Laboratories (Detroit, U.S.A.), D-galactose (glucose-free) from Thomas Kerfoot Co. (Vale of Bardsley, Lancs, England). D (-) threo- and L (+) threo-chloramphenicol were donated to us by Parke-Davis Co*. (Detroit, Michigan). "Glucostat" and "Galactostat" reagents were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Glucose-6-phosphate, phosphoenolpyruvate (disodium salt) pyruvate and all other enzyme utilized in this study were obtained from C. F. Boehringer & Soehne (New York, U.S.A.). All other chemicals were of the highest grade available.

Medium Inorganic Salts

KH_2PO_4	9.0 gm/l
$(NH_4)_2SO_4$	6.0 gm/l
$MgCl_26H_2O$	0.5 gm/l
$CaCl_2$ (anhydrons)	0.3 gm/l

* We are indebted to Dr. H. E. Machamer for the generous gift of the D (-) and L (+) *threo* isomers of chloramphenicol.

CHAPTER 2

Preliminary Experiments

(i) The Growth Characteristics of S. Cerevisiae

Anaerobic Growth

Growth on 2.7% glucose anaerobic medium (YAn.N_2) is characterized by a 9.0 hr lag period, followed by a fast, 3.0 hrs long fermentatative phase. At this point glucose becomes exhausted and the cells enter a terminal stationary phase. On 2.7% galactose (YAn.N_2) the lag phase is 14.0 hrs long and the fermentative growth rate is slower. Galactose becomes exhausted after 6.0 hrs of exponential growth and the cells enter stationary phase. The dry weight yield/unit carbon source is the same for both sugars [Fig. 2(i)].

Aerobic Growth

The rate of increase in dry weight with time varies with the carbon source. A pronounced di-phasic curve is observed when yeast are grown on 2.7% glucose, sucrose or fructose, Fig. 2(ii)A. The lag period is 7.5 hrs long, followed by a fast logarithmic phase of 3.5 hrs in which glucose is completely fermented to ethanol and carbon dioxide. The cells then enter a plateau or lag period before logarithmic growth is resumed. This second growth phase is respir-

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Fig. 2(I).

Yeast grown on maintenance slopes for 48 hrs. at 30°C. were inoculated into Y.An. medium to a concentration of 0.4 mg. dry weight/ml. Oxygen pre-nitrogen was bubbled through the cultures which were contained in tightly stoppered flasks. Cells were kept suspended by gentle shaking. Incubation was at 30°C.

Fig 2(i) Anaerobic growth curves, Y.An.N2
2.7% glucose,
2.7% galactose
dry weight - mg./10.0 mls culture
48.0
40.0
32.0
24.0
16.0
8.0
12.0
16.0
20.0
24.0
time after inoculation - hours

LEGEND

Fig. 2(II) A and B.

Yeast grown on maintenance slopes for 48 hrs. at 30°C. were inoculated into Y.An. medium to a concentration of 0.4 mg. dry weight/ml. Cultures were aerated by vigorous shaking and the temperature was maintained at 30°C.

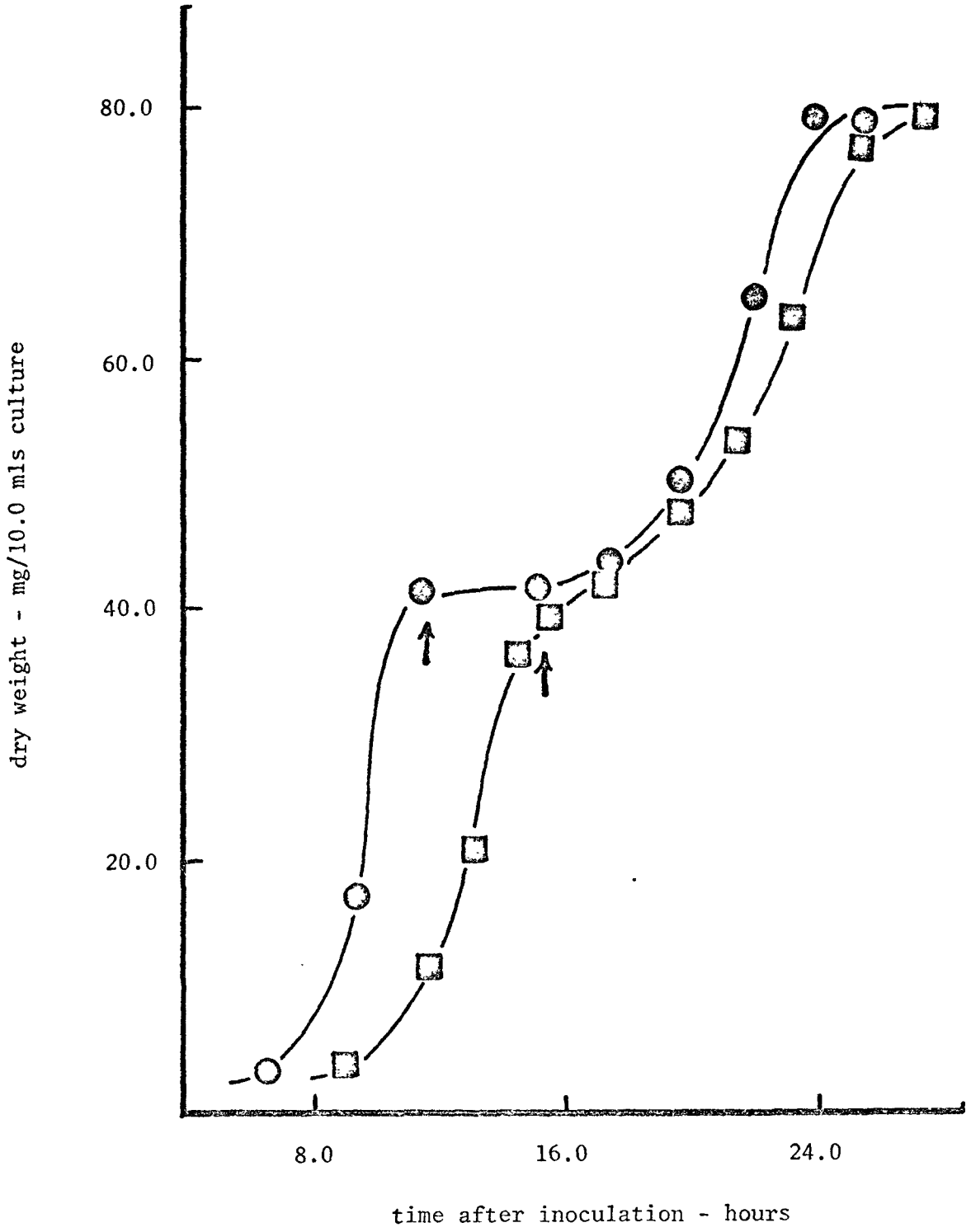

Fig 2(ii) A Aerobic Growth Curves

2.7% glucose - ○ - 2.7% maltose - □ -

↑ primary carbon source exhausted

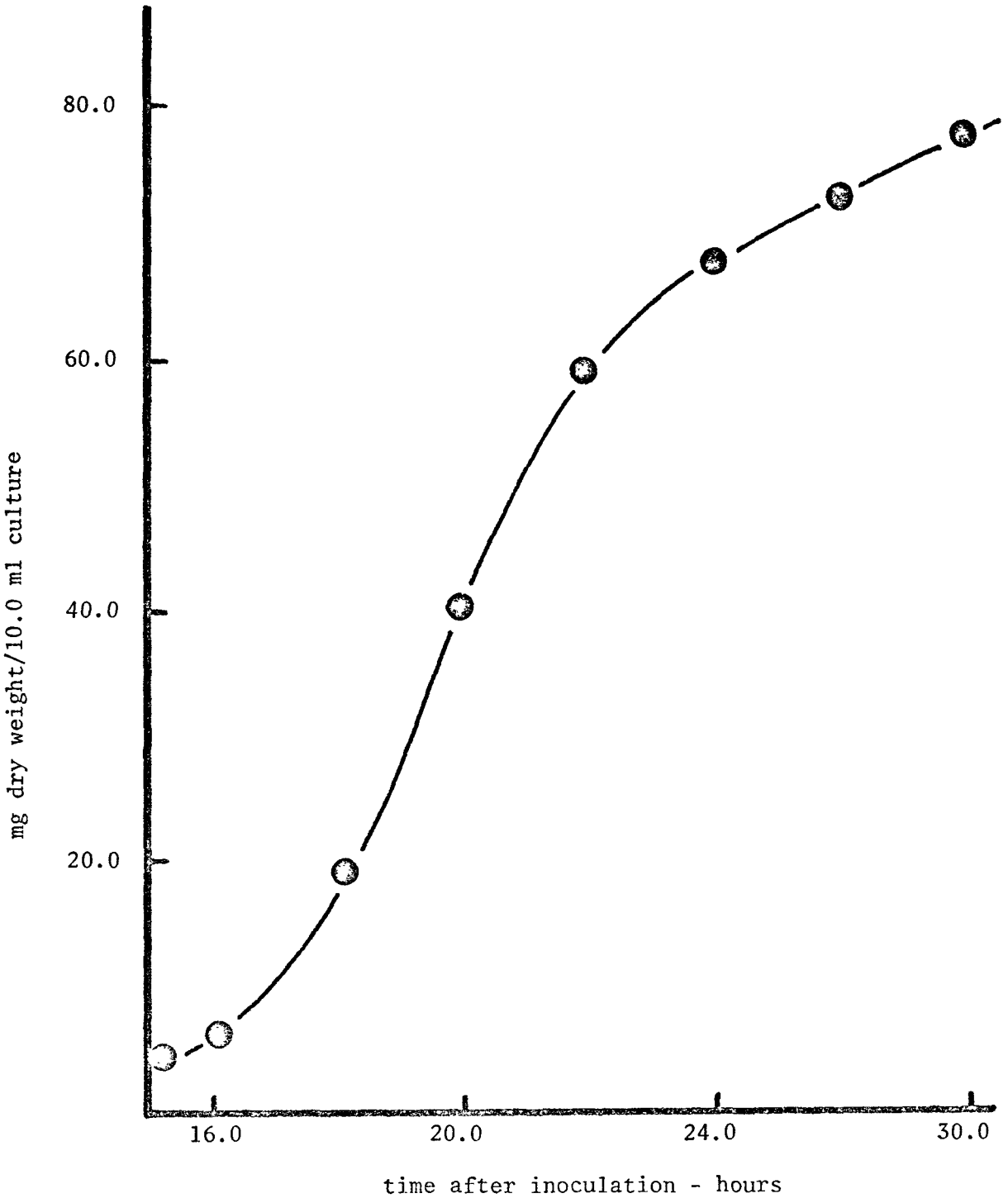

Fig 2(ii) B Growth curve: 2.7% galactose Y.An.O_2

atory in nature, the yeast utilizing the products of the earlier fermentation as carbon-energy source. Galactose grown yeast also exhibit a di-phasic growth curve but the transition from the fermentative mode to the respiratory mode is achieved gradually and the transition is initiated before galactose is completely utilized (22 hrs) [Fig. 2(ii) B]. Both the lag period, 14.5 hours, and the dry weight yield/unit carbon source in the fermentative phase are much greater than those observed for the glucose grown culture. Yeast grown on 2.7% maltose, Fig. 2(ii) A, show intermediary characteristics. The lag period is 10.5 hrs. long, and the fermentative → respiratory transition is achieved with a minimal lag period. The intermediacy of the maltose grown yeast between the alternatives represented by glucose and galactose cells also appears in the other general parameters of growth. The final dry weight yield/unit carbon source is similar for all cultures at the time of ethanol exhaustion.

Ethanol metabolism

Anaerobically, the rate of production of ethanol is proportional to the growth rate and the dry weight yield is similar for both galactose and glucose grown yeast. Aerobically, glucose grown cells produce ethanol much more rapidly than do galactose grown cells [Fig. 2(iii)]. The yield of ethanol for glucose cells is 37.5 µmoles/ml., whereas the yield for galactose cells is only 23 µmoles/ml. The maltose culture exhibits an intermediate rate of ethanol production. Ethanol production continues for a short while after the primary

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Fig. 2(III)

Ethanol analysis was performed on supernatants derived from the growth curves shown in Figs. 2(II)A and B.

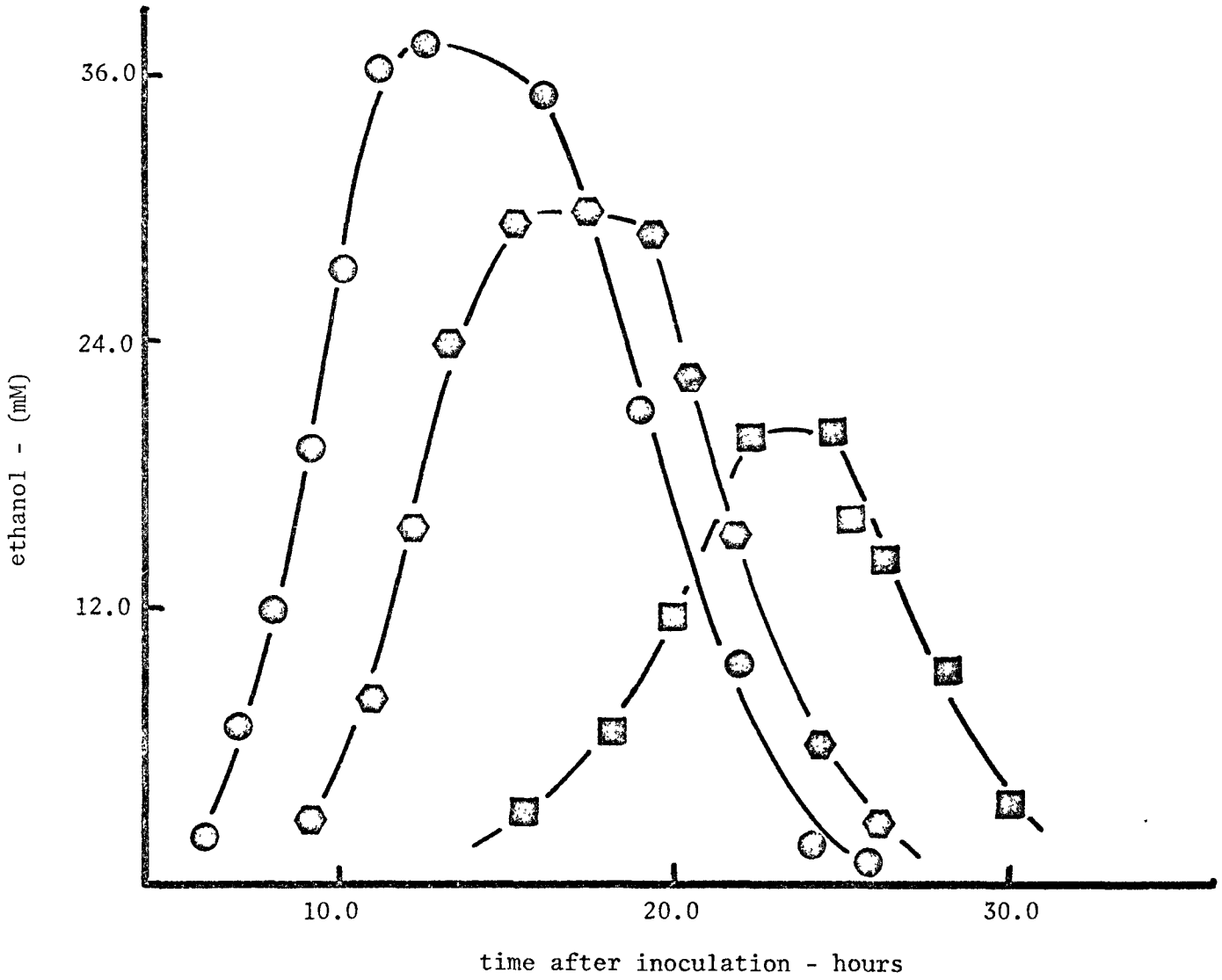

Fig 2 (iii) Aerobic ethanol metabolism: 2.7% glucose, -●- 2.7% maltose, -⬢- 2.7% galactose -■-

carbon source has been exhausted from the medium and utilization commences just prior to the initiation of the second growth phase [cf. Fig. 2(ii) A and B and Fig. 2(iii)].

Respiration

The *in situ* respiratory ability (Q_{O_2}) Fig. 2(iv) illustrates the most striking difference between the three cultures. The Q_{O_2} at the beginning of logrithmic growth (see also Table I) is very different in each case; Q_{O_2} galactose > Q_{O_2} maltose > Q_{O_2} glucose. This difference is reflected in the total respiratory ability Fig. 2(v). However, the rates of increase in total respiratory ability for all the carbon sources tested, as shown by the parallel slopes in this semi-logarithmic plot are the same. Perhaps the most striking fact is the coincidence of the curves in time, as measured from the time of inoculation (data for sucrose and fructose are omitted for clarity).

Table I shows the Q_{O_2} at various times for cultures grown aerobically on 2.7% glucose, 2.7% maltose, and 2.7% galactose.

TABLE 2 (I)

Respiratory ability (Q_{O_2}) at various times during aerobic growth on glucose, galactose and maltose

	Inoculum	Early Log	Plateau	Late Log
glucose	0.074*	0.005	0.013	0.07
maltose	0.072	0.025	0.30	0.125
galactose	0.075	0.073	0.85	0.150

* µmoles O_2/min/mg. dry weight

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Fig. 2(iv) and 2(v)

The oxygen consumption data shown in these two figures (QO_2 and total respiration) were determined on samples taken from aerobic yeast cultures. The culture conditions were Y.An. medium, 30° C and aeration by vigorous shaking. Carbon source at 2.7% was added as indicated.

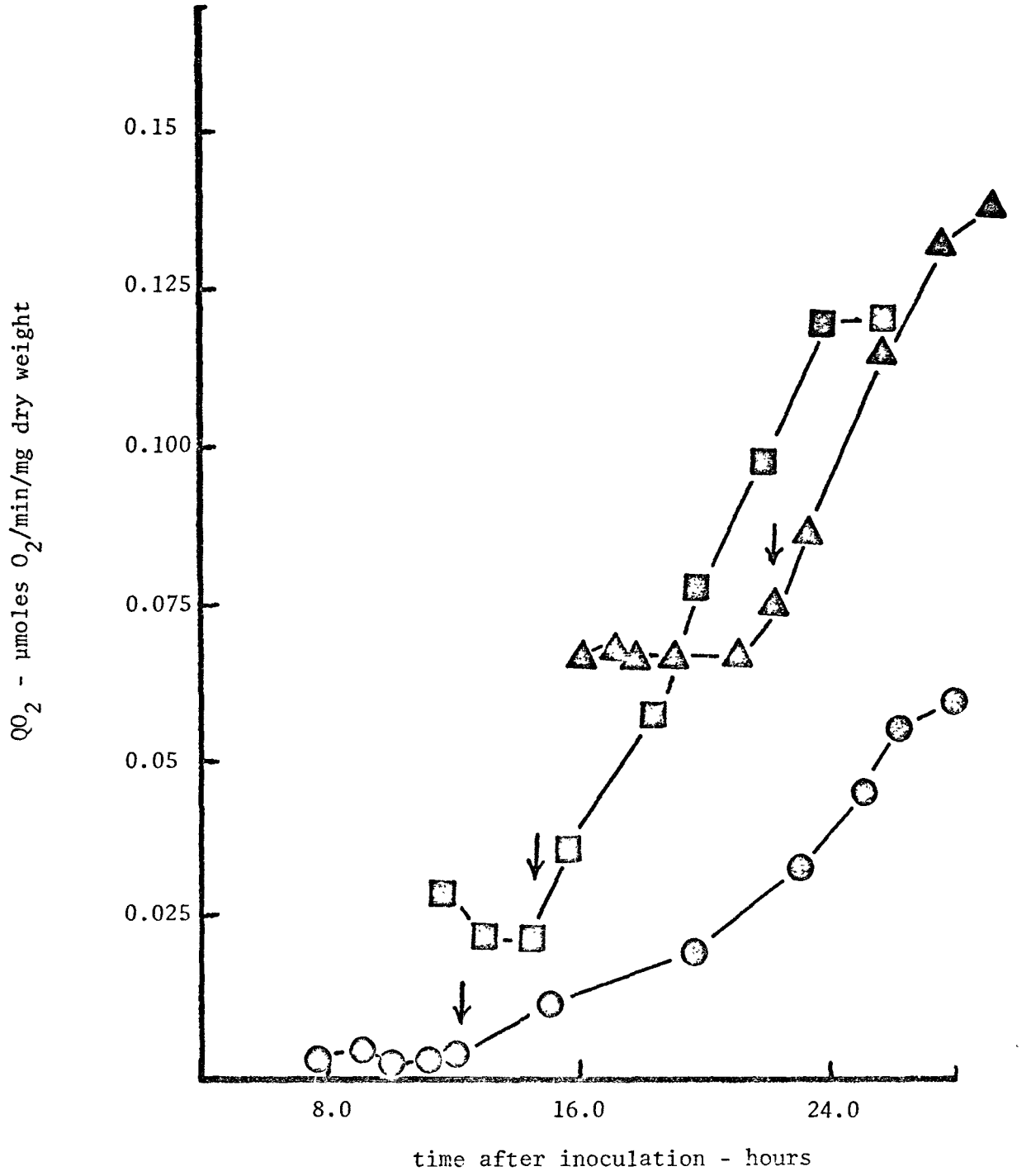

Fig 2(iv) Changes in Respiratory Ability Under Aerobic Conditions

glucose -○- maltose -□- galactose -△-

↓ primary carbon source exhausted

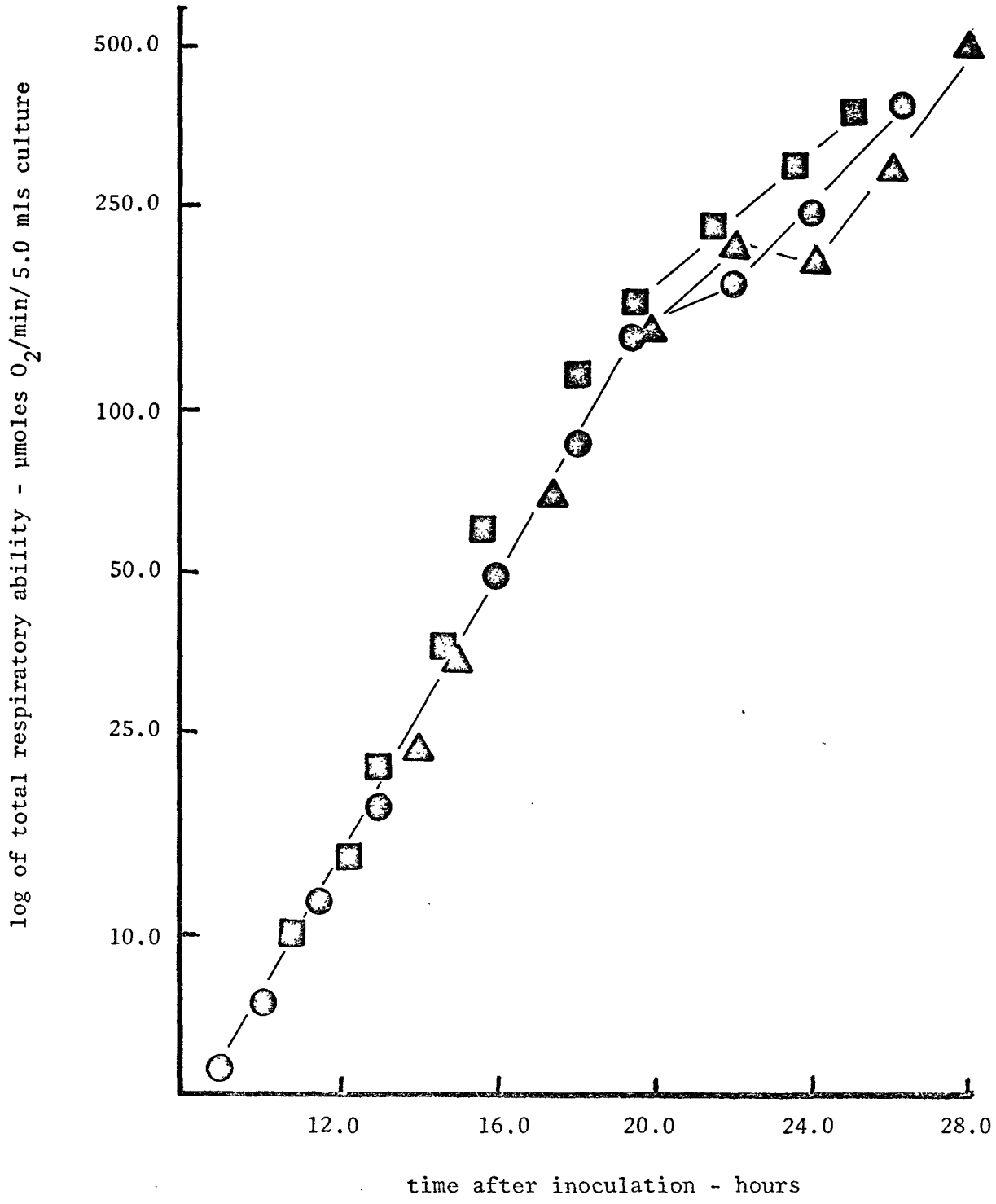

Fig 2 (v) Total Respiratory Ability During Aerobic Growth

2.7% glucose -○- 2.7% maltose -□- 2.7% galactose -△-

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Fig. 2(vi)

Growth conditions were the same as those for Fig. 2(iv). These observations illustrate the change in QO_2 between the onset of glucose utilization and the mid-fermentative phase. The dilution curve was calculated on the premise that there would be no further increase in the total respiratory ability after 4.0 hrs.

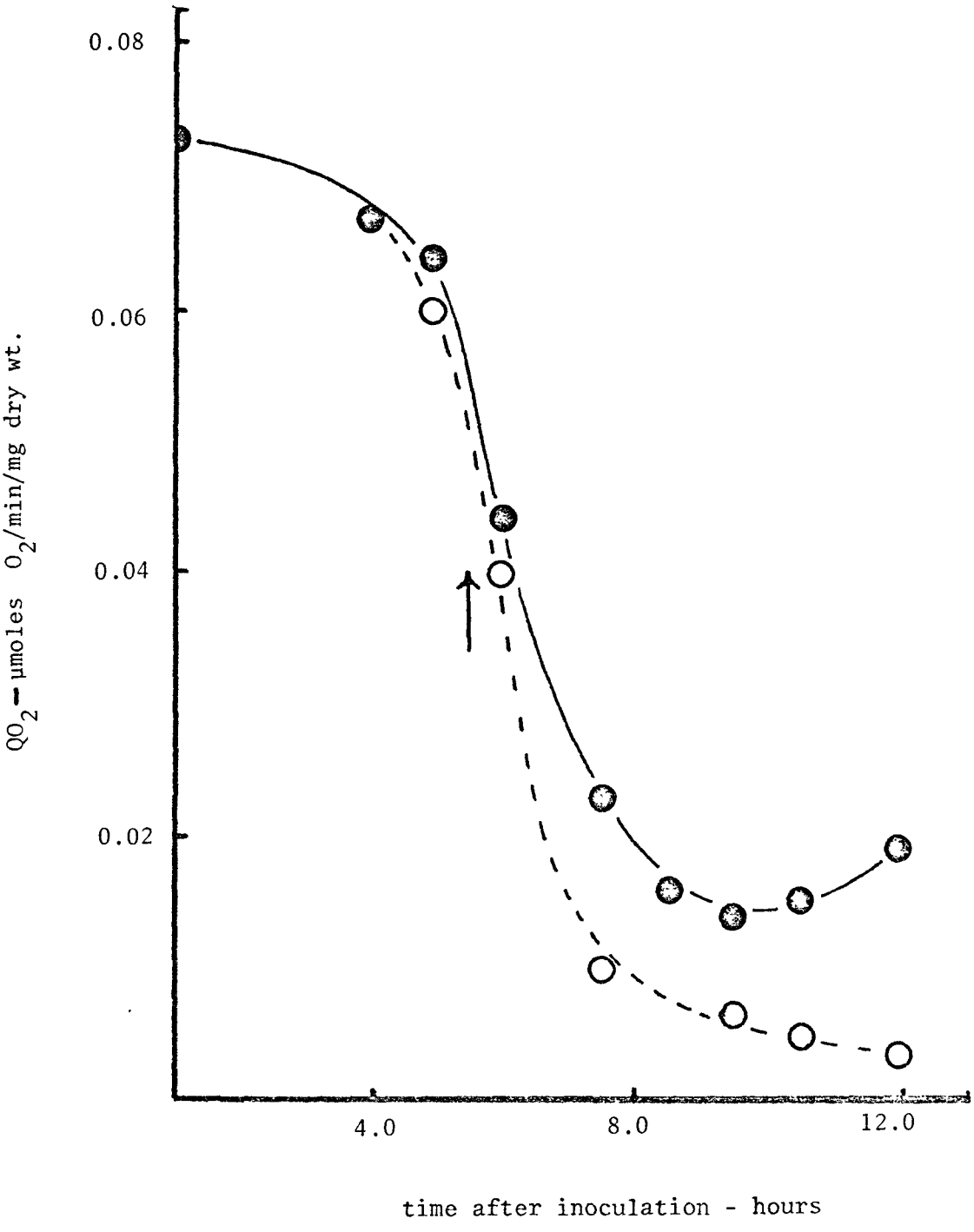

Fig 2 (vi) Lag phase respiration of glucose aerobic cells

— ○ — observed QO_2 - - ○ - - dilution curve

↑ glucose utilization commences

The observed curve in Fig. 2(vi) shows the way in which the glucose Q_{O_2} is reduced during the latter part of the lag period. The theoretical curve is calculated on the assumption that there would be no net synthesis of respiratory ability, and that the Q_{O_2} would be reduced in proportion to the increase in dry weight. Obviously there is a net synthesis of respiratory ability during this phase, although the data gives no insight into whether this total respiratory potential is being expressed as respiratory ability or not [see also Chapter 3, Fig. 3(ii)].

Discussion

The three characteristic growth curves Fig. 2(ii) A and B are conditioned by the rate at which the primary carbon source can be fermented. Utilization time is 3.5 hrs., 6.0 hrs., 10.0 hrs., for glucose, maltose and galactose respectively. The ethanol production is a result of the balance between the pyruvate → alcohol, pyruvate → acetyl CoA and pyruvate → stored carbohydrate reactions. When compared to glucose grown yeast, maltose and galactose grown yeast show a slower rate of ethanol production and a lower ethanol yield, and these changes are probably due to the added respiratory ability possessed by these cells.

The Q_{O_2} is a ratio, µmoles O_2/min/mg dry weight. All the growth rates are different but logarithmic, and the rates of increase in total respiratory ability are also logarithmic but similar. Using this information it is possible to calculate the changes in the Q_{O_2} which would result from changes in the growth rate while the rate of

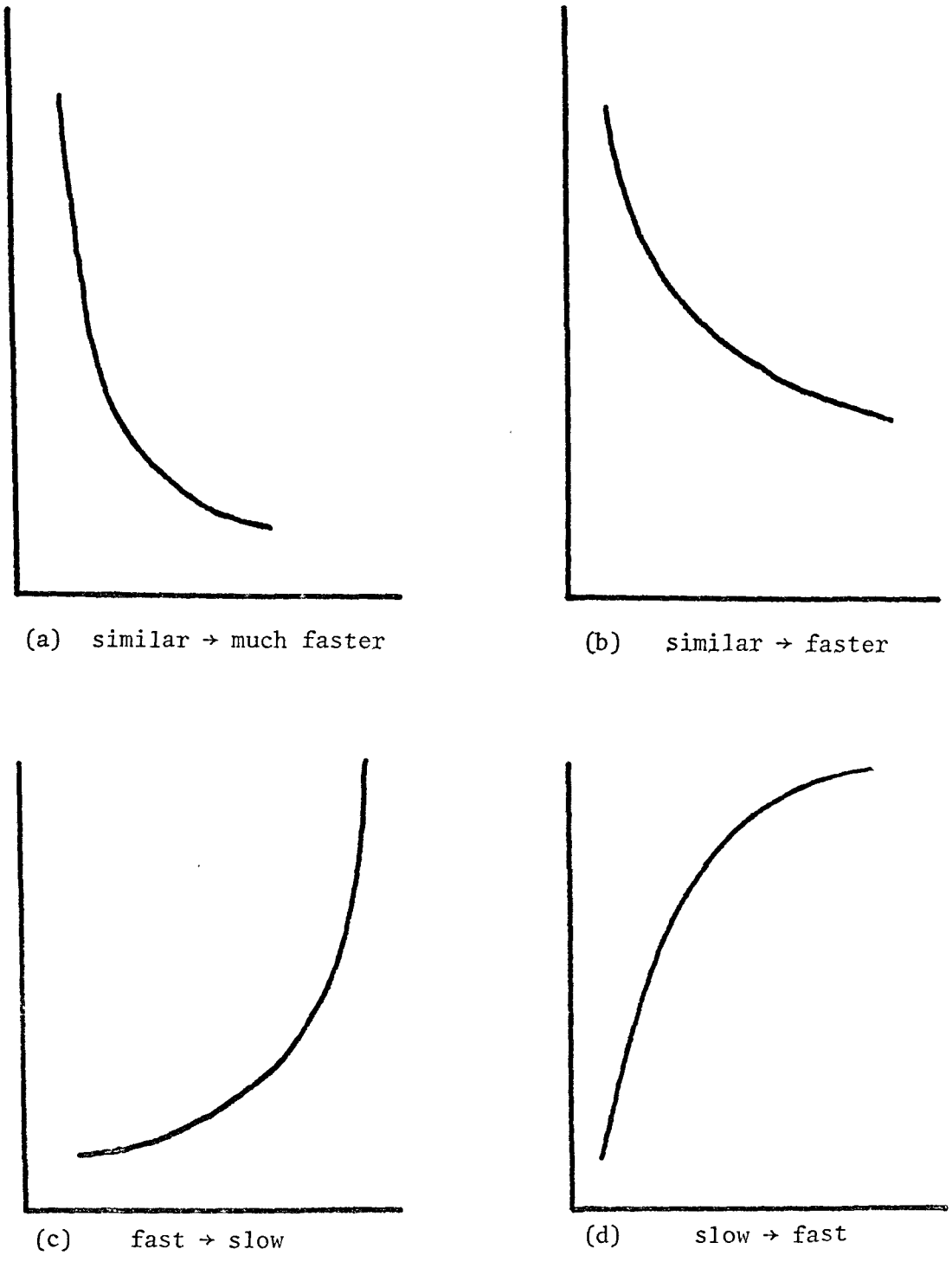

Fig 2(vii) Theoretical QO_2/time profiles: showing the changes in the QO_2/time when various changes occur in the growth rate during the maintenance of a constant rate of increase in total respiratory ability.

increase in total respiratory ability remains constant. Some of these are illustrated in Fig. 2(vii). (a) shows the transition from similar rates to a relatively very fast growth rate (glucose early log.), (b) shows this same transition for a relatively fast growth rate (maltose early log.), (c) shows the transition from a fast growth rate to a very slow one (glucose, plateau phase) and (d) shows a transition from a very slow growth rate to a much faster one (glucose, end of plateau phase). Using these calculated curves it is possible to construct curves similar to those observed in Fig. 2(iv). The Q_{O_2} values for galactose cells Table I, show that under this condition the rate of growth and the rate of total respiratory ability production are the same (Q_{O_2} maintained) until the yeast cell becomes dependent upon mitochondrial energy generation and the total growth rate decreases.

Glucose has been shown to repress the function of the respiratory chain enzymes (Crabtree Effect) and it is uncertain as to what role this repression plays in the experiments described above. For this reason it is not possible to draw any real conclusions about the synthesis of mitochondrial proteins from these data. However, the constant logarithmic rate of respiratory ability increase may indicate that some limiting substance in the mitochondrion is being synthesized at a steady logarithmic rate. If this is so, then this synthesis is not responding to the exhaustion of the primary carbon source or the transition to ethanol metabolism. The rate change in Fig. 2(v) occurs at similar times in all the cultures, suggesting that this is the point at which the yeast finally becomes

dependent on mitochondrial energy production, and therefore the point at which the fermentative utilization of the primary carbon source finally ceases. Other workers[64] have observed that the amount of stored carbohydrates varied with carbon source, and that the fastest fermentative growth condition (glucose) gives rise to the greatest deposition of carbohydrates. This phenomenon may explain the apparent inconsistency between the rate transition in Fig. 2(v) and the ethanol utilization curves show in Fig. 2(iii).

In summary, it is clear that the fermentative carbon source has a very great effect upon the physiological characteristics of yeast growth, and that these differences are further compounded by the anaerobic/aerobic modes of growth available to the yeast.

CHAPTER 3

The Effect of Carbon Source and Anaerobiosis on the Loss of Respiratory Functions.

(i) Preliminary experiments

In these experiments yeast were grown aerobically to mid-fermentative phase, harvested, and then re-inoculated for anaerobic growth on the same or a different carbon source. After transfer the changes in dry weight, Q_{O_2}, malic dehydrogenase (MDH) and cytochrome oxidase (cyt. c. oxid.) were monitored. The Q_{O_2} was taken to be a measure of the functional integrity of those mitochondria present in the yeast before transfer and was determined on aerated culture samples as described in the Methods. MDH was chosen as being an enzyme representative of those which are most probably coded for by nuclear DNA, synthesized in the cytoplasm and then integrated into the mitochondrion.

TABLE 3 (I)

Transfer	1st growth condition	2nd growth condition	Condition tested
1	2.7% galactose aerobic	2.7% galactose aerobic	slight repression aerobic control
2	2.7% galactose aerobic	2.7% galactose anaerobic	slight repression + anaerobiosis
3	2.7% galactose aerobic	2.7% glucose anaerobic	strong repression + anaerobiosis
4	2.7% glucose aerobic	2.7% glucose anaerobic	anaerobiosis on repressed cells

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Fig. 3(I), 3(II), 3(III) and 3(Iv).

Yeast were initially grown aerobically to mid exponential phase on 2.7% galactose Y.An. medium. At this point the cells were harvested and inoculated into a second growth condition to a concentration of 4 gms. wet weight/litre. The initial and secondary growth conditions, together with the conditions tested are listed in Table 3(I) p. 42.

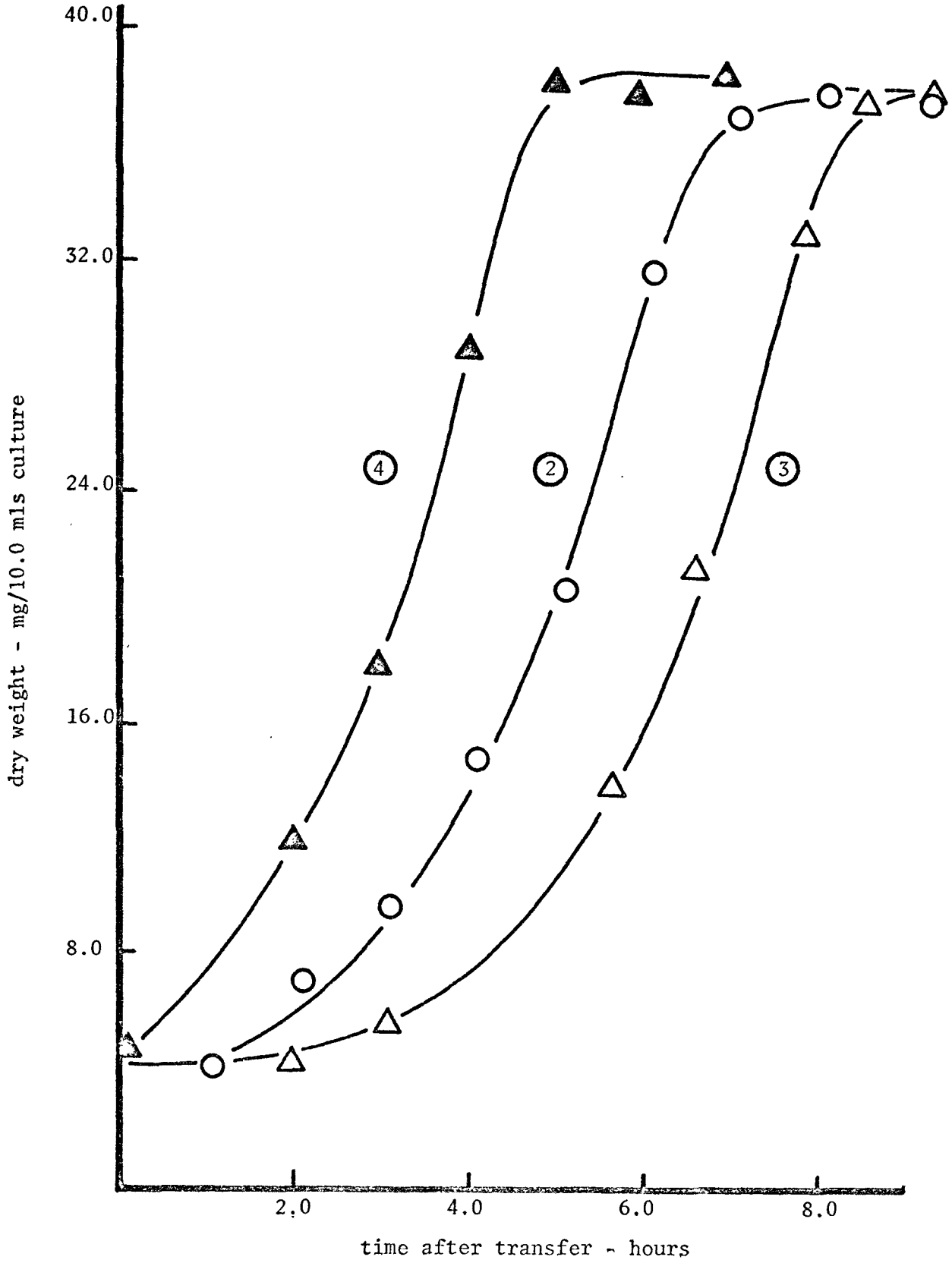

Fig. 3(i) Anaerobic growth curves: transfers 2,3, and 4.

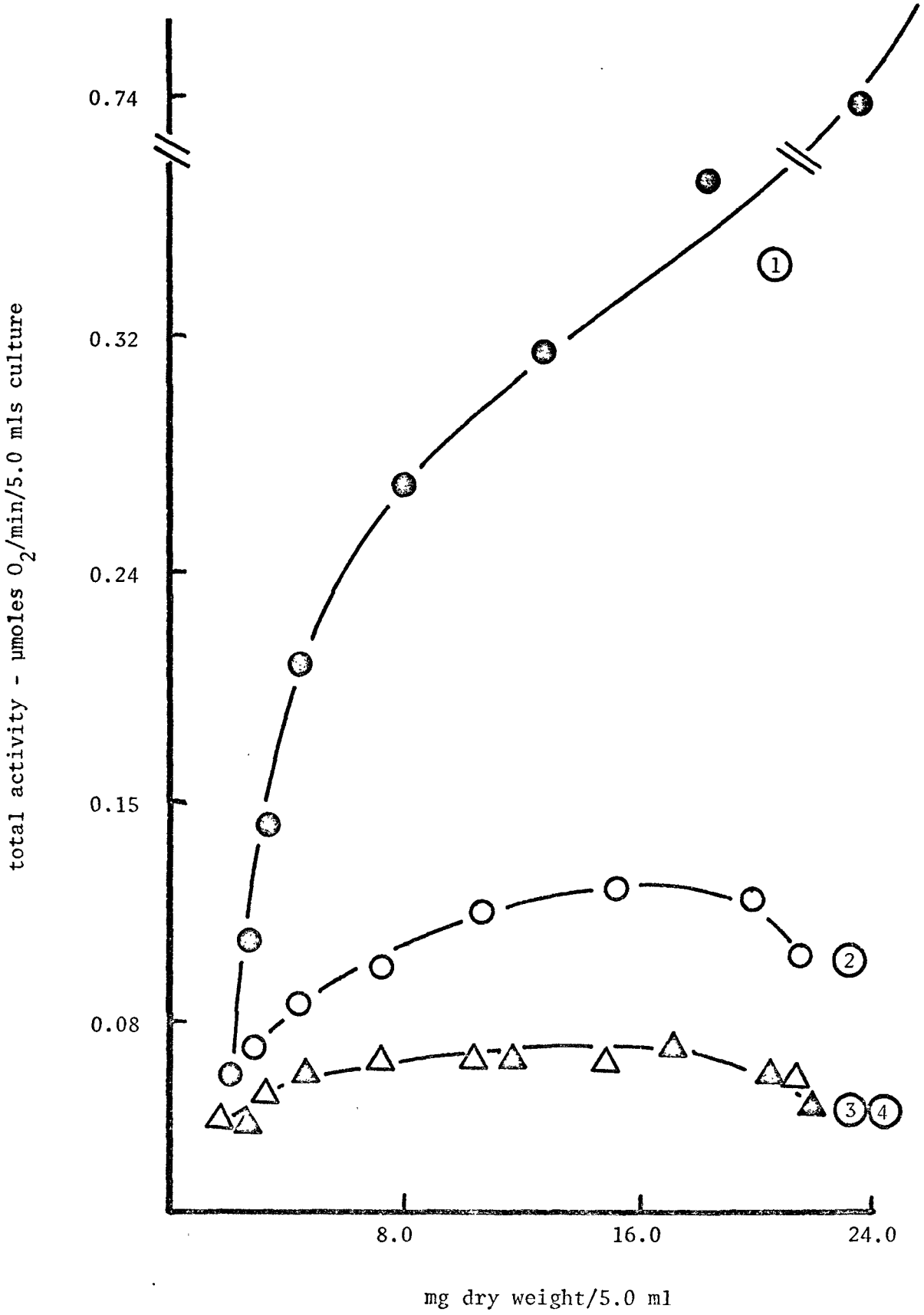

Fig 3(ii) Aerobic → Anaerobic Transfers: Total Respiratory Ability

Cyt. c. oxidase was chosen as representative of those enzymes of mitochondrial origin which are coded for by mitochondrial DNA and synthesized in the mitochondrion . The transfers performed are summarized in the Table above.

Since yeast utilize glucose and galactose at different rates the results of the transfers are compared in terms of dry weight in order to normalize the activities to the same stage of physiological development [cf. Fig. 3(i)]. All curves show an increase in total respiratory ability, indicating that there is no net destruction of oxidative activity (i.e. mitochondria). The initial Q_{O_2} and subsequent total respiratory ability of the galactose aerobic cells transferred to anaerobic conditions are both conditioned by the carbon source in the second growth condition. The initial Q_{O_2}'s in the control and galactose aerobic → galactose anaerobic transfers are however almost identical, suggesting that growth is required to initiate the anaerobic conditioning [Fig. 3(ii)].

The specific activities for cyt. c. oxid. activity are shown in Fig. 3(iii). The two curves for transfers 2 and 3 are parallel and the differences between the initial and final activities are very similar, suggesting that the atmospheric condition, rather than the carbon source, is controlling the synthesis of this enzyme. There is a maintenance of specific activity in transfer 4 which suggests that cyt. c. oxidase is being synthesized under anaerobic-repressed growth conditions.

Fig. 3(iv) shows the change in the specific activity of MDH

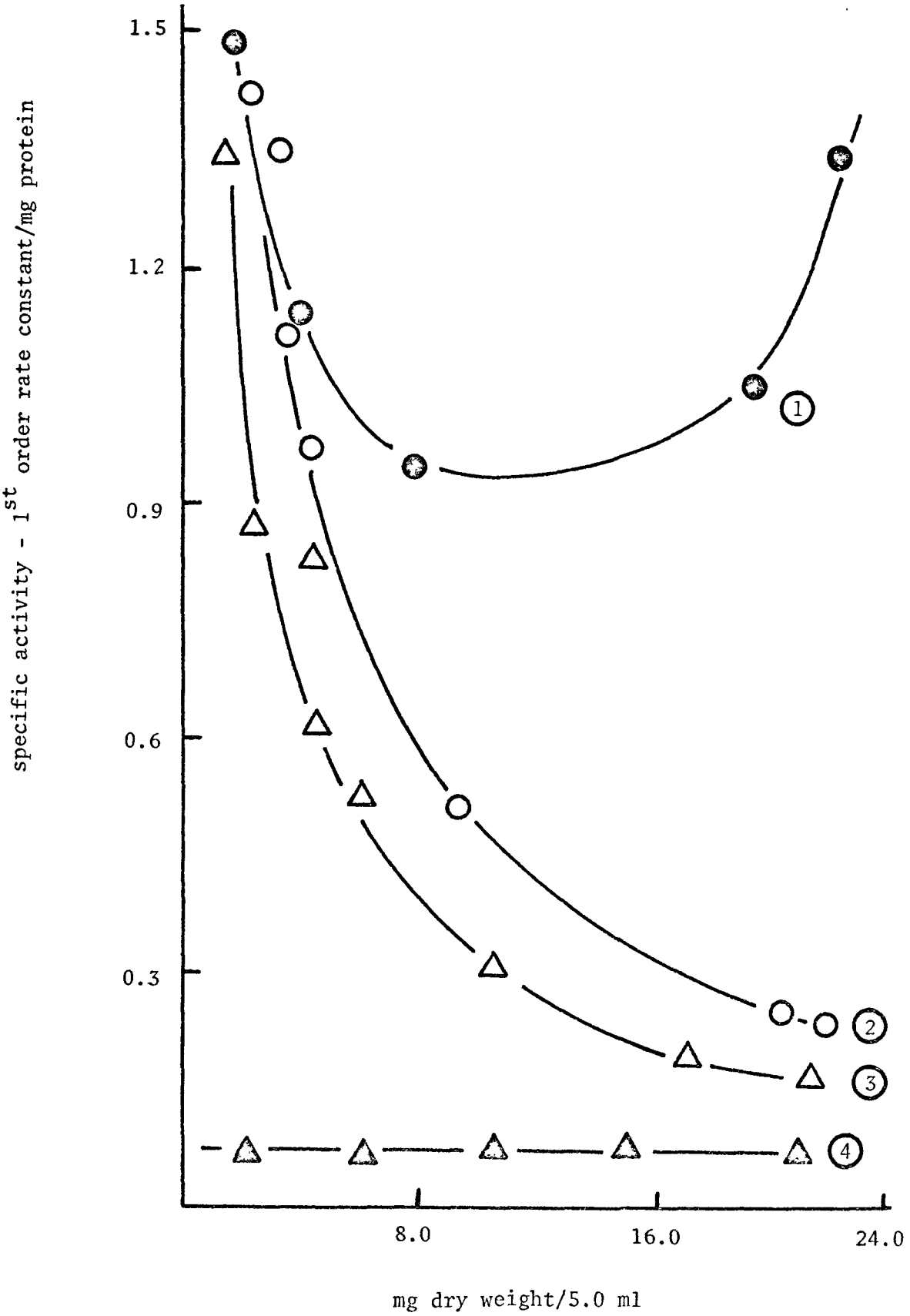

Fig 3(iii) Aerobic → Anaerobic Transfers: Cytochrome Oxidase

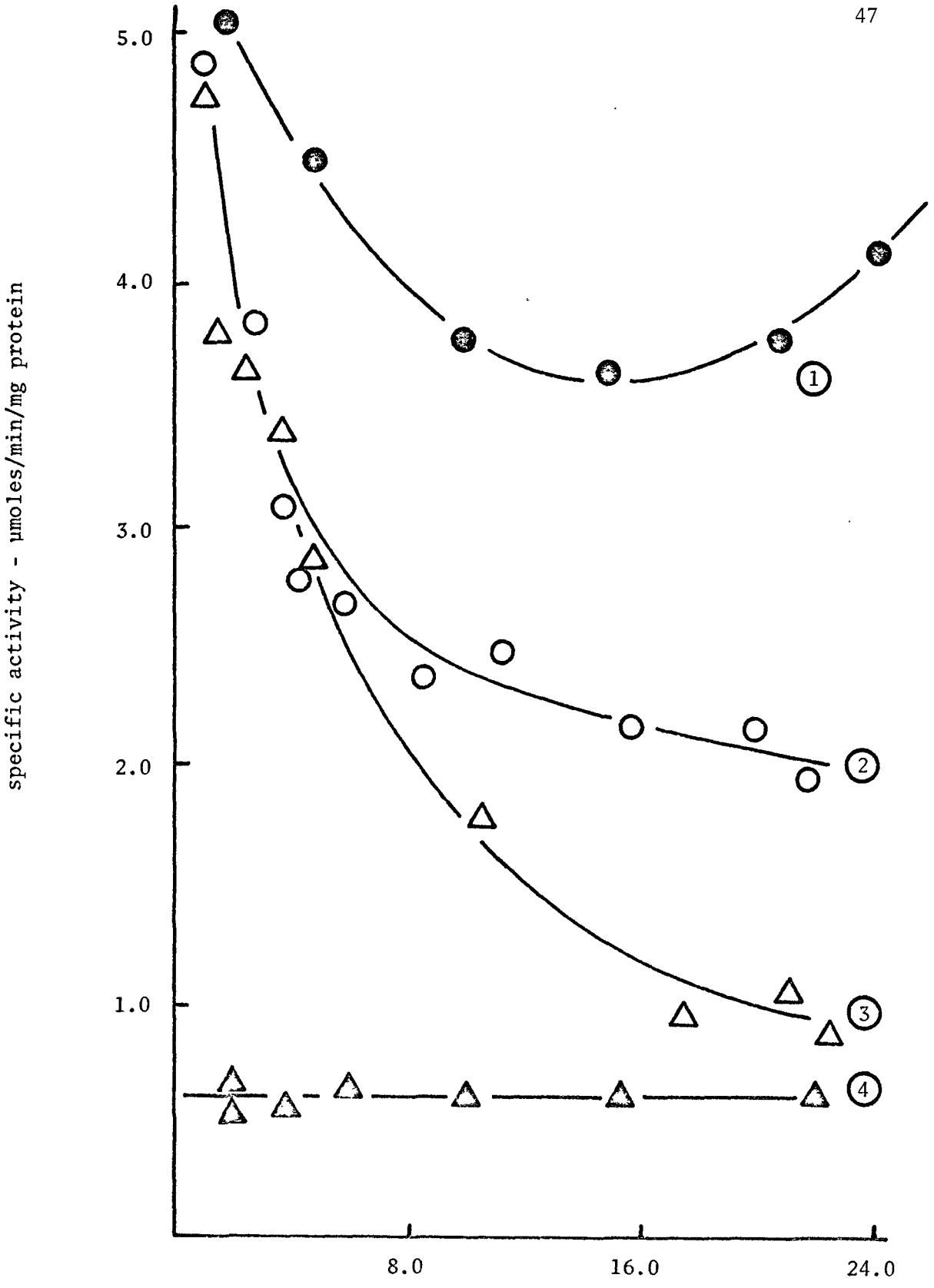

Fig 3(iv) Aerobic → Anaerobic Transfers: Malic Dehydrogenase

for the same experiments. There is an obvious difference between all of the conditions. The degree to which the specific activity is repressed is 3 > 2 > 1. The maintenance of specific activity in transfer 4 strongly suggests that synthesis of this enzyme is also taking place under anaerobic-repressed growth conditions.

(ii) A Further Study of Transfer 4

It was thought that one way to prove that synthesis is taking place under anaerobic-repressed conditions was to determine the total activity of the mitochondrial enzymes in the culture. To this end, transfer 4 was repeated and total activities of several other key enzymes were also determined. Pyruvate decarboxylase, hexokinase and glucose-6-phosphate dehydrogenase were determined in addition to MDH and cyt. c oxidase in order to provide a control on the methodology. Glucose-6-phosphate dehydrogenase is plotted in both Figs. 3(v) A and B and Figs, 3 (vi) A and B to illustrate the scale involved in the rates of synthesis of the two fermentative enzymes [Fig (v) B] compared to the rates of synthesis of the two mitochondrial enzymes [Fig. (vi) B] under anaerobic-repressed conditions. In contrast to the transfer 4 previously described in this experiment the aerobic cells were harvested about 0.5 hours after de-repression had commenced [cf. Fig. 3 (vi) A, MDH curves]. The correlation between specific activity and synthetic rates amply illustrate the validity of this experimental approach.

In Fig (v) B pyruvate decarboxylase synthesis accelerates to a very fast and steady rate. This is reflected in the specific

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Fig. 3(v) A and B and Fig. 3(vi) A and B.

In a more detailed investigation of transfer 4 [Table 3(i)], cells were grown initially on 2.7% glucose, Y.An.O_2 to the end of the fermentative phase, harvested and transferred into 2.7% glucose, Y.An.N_2 growth conditions. Malic dehydrogenase curve #1 derives from Fig. 3(iv) #4 and is included in Fig. 3(vi) A for purposes of comparison.

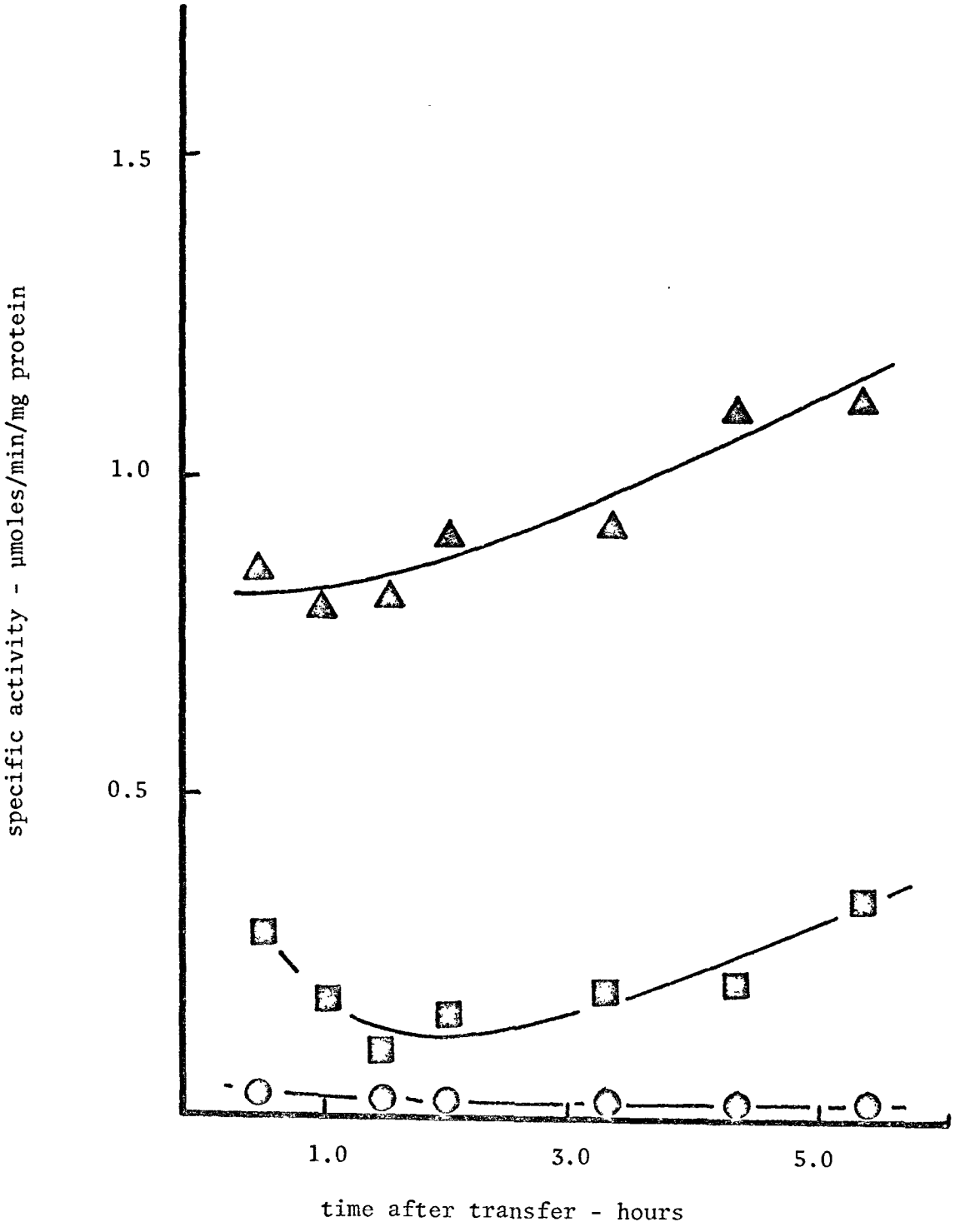

Fig 3(v) A Transfer 4 - specific activity

pyruvate decarboxylase – △ – hexokinase - □ -

glucose-6-PO_4 dehydrogenase - ○ -

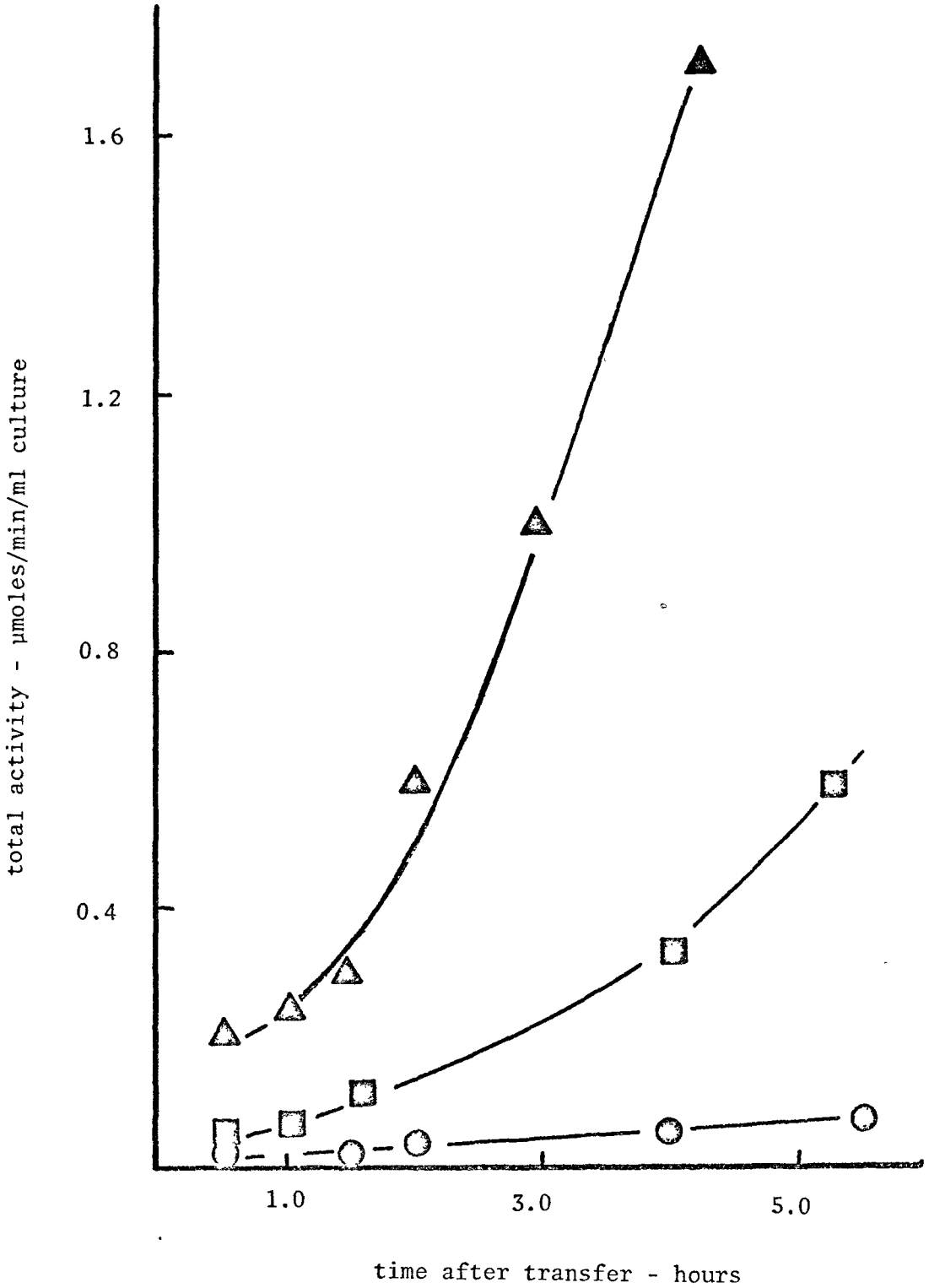

Fig 3(v) B Transfer 4, total activities

pyruvate decarboxylase - △ - - □ - hexokinase

glucose-6-PO_4 dehydrogenase - ○ -

activity as an initially steady state followed by an increasing specific activity. At a later stage of anaerobic growth (3.0 hrs) the synthetic rate for hexokinase is increased, halting the loss of specific activity and returning this activity to its initial level. The same changes on a smaller scale, are undergone by glucose-6-phosphate dehydrogenase [Fig. (v) and (vi)].

Similar interpretations may be applied to the curves shown in Fig. (vi) A and B for MDH and cyt. c. oxidase. As synthesis of MDH adjusts to the new conditions, the specific activity of the enzyme approaches the steady state level appropriate to this physiological condition. The MDH curve from Fig. 3 (iii) is included for comparison. The cyt. c. oxidase activities are expressed as 1^{st} order rate constant and are not directly comparable to those for MDH, but the kinetics observed demonstrate that synthesis of cyt. c. oxidase is taking place under anaerobic-repressed growth conditions.

Discussion

The data from the comparative transfers [Figs. 3 (i) → (iv)] suggests that there are two factors which are exercising independent control over the synthesis of mitochondrial enzymes. MDH is an enzyme which is involved in various anapleurotic functions, and it seems reasonable to suppose that the synthesis of this, and similar mitochondrial enzymes should be sensitive to the general metabolic requirements of the cell. In contrast to this, cyt. c. oxid. is a specific enzyme, concerned with the transfer of electrons to oxygen,

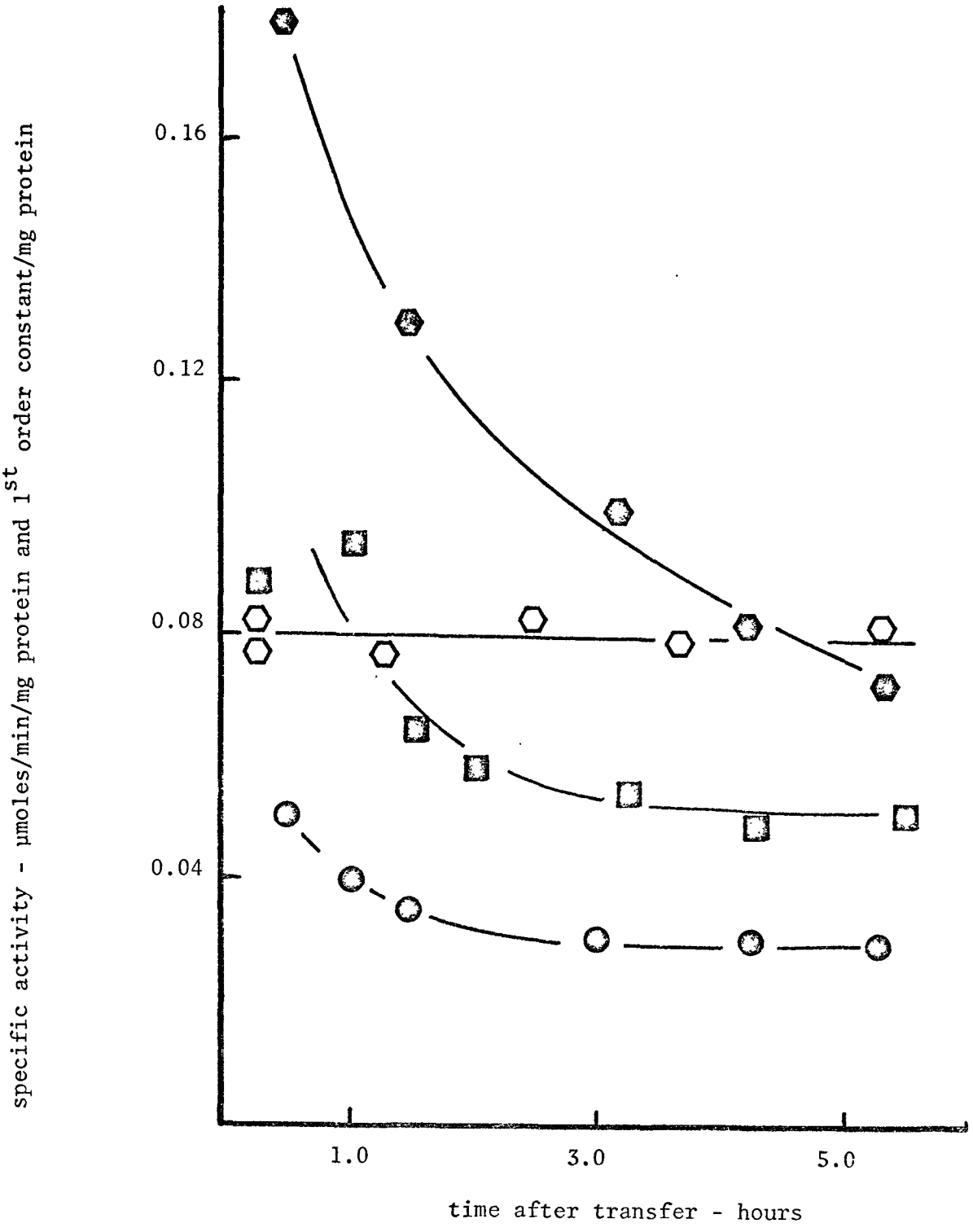

Fig 3(vi) A Transfer 4 - specific activities

- ○ - glucose-6-PO_4 dehydrogenase - □ - cytochrome c oxidase

- ⬡ - malic dehydrogenase - expt #1, - ⬡ - malic dehydrogenase - expt #2

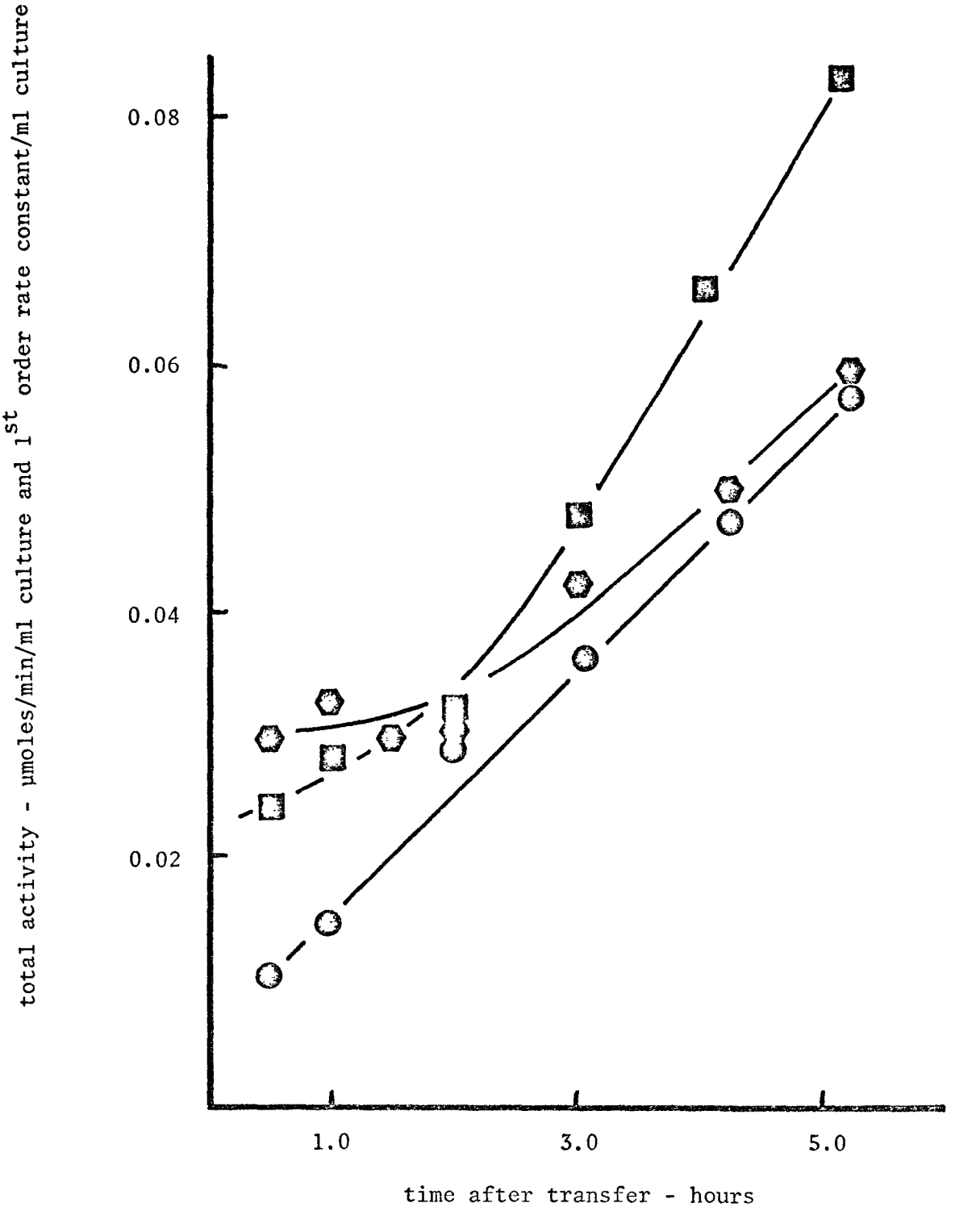

Fig 3(vi) B Total activities, transfer 4

- malic dehydrogenase
- cytochrome c oxidase
- glucose-6-PO_4 dehydrogenase

At first sight it would appear that the synthesis of cyt. c. oxidase is induced by oxygen, but this does not explain the data for transfer 4 [Figs. 3(vi) A and B]. The alternative explanation is that the cyt. c. oxidase synthesizing system is responding to its immediate metabolic environment, and that its functioning under aerobic conditions alters this environment in such a way as to make it more favourable to cyt. c. oxidase synthesis. This in turn implies that the cyt. c. oxidase synthesizing system is free of direct nuclear control and is not responding to the specific metabolic needs of the cell, as is the MDH synthesizing system. Thus one may conclude that oxygen induction is not a necessary condition for the synthesizing of cyt. c. oxid., but that the mitochondrial information - synthetic system responds to the cellular environment in which it is functioning.

CHAPTER 4

The Direct Effects of Chloramphenicol on Cellular Metabolism

(i) The Effect of Chloramphenicol on Growth Kinetics and Respiration in Whole Yeast and Isolated Mitochondria.

The experiments described below were designed to describe and delineate the effects of the D(-) and L(+) *threo* isomers of chloramphenicol (CAP) on growth and *in vivo* respiration in whole yeast, and the *in vitro* respiration of isolated yeast mitochondria.

5% glucose $YAnO_2$ growth conditions were chosen for these experiments. Clark-Walker and Linnane[65] published experiments indicating that under their experimental conditions, CAP did not cause any further inhibition in glucose repressed (5%) yeast. Preliminary experiments in this laboratory utilizing 2.7% glucose $YAnO_2$ conditions suggested that this might not be true. Also, the yield/ml of culture is much higher on 5% glucose and this facilitated the fast sampling necessary for the metabolic intermediate study reported in this Chapter. Experimentally, the 5% glucose $YAnO_2$ condition was the most convenient and it is also the most repressed condition previously studied. The differential effects of CAP are apparent throughout the aerobic growth cycle, but in order to facilitate the expression of results, only that part of the growth curve from mid-fermentative phase → stationary phase is presented here.

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Figs. 4(I) to 4(III) B.

Yeast were harvested from 48 hr. maintenance slopes and inoculated into 5% glucose, Y.An. medium. Aeration was by vigorous shaking: D (-) and L (+) chloramphenicol (CAP) were added at 4.0 mg/ml as indicated.

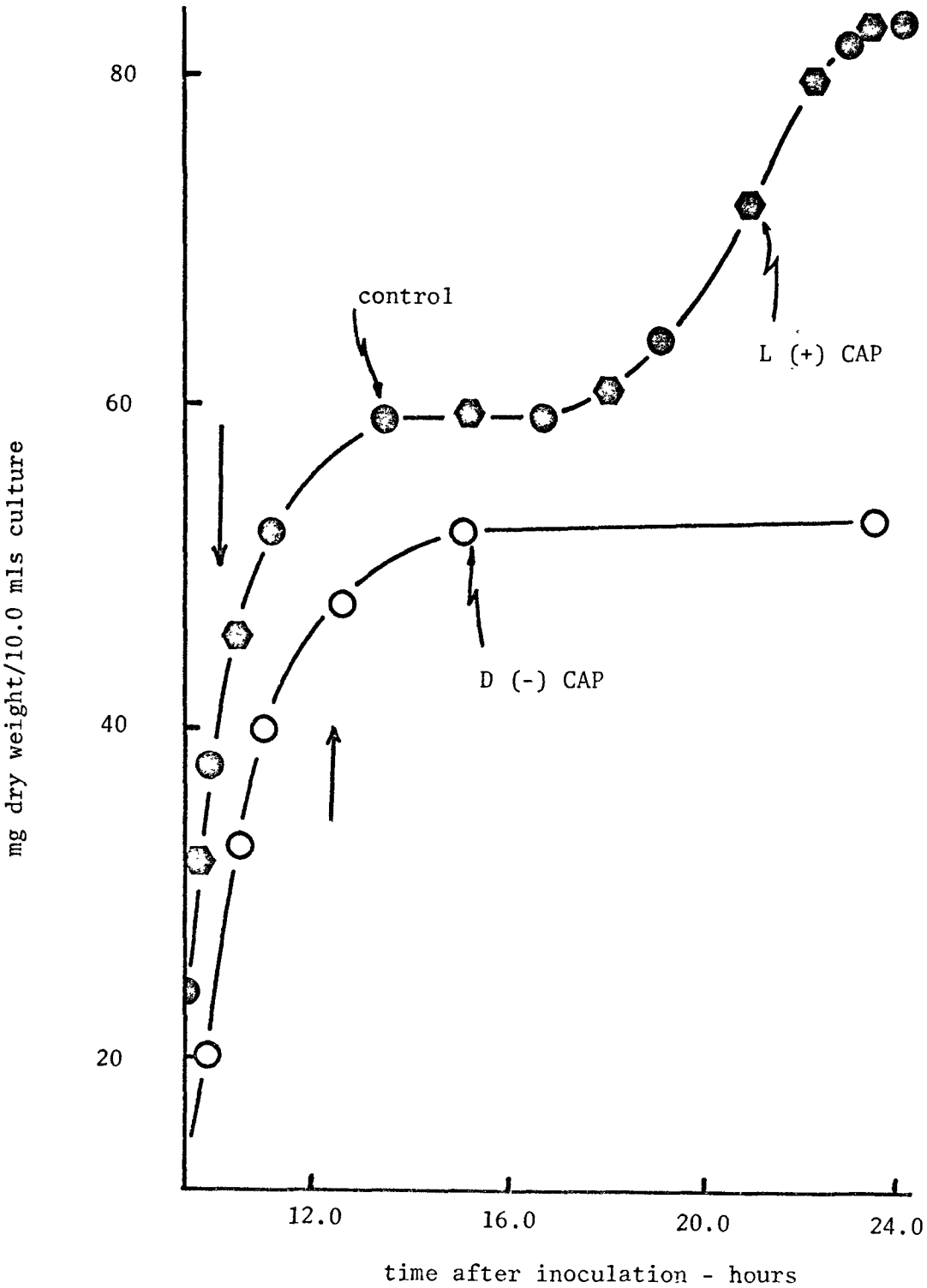

Fig 4(i) Aerobic growth curve: 5% glucose Y.An.O_2

in all the following Fig 4 diagrams the arrows indicate glucose exhaustion.

Effects of Chloramphenicol on Aerobic Growth

A comparison of the growth curves [Fig. 4(i)] of yeast grown in the absence and presence of either *threo* isomer of chloramphenicol at a concentration of 4.0 mg/ml of medium revealed striking differences (contrast Fig. I, Clark-Walker and Linnane[65]). Cells grown in the presence of D(-) chloramphenicol demonstrated a longer lag phase (by I 1/4 hr) than either the control or L(+) chloramphenicol grown cells. This difference was further emphasized by the fact that complete glucose utilization was similarly out of phase. It will be noted that the control curve is diphasic. The initial exponential phase reflects growth which occurs at the expense of fermentation of glucose to ethanol [cf. Fig. 4(ii)]. When glucose is exhausted from the medium, an intermediate lag phase in growth occurs, during which time more ethanol accumulated but apparently could not be utilized as a primary energy source [cf. Fig. 2(iii)]. Further growth, exemplified by the secondary exponential phase, commences only when a new oxidative enzymic potential is achieved (contre-Pasteur effect[66]) and then the accumulated alcohol is metabolized [cf. Fig. 2(iii)]. This metabolism promotes further cell proliferation until final stationary phase conditions are achieved (22 hrs). Although ethanol is still present in the medium further cell growth is "shut off" due to the exhaustion of some other medium constituent.

In the presence of D(-) chloramphenicol the secondary respiratory phase of cell growth is never reached and the shape of this curve is similar to a classical anaerobic growth curve [Fig. 2(i)]. The yield of growth of these inhibited cells was approximately 65%

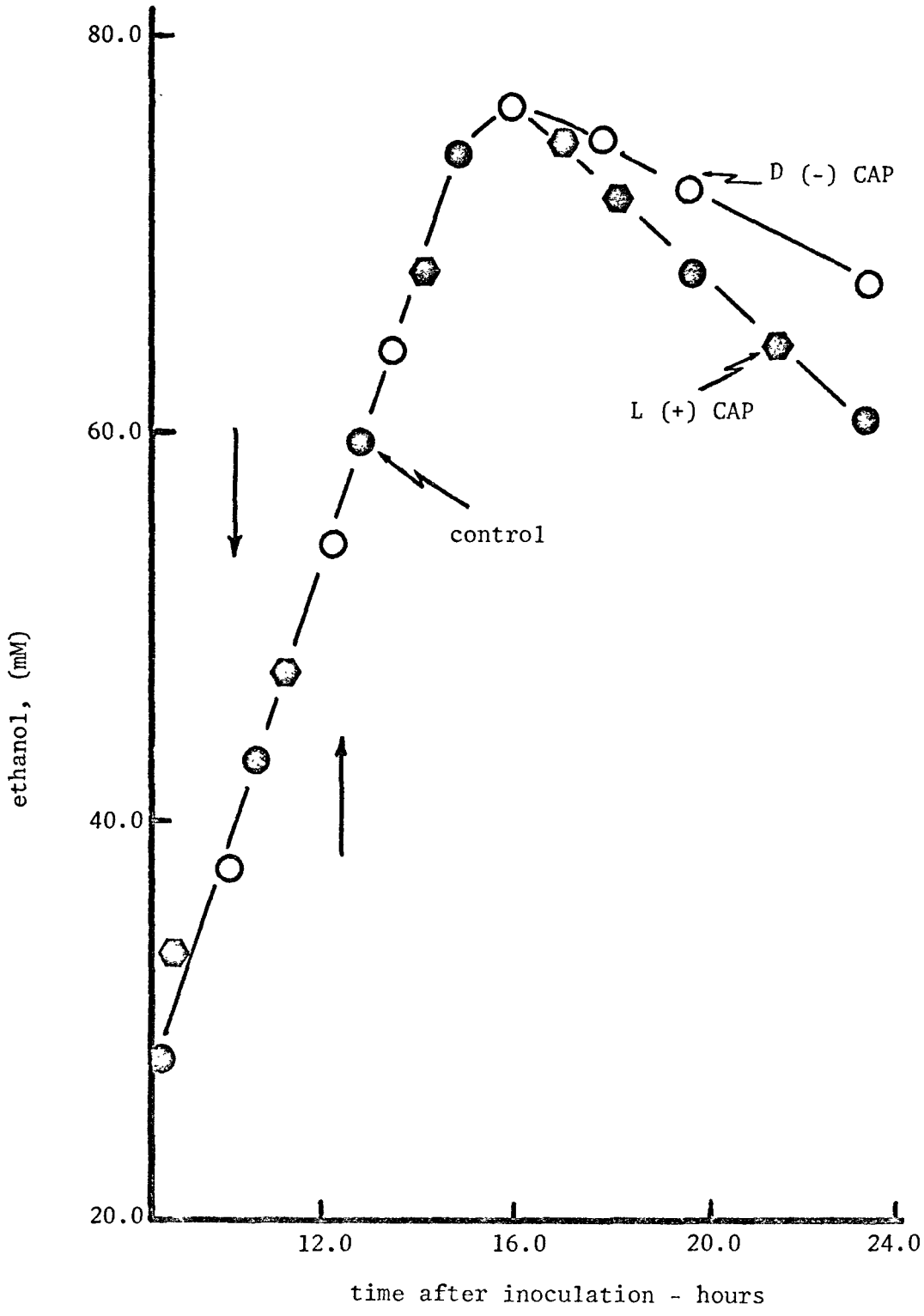

Fig 4(ii) Ethanol metabolism, 5% glucose Y.An.O_2

of the control stationary cells. This isomer of the antibiotic also caused the yeast to produce more ethanol per unit time than for the control, and then inhibited further ethanol metabolism. In contrast the L(+) *threo* isomer of chloramphenicol did not affect either the growth kinetics or the production and utilization of ethanol.

The respiratory ability of these different cell populations is illustrated in Fig. 4(iii) A and B. From the Q_{O_2} data [Fig. 4 (iii) A] it can be seen that cells grown on glucose alone are fully repressed (cf. Utter *et al*[21]) during their fermentative phase [cf. Fig. 2(iv)] and then become oxidatively functional when glucose is exhausted from the medium. This de-repression phenomenon occurs late in the initial exponential phase of growth and maximinizes during the secondary lag phase. When final stationary growth conditions are attained (22 hrs) the respiratory ability of these cells is markedly decreased and is of the same magnitude as repressed or fermenting cells. In the presence of D(-) chloramphenicol on the other hand, the respiratory rate associated with fermentation is only a third of the control rate. The anticipated rise in Q_{O_2} associated with carbon source de-repression does not develop and on the contrary, the Q_{O_2} drops to almost zero. When this data is expressed as total activity [Fig. 4(iii) B] it will be seen that these inhibited cells do in fact respire at a steady rate, indicating that the Q_{O_2} decrease is due to dilution by increasing cell mass. Control cells maintain net synthetic kinetics (upward slope of curve) followed by "degradation" of oxidative function which is conditioned by stationary phase con-

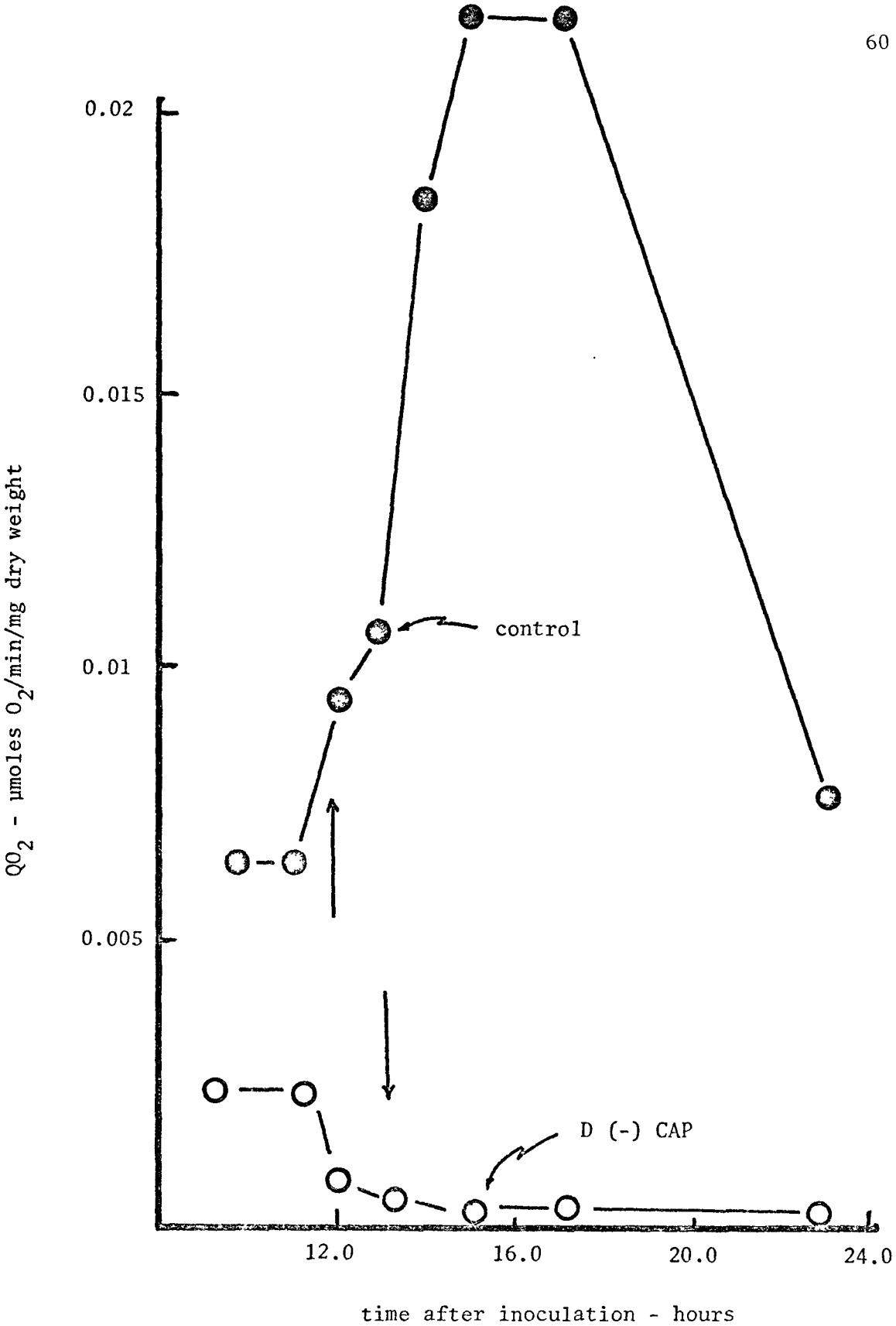

Fig 4(iii) A Respiratory ability (QO_2),
5% glucose Y.An.O_2

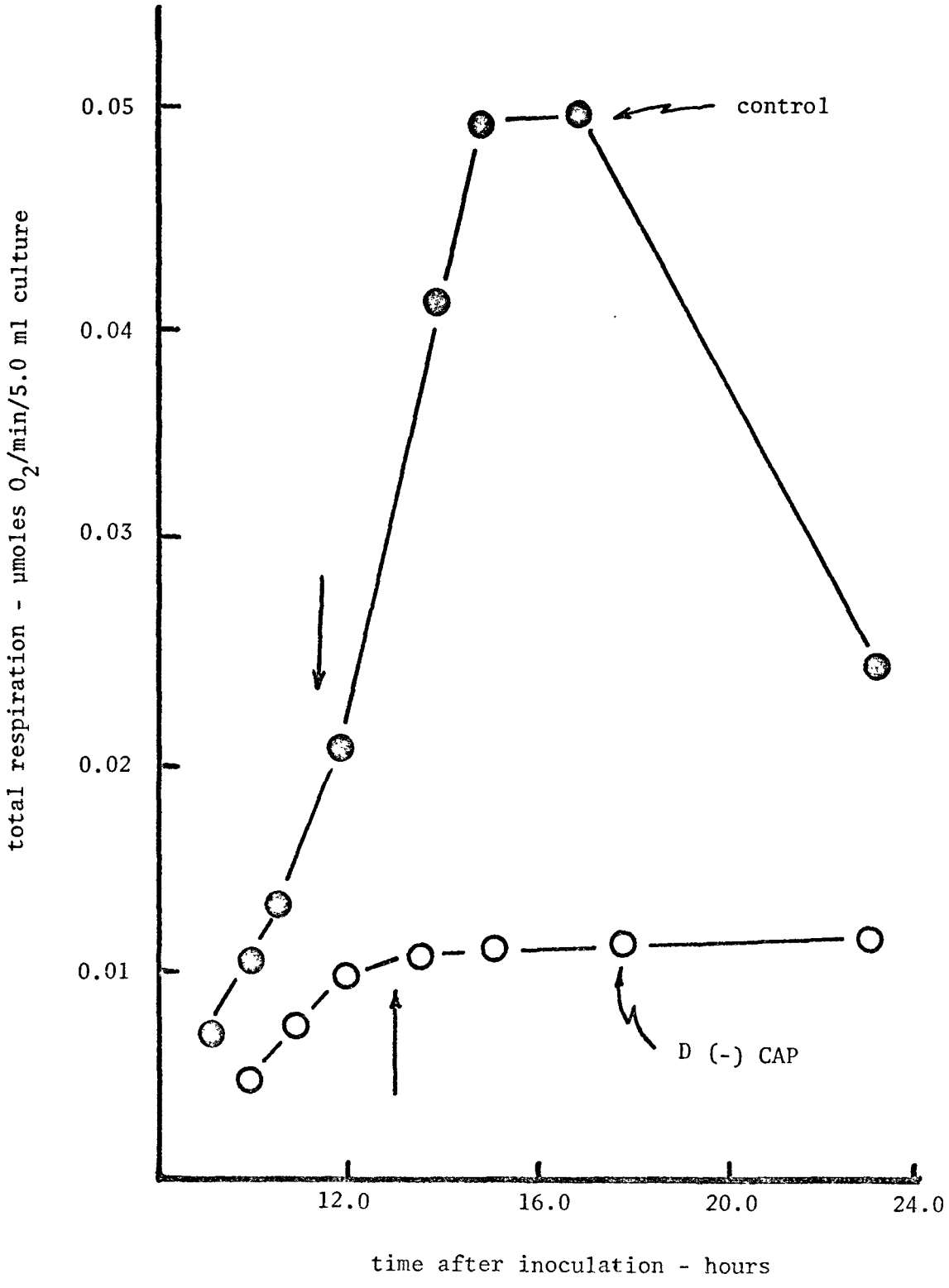

Fig 4(iii) B Total respiratory ability, 5% glucose Y.An.O_2

ditions.

Effect of ethanol transfer on growth kinetics and respiration

The inhibition of respiration of yeast grown on glucose in the presence of D(-) chloramphenicol may be due to either a direct effect on the respiratory apparatus or to an indirect effect upon the assembly of this respiratory apparatus (i.e. protein synthesis). In order to resolve this paradigm, chloramphenicol was added to a yeast population which was de-repressed and had functioning oxidative organelles. To this end yeast were grown on 5% glucose medium into their secondary lag phase and then harvested at 30°. These de-repressed cells were used to inoculate two flasks both containing 5% ethanol anaerobic media, with one of the flasks supplemented with 4.0 mg/ml D(-) chloramphenicol. The results of these experiments are shown in Fig. 4(iv) and Fig. 4(v) A and B. Fig. 4(iv) clearly shows that these de-repressed transferred cells accommodate very slowly to ethanol under the experimental conditions and that in the presence of the antibiotic growth is further suppressed. The respiratory ability of the control cells [Fig. 4(iv) A] after transfer to ethanol continued to increase and showed that de-repression is fully affected in the presence of this end product of fermentation. Cells transferred to the ethanol medium containing the drug began to lose their respiratory capability (Q_{O_2}) within 4 hours. When these results are replotted as total respiratory ability, rather than as Q_{O_2} [Fig. 4(v) A], cells grown in the ethanol medium rapidly demonstrated a positive synthetic response to their environment which continued for 7 hours and then leveled off. Chloramphenicol-grown

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Figs. 4(iv) to 4(v) B.

Yeast were grown aerobically for 16.0 hrs. in 5% glucose Y.An. medium, harvested and re-inoculated at a concentration of 4.0 mg. wet weight/litre into 5% ethanol Y.An. medium. Growth was continued aerobically. This constitutes a transfer of partially de-repressed glucose-grown yeast into aerobic, non-fermentable conditions. D (-) chloramphenicol (CAP) was added to a concentration of 4.0 mg/ml.

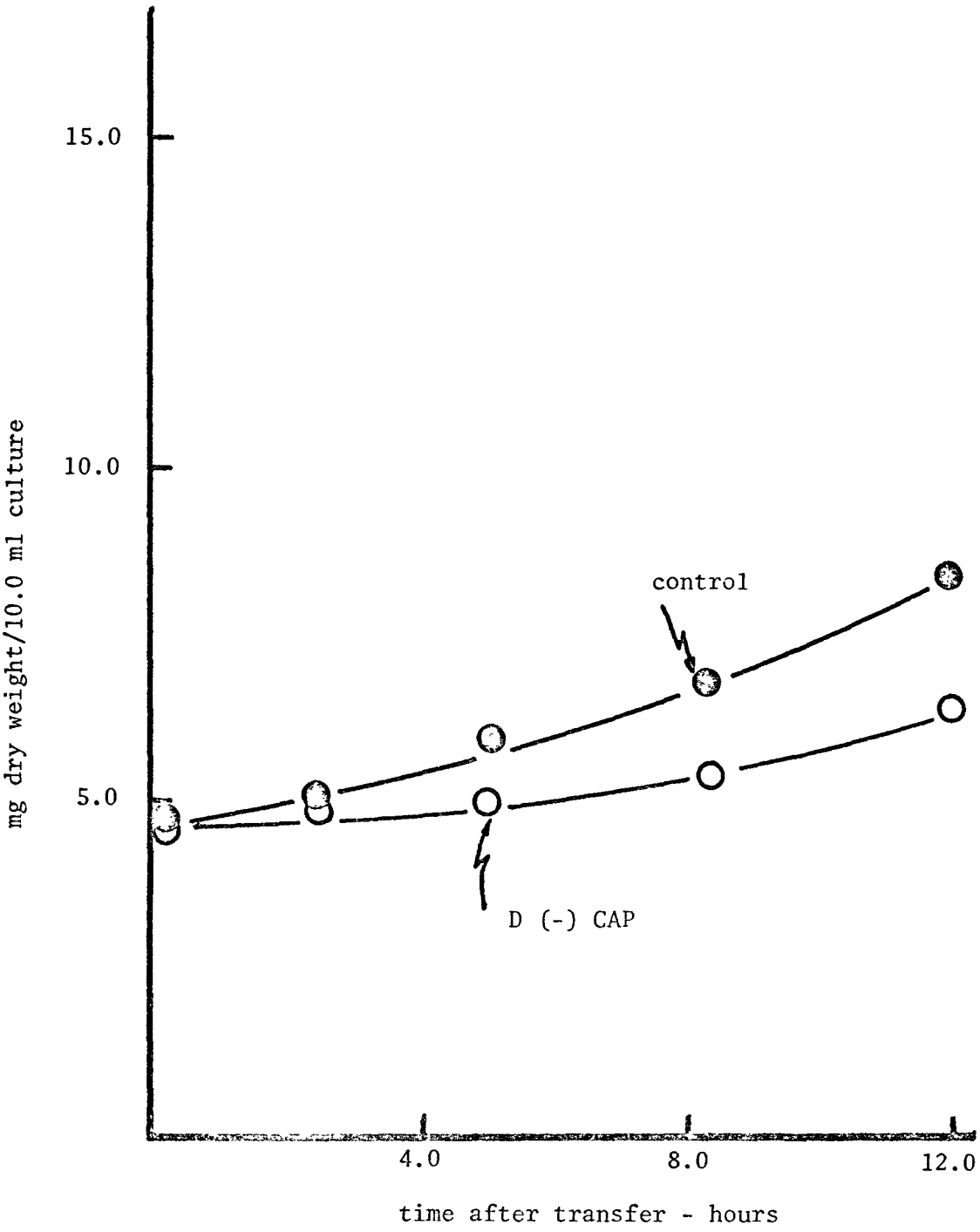

Fig 4(iv) Growth curve, 5% glucose Y.An.O_2 ⟶

5% ethanol Y.An.O_2

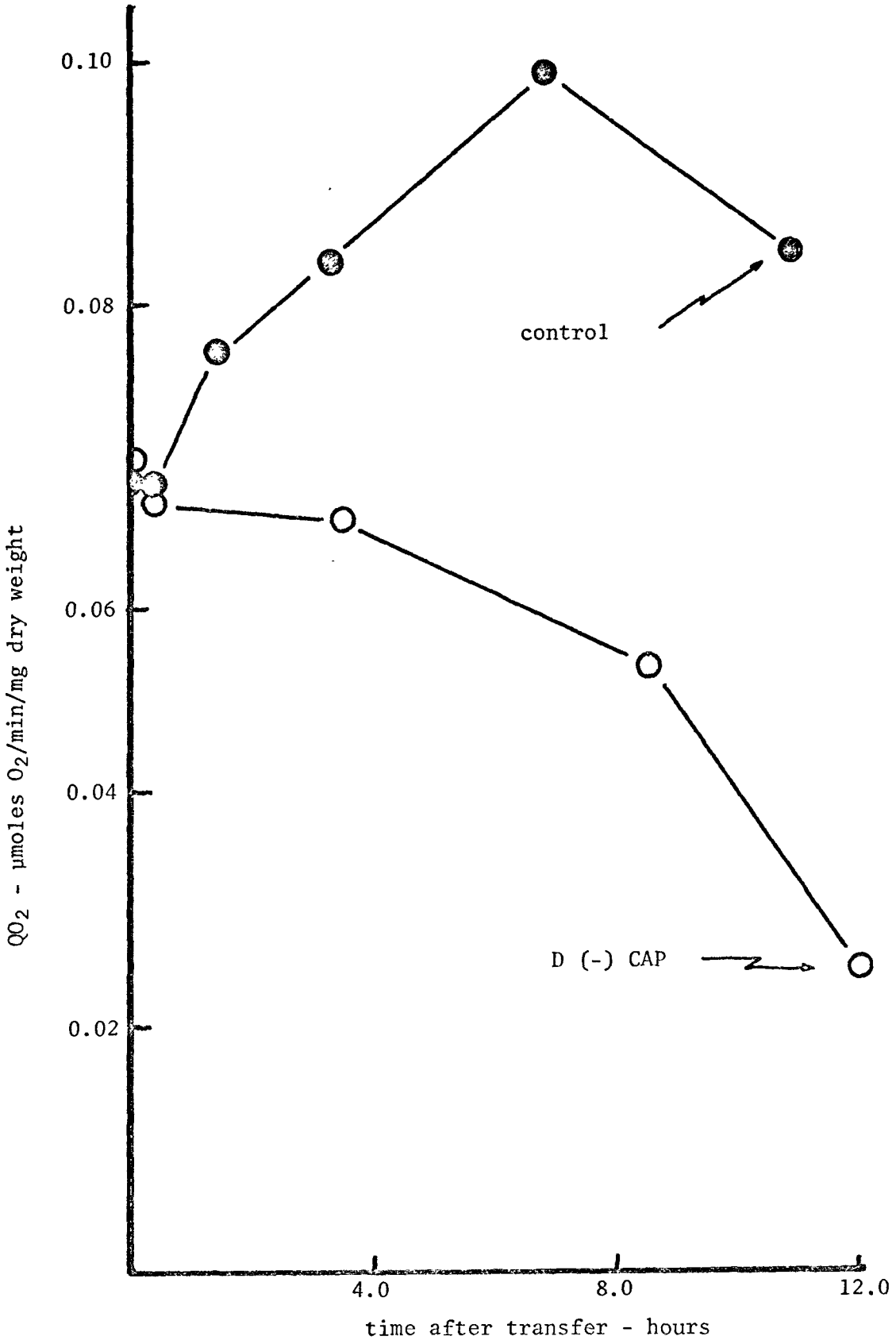

Fig 4(v) A Respiratory ability (QO_2), glucose →ethanol transfer

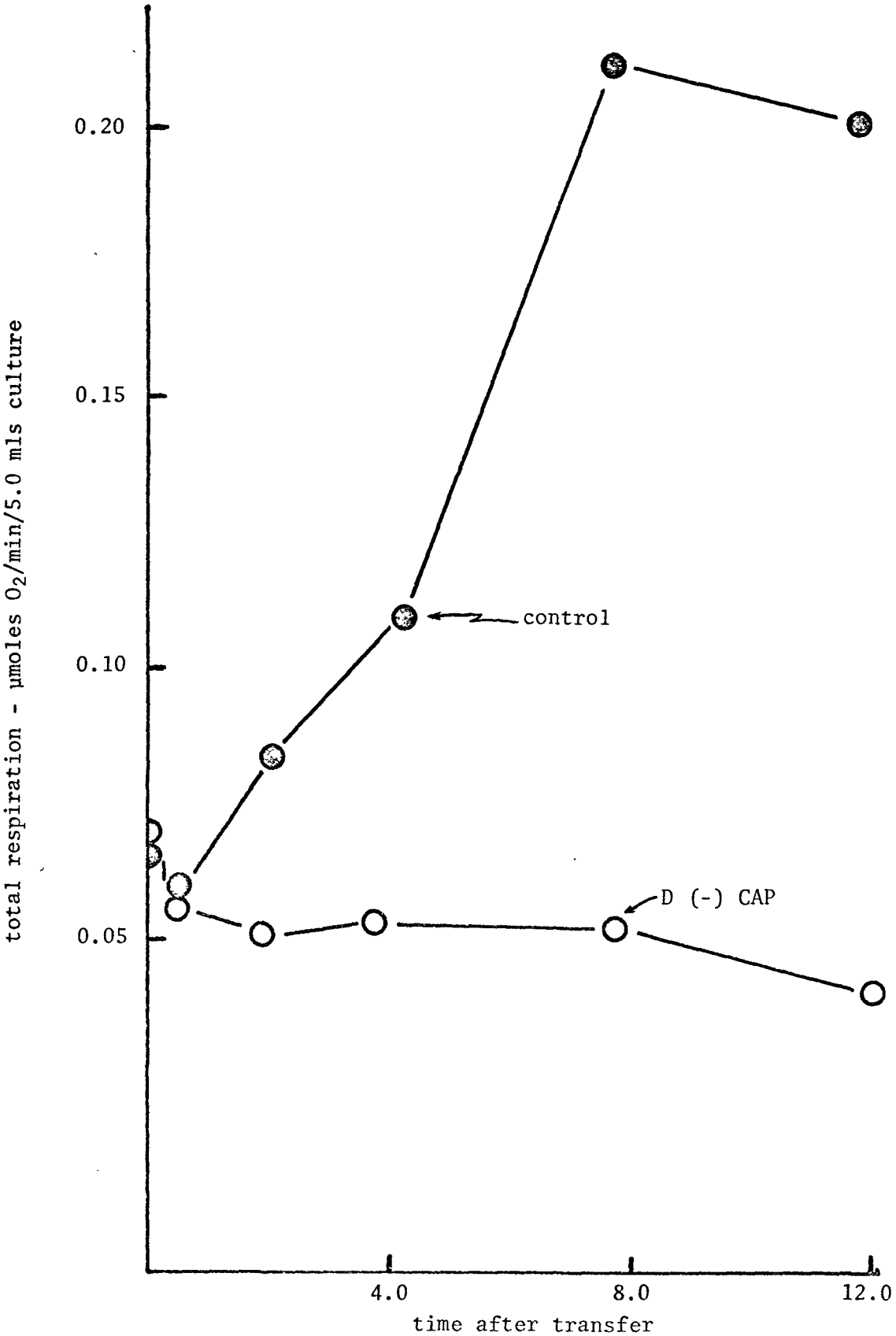

Fig 4(v) B Total respiratory ability, glucose → ethanol transfer

cells on the other hand maintained a constant rate of respiration with no net increase in activity. These results are identical to those obtained from cells grown initially in the presence of chloramphenicol utilizing glucose as the primary carbon source [Fig. 4 (iii) A and B]. These results tend to support the hypothesis that the assembly of the respiratory apparatus is being affected by the drug.

The effect of chloramphenicol on the oxidative and phosphorylative activity of isolated mitochondria.

In order to localize the site of activity of chloramphenicol, experiments were carried out with isolated yeast mitochondria. The oxidation of various NAD- linked citric acid cycle substrates were measured polarographically and the results of some of these experiments are shown in Table 4I. These mitochondria [Fig. 4(ix)] are coupled and functionally similar to those found by Ohnishi *et al.*[67]. The ADP/O ratios and respiratory control (R.C.) values for yeast oxidizing NADH, succinate, isocitrate, oxo-glutarate and ethanol were found to be 1.4, 2.2; 1.5, 1.7; 1.5, 1.1; 2.5, 5.6; and 1.6, 1.4. The *threo* isomers of chloramphenicol, D(-) and L(+) did not alter the oxidative rate of any of the substrates tested, even though the relative concentrations of the antibiotics were much higher than those used in the whole cell experiments. The data for succinate and NADH oxidation are reported in Table 4 (I), the other substrates tested gave similar results. The stimulation of oxygen uptake by ethanol reflects mitochondrial ethanol oxidase activity

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Table 4(I) and Fig. 4(ix).

Cells were grown aerobically in 2.7% glucose for 20 hrs. (late respiratory phase) before harvesting. Mitochondria were isolated immediately, using the snail-gut enzyme - spheroplast technique. Table 4(I) shows the effects of D (-) and L (+) chloramphenicol (CAP) on the oxidation of NADH and succinate by these mitochondria. Fig. 4(ix) shows a typical polarographic technique from which ADP:O and R.C. were calculated.

TABLE 4 (I)

Effect of Chloramphenicol on the Oxidative Rates of Isolated Mitochondria

Yeast mitochondria were isolated and assayed for oxidative capacity polarographically as described in the method's section.

Addition	µAtoms O_2/min /mg protein
Succinate (3.4 mM)	0.34
" + ADP (0.6 mM)	0.52
" " + EtOH (55.0 mM)	0.61
" " " + D(-) CAP (2 mg/ml)	0.61
" " " + L(+) CAP (2 mg/ml)	0.60
NADH (0.5 mM)	0.49
" + ADP (0.6 mM)	1.13
" " + EtOH (55.0 mM)	1.47
" " " + D(-) CAP (2 mg/ml)	1.44
" " " + L(+) CAP (2 mg/ml)	1.44
Endogenous respiration	0.06
EtOH (55.0 mM) ADP (0.6 mM)	0.29

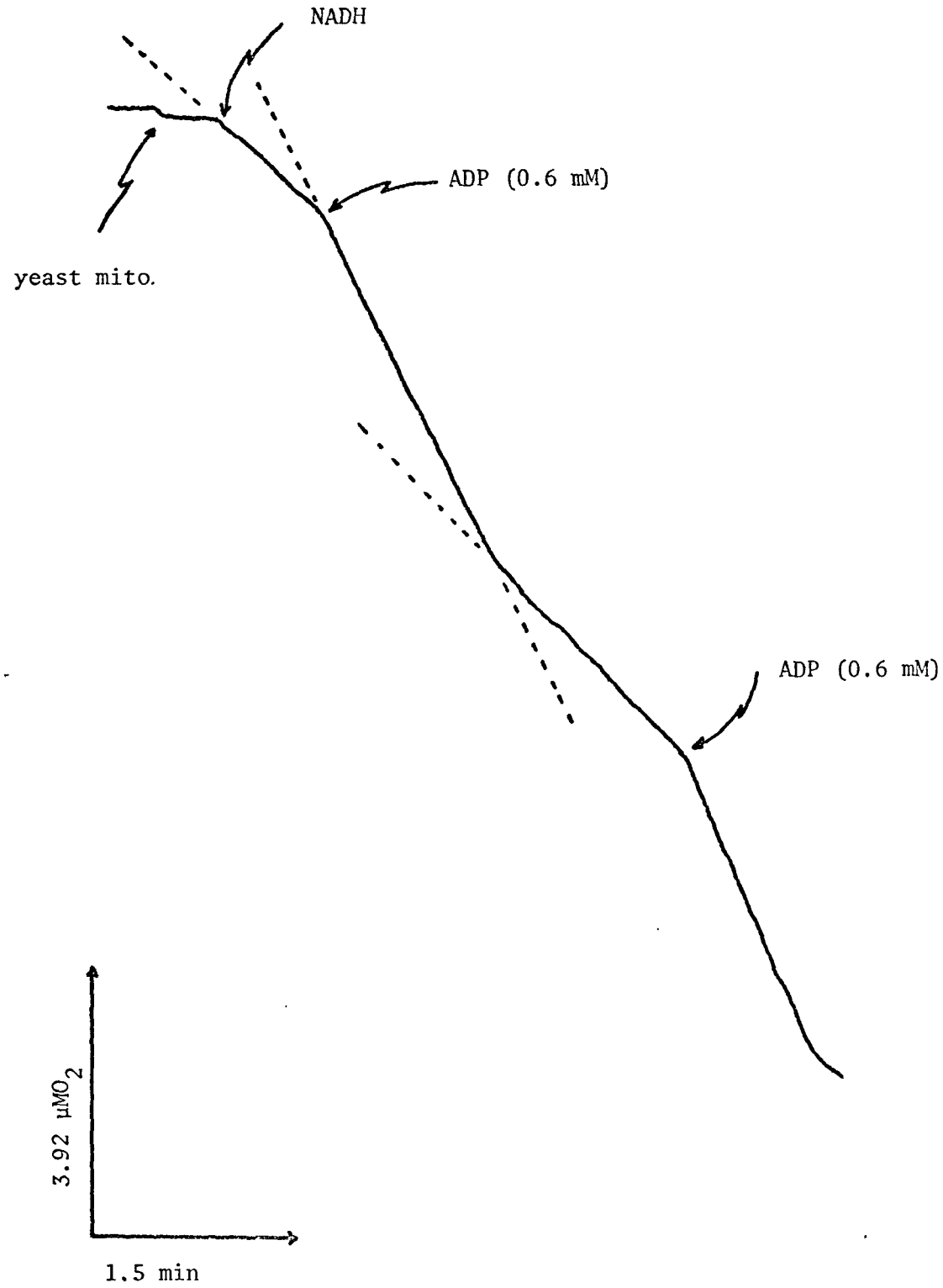

Fig 4(ix) Oxygen electrode trace - isolated mitochondria exhibiting respiratory control while oxidizing NADH

described by Ohnishi *et al*[67].

Discussion

Chloramphenicol has a dual effect on the physiologic response of yeast to glucose. During the fermentative or primary phase the antibiotic causes a prolongation of the lag phase of growth, loss of respiratory ability and a decreased production of cell mass per unit of fermentable substrate. This decreased respiratory ability [Fig. 4(iii) A] can be due to a direct or indirect effect of chloramphenicol and this type of experiment gives little or no insight into the mechanics of action of this drug. When "petite" strains of yeast are grown without this drug under similar conditions[68] the lag phase is increased and the yield decreased in comparison to respiratory competent strains. It is of interest that metabolic inhibition and genetic pre-emption of mitochondrial function elecit similar physiological responses. The D(-) chloramphenicol inhibited cells do not have a good respiratory capacity and this is reflected in the higher rate of ethanol production (contre-Pasteur effect) [Fig. 4(ii)]. As suggested by Holzer[69] this is probably mediated via competition between pyruvate oxidase and decarboxylase. The rate of utilization of glucose is similar and this in conjunction with the slower rate of oxidative removal of pyruvate in the inhibited cells would cause an increase in the intracellular concentration of pyruvate [Fig 4(vii)] thus favouring the decarboxylase reaction. The decreased yield also reflects the amount of oxidation being carried out in the culture, oxidation producing much

more energy per unit of hexose than fermentation.

When wild type yeast cultures enter their final stationary phase, respiration decreases precipitously. This phenomenon is absent in the inhibited cells [Fig. 4(iii) A] demonstrating that the plateau induced by chloramphenicol is not a true stationary phase. From a comparison of Fig. 4(v) A and B it can be seen that the increase in the control Q_{O_2} is due to continued synthesis of respiratory capacity, whereas the decrease in the inhibited culture is due to continued proliferation of cell mass. Under these conditions then, there is no degradation of mitochondrial activity.

The ethanol transfer experiments were performed in order to test the effects of chloramphenicol upon cells undergoing de-repression. The transfer method was selected so that any effects due to the residual medium i.e. accumulated extracellular end-products, nearly exhausted medium etc., would be minimized. The slow reproducible growth rate [Fig. 4(iv)] was unexpected since the oxidative capacity of these cells was very high. What clearly emerges from these transfer experiments is that the antibiotic *in vivo* prevents any further increase in respiratory ability without impairing the function of pre-constituted enzyme systems [Fig. 4(iii) B and Fig. 4(v) B], and that the drug is not altering the function of the cellular mechanisms which control the "de-repression" phenomenon.

The evidence accumulated demonstrates that only the D(-) *threo* isomer of chloramphenicol affects the growth characteristics of yeast. The respiratory capacity of these inhibited cells is reduced in all physiologic stages of aerobic growth on 5% glucose

[Fig. 4(iii) A] and the other changes in growth parameters [Fig. 4 (i) and (ii)] result directly from the loss of respiratory ability. The ethanol transfers eliminate the possibility that inhibition by chloramphenicol is a secondary manifestation resulting from a primary inhibition of electron transport[70]. This substantiates the suggestion of Linnane[65] that the inhibition of cytochrome a+ a_3, b and c_1 synthesis is due to a direct effect by the antibiotic during mitochondrial genesis.

The experiments with coupled mitochondria isolated from respiratory competent yeast (Table I) demonstrate quite conclusively that *in vitro* neither D(-) or L(+) *threo*-isomers of chloramphenicol affect the oxidation of various substrates tested. The sum of this evidence indicts protein synthesis as the most likely site at which D(-) chloramphenicol interacts with cellular metabolism.

(ii) The effects of chloramphenicol on some intermediary metabolites

The measurement of nicotinamide adenine nucleotides [oxidized (NAD) and reduced (NADH) forms], adenosine phosphates (ATP, ADP, AMP), inorganic phosphate (Pi), pyruvate, and glucose-6-PO_4 was carried out on the same aerobic 5% glucose-grown cultures described in the previous section [Chapter 4(i)]. Preliminary experiments showed that the levels of the reduced and oxidized nicotinamide adenine phosphates (NADPH and $NADP^+$) were ten times lower than those observed for NAD^+ and NADH (cf. Polakis and Bartley[60]) and it was decided not to study these compounds in detail. $NADP^+$ and NADPH are not directly involved in the major energy-generating pathways operative under the growth conditions studied

here, and it seems reasonable to suppose that any fluctuations in these minor intermediates would not affect the respiration:fermentation energy-balance to any great extent.

Changes in the levels of pyruvate and the nicotinamide nucleotides

Changes in nicotinamide nucleotide levels [Fig. 4(vi)] mirror the general pattern already observed. The ratio of [NADH] / [NADH] + [NAD^+] in cells grown in the glucose medium begins to increase when the glucose is completely utilized (10 hrs) indicating that the production of reduced equivalents of this nucleotide overshadows its consumption. De-repression of the oxidative system begins to occur at this time. As the Q_{O_2} of the yeast suspension increases [cf. Fig. 4(iii) A] the magnitude of the nucleotide ratio drops reflecting a maximum rate of oxidation of reduced NAD^+. When secondary exponential conditions are reached the ratio increases until equilibrium conditions are attained. The concentration of [NAD^+] increases as stationary phase conditions are approached and the cells become conditioned for metabolic "shut-down".

In the presence of chloramphenicol the ratio of [NADH] / [NAD^+] + [NADH] initially is much higher than in the control cells. This is reflected in the respiratory potential of these cells since their oxidative capacity is much lower than glucose-grown cells even though they both share the same pathways for NADH production. When glucose becomes exhausted from the medium a slightly lower ratio is established which is higher than any control value. This high ratio results from the metabolism of tricarboxylic acid cycle intermediates by NAD^+-mediated

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Fig. 4(vi) to 4(viii) C.

Pyruvate, glucose-6-P, nicotinamide and adenine phosphate nucleotides were determined on yeast growing aerobically in 5% glucose Y.An. medium. The conditions of growth were as described for Fig. 4(i). Assays were performed fluorometrically as described in the Method's section.

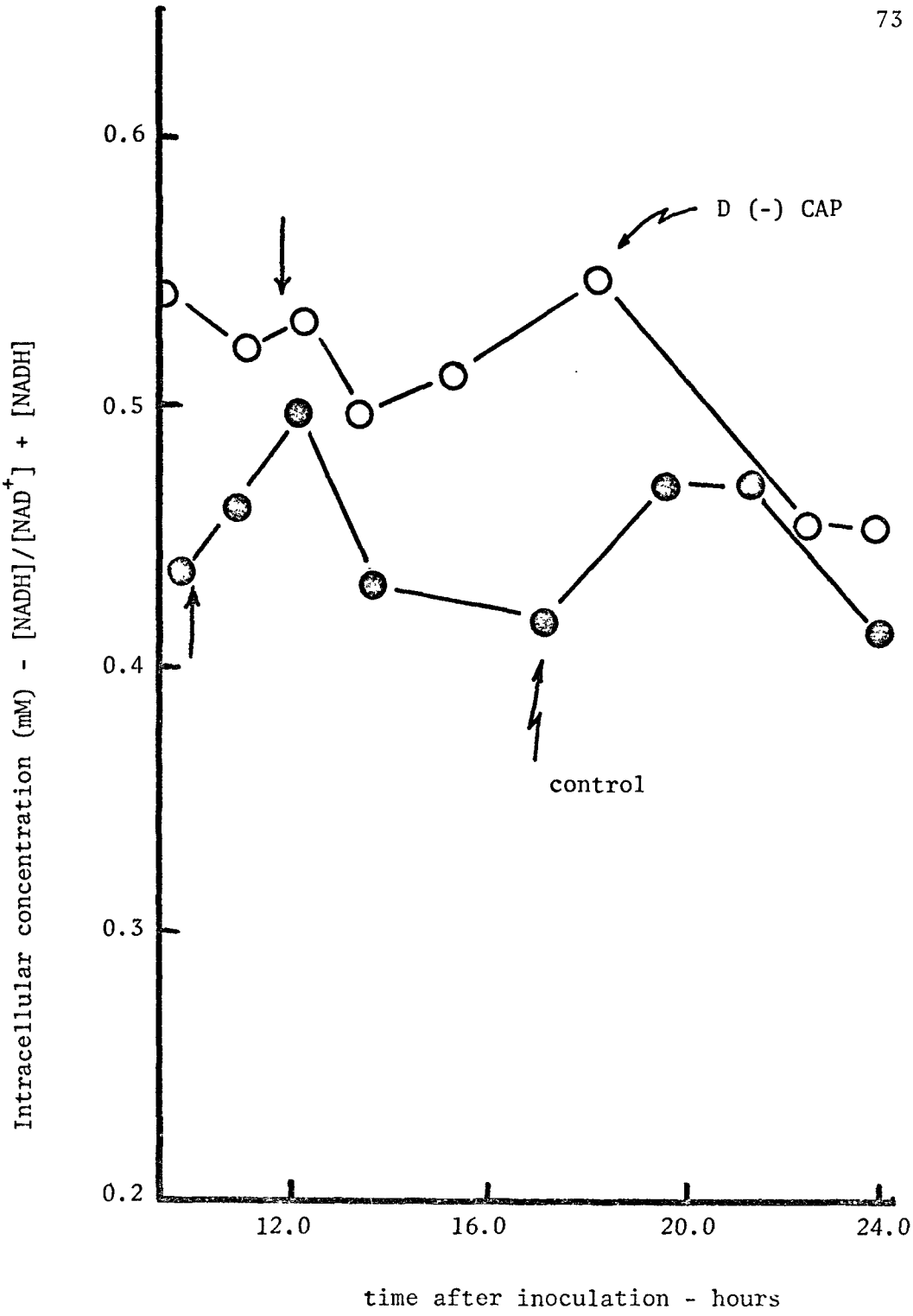

Fig 4(vi) Intracellular changes in nicotinamide nucleotide concentrations: 5.0% glucose Y.An.O_2

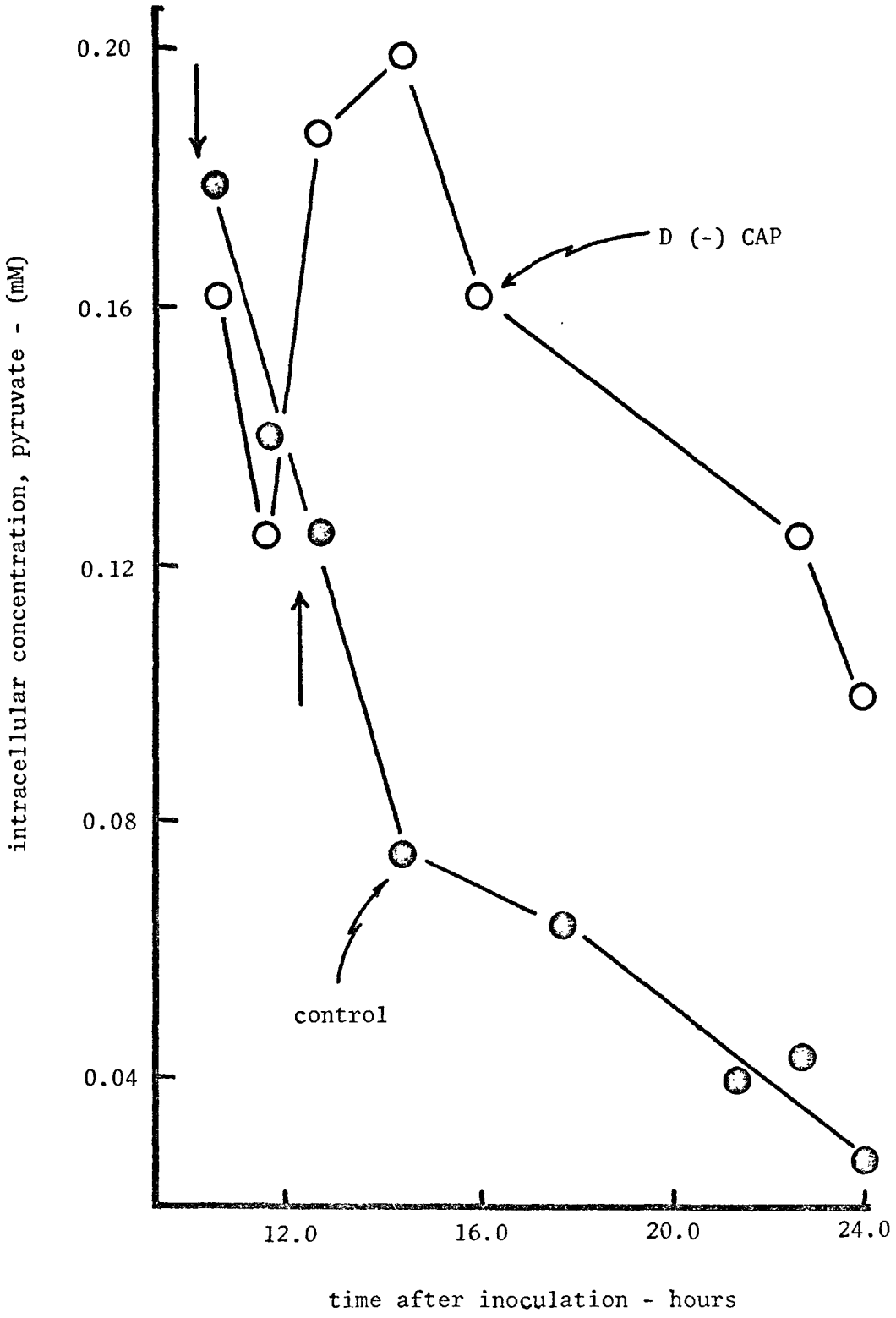

Fig 4(vii) Changes in intracellular pyruvate: 5% glucose Y.An.O_2.

enzyme systems producing NADH which is not utilized. As this coenzyme accumulates a negative feedback becomes operative and less cycle substrates are metabolized (curve drops).

The levels of reduced and oxidized nicotinamide nucleotides affect the pyruvate concentration in yeast. Holzer and Goedde[49] reported that pyruvate decarboxylase occurs in the cytoplasm while pyruvate oxidase is found in the mitochondrion. Both enzyme activities account for the removal of pyruvate in the control system and each is contingent upon the supply of NADH and NAD^+ respectively. When glucose disappears the NAD^+ levels are high and this supports oxidase activity [Fig. 4(vi)] and when NADH levels rise decarboxylase activity is potentiated and more ethanol is produced. The curve representing pyruvate levels at various physiological stages of cell growth is basically diphasic with a break occuring at 14 hours. This coincides both with the maximum Q_{O_2} attained by these cells and the mid point of the secondary lag phase.

In the presence of D(-) chloramphenicol the concentration of pyruvate initially follows that of the control. However, when glucose disappears from the medium, pyruvate accumulates intracellularly for 3 hours and then is metabolized at nearly the same rate as that of the control. The rate associated with de-repression is maintained for a much shorter period, but the slopes of these two curves are basically the same.

Changes in the levels of the adenosine phosphates, inorganic phosphate and glucose-6-phosphate

Changes in cellular adenosine phosphates during the various

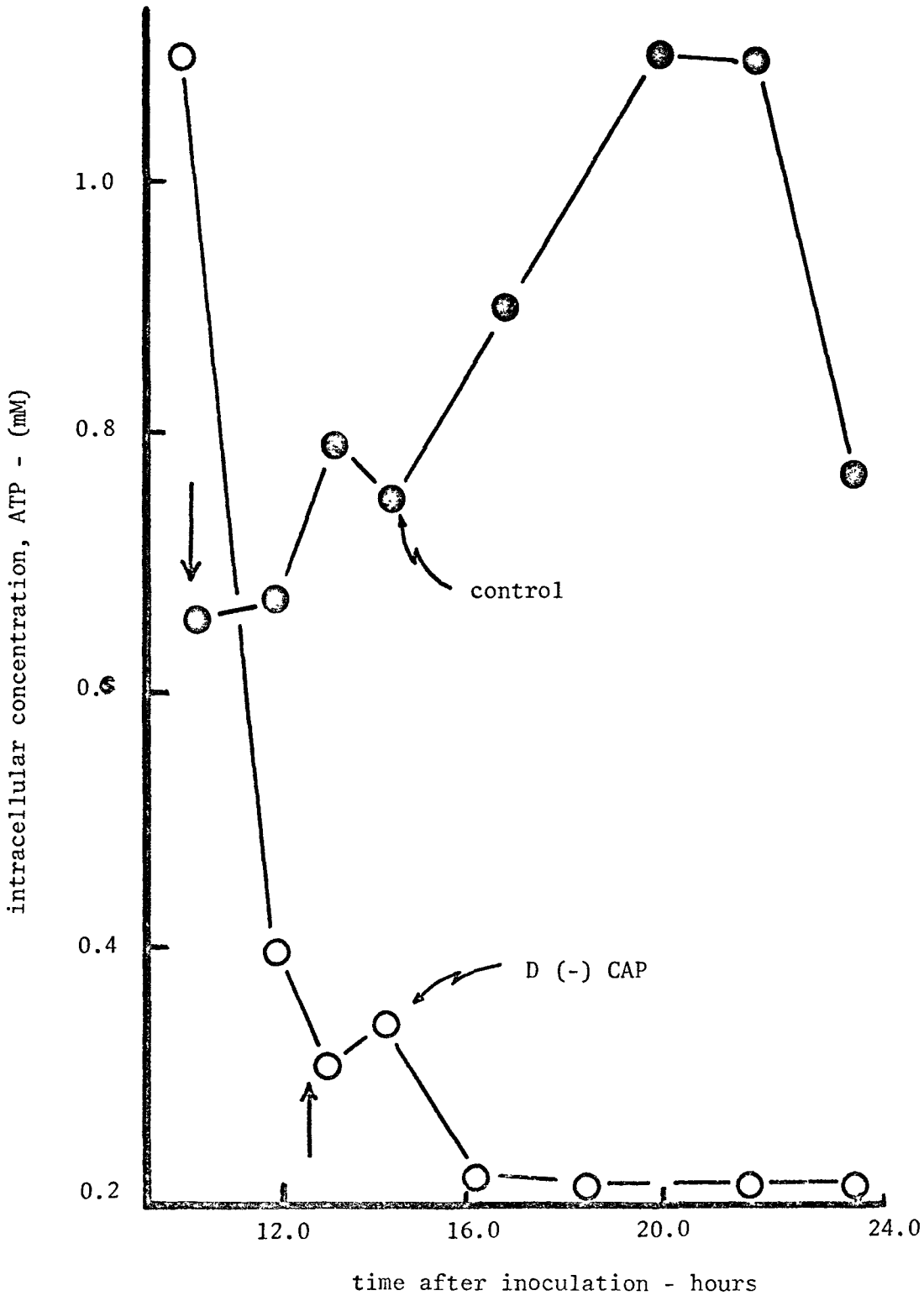

Fig 4(viii) A Changes in intracellular ATP:
5% glucose Y.An.O_2

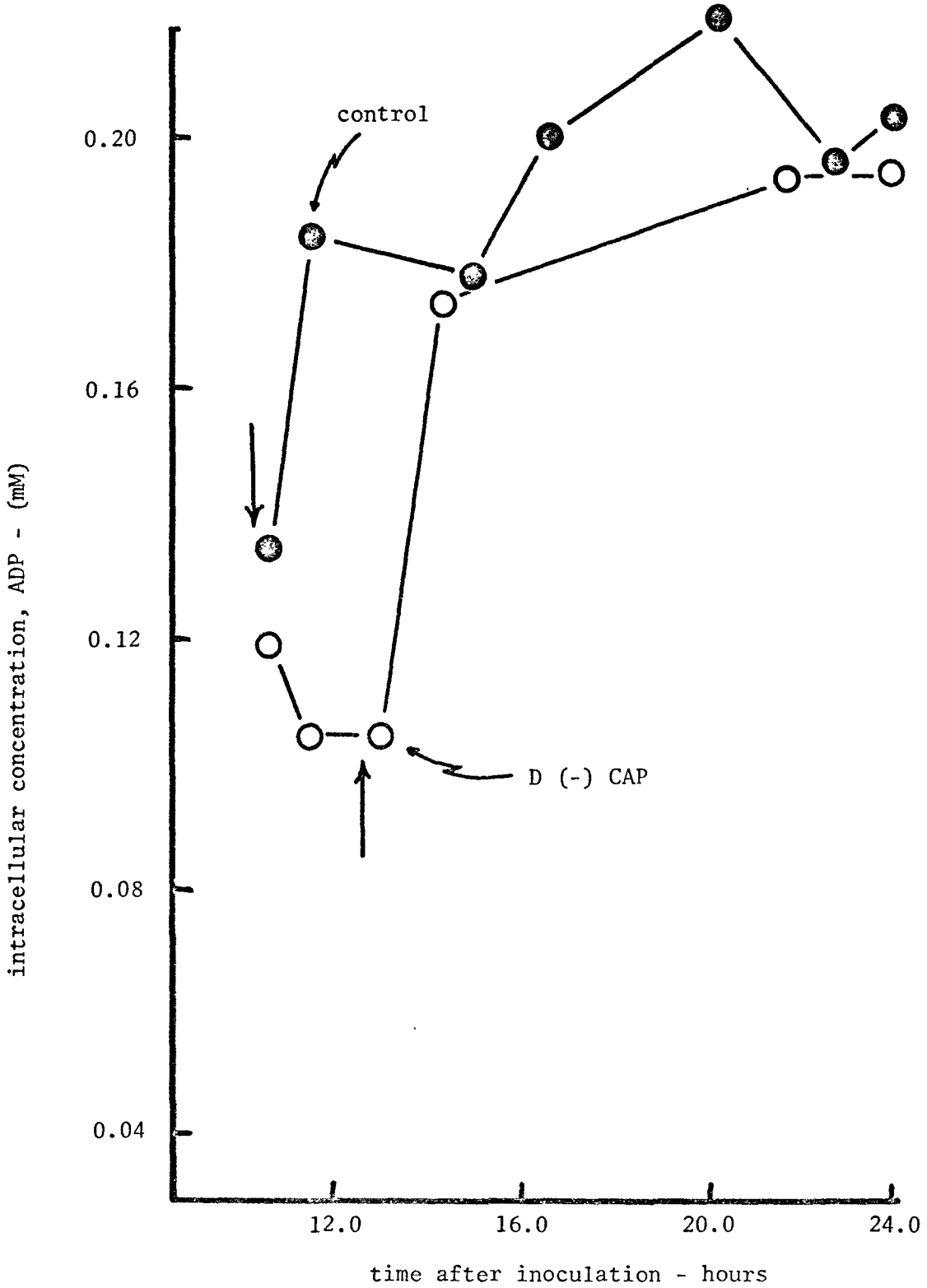

Fig 4(viii) B Changes in intracellular ADP:
5% glucose Y.An.O_2

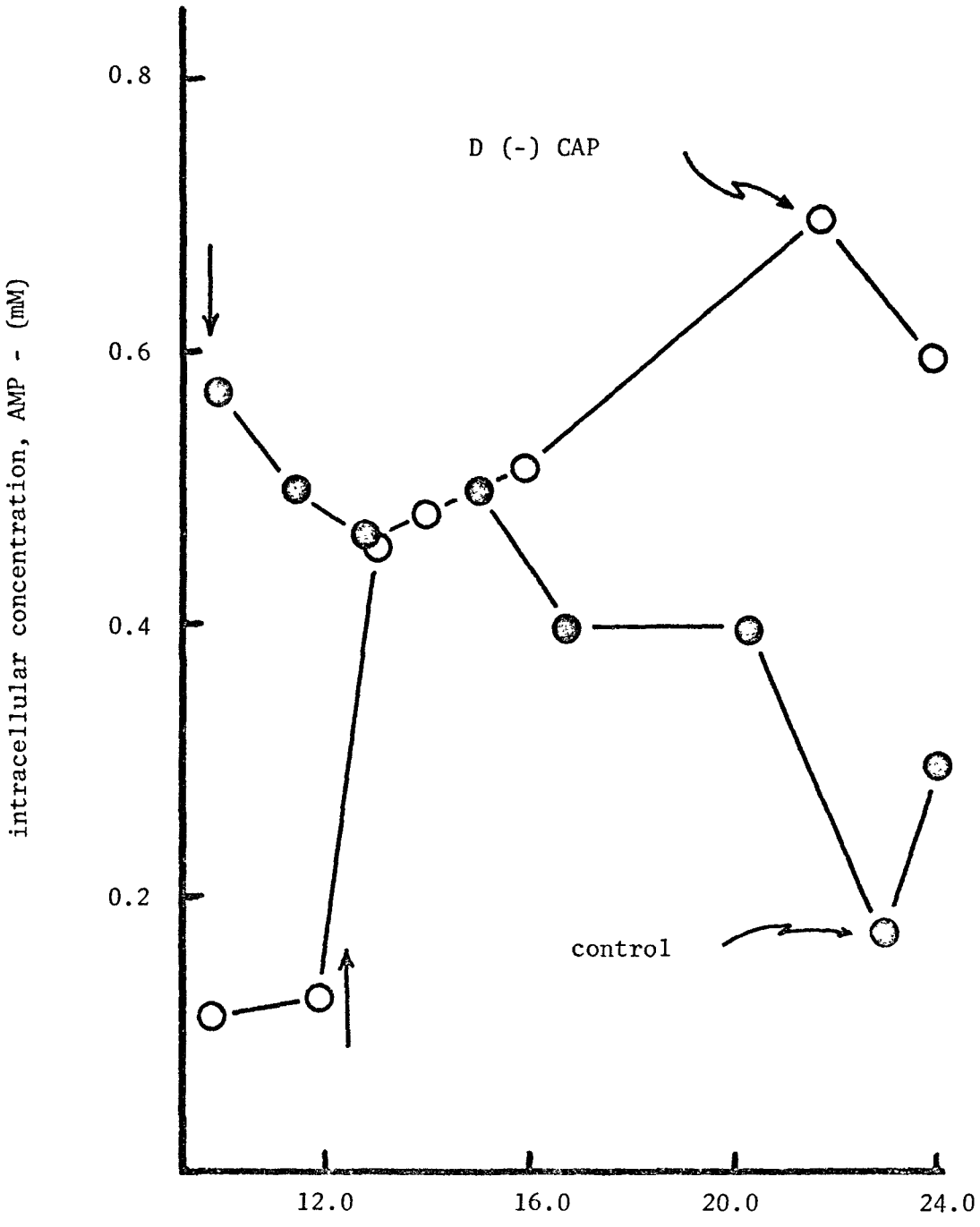

Fig 4(viii) C Changes in intracellular AMP:
5% glucose Y.An.O_2

physiological growth phases of yeast cells metabolizing glucose as carbon source are illustrated in Figs. 4(viii) A, B and C. When glucose is totally consumed from the medium the level of ADP rises slightly while AMP drops markedly and ATP maintains a slow rise. These changes are associated with the secondary lag phase where the most active respiration occurs. When secondary exponential phase conditions prevail a sharp increase in ATP concentration occurs and this rise is complemented by a drop in AMP and a static ADP state. During the secondary lag phase there is a rapid uptake of inorganic phosphate from the medium. Thus it appears that the phosphorylation sequence in yeast is $P_i \rightarrow$ AMP $\rightarrow$ ATP and the AMP $\rightarrow$ ADP $\rightarrow$ route is secondary. After 20 hours the inorganic phosphate uptake reaches equilibrium and slows down considerably without becoming rate limiting. The concentration of ATP levels off whereas AMP drops. When stationary phase conditions are achieved ATP levels fall to those observed prior to glucose disappearance and AMP values increase,

In the presence of chloramphenicol, inorganic phosphate uptake abruptly stops after complete utilization of glucose. Glucose-6-phosphate was detected for a short period after this time. ATP levels, which were very high prior to glucose disappearance, dropped precipitously and reached a low level while both AMP and ADP increased indicating an almost complete cessation of phosphorylation in the presence of this drug.

Discussion

The data on the pyridine and adenine nucleotides [Fig. 4(vi)

and (viii) A, B, C] and pyruvate [Fig. 4(vii)] illustrates the metabolic equilibration involved in a transition from a fermentative to respiratory mode of metabolism. The inhibited culture shows the effect of a reduced respiratory capacity on the way in which these equilibria are attained. The pyruvate and pyridine nucleotide data [Fig. 4(vi) and (vii)] are remarkable for the parallelism of the responses exhibited in the contrasting cultures, reactivity being governed by the ratio of the catalytic systems responsible for enacting these changes. While glucose is still being metabolized, the primary source of NADH is fermentation. This NADH will be oxidized via cell growth respiration, and alcohol production. The high level of NADH in the inhibited cells reflects the lowered oxidative capacity for regenerating NAD^+. Under aerobic-glucose growth conditions the fermentative capacity of the yeast is very high when compared to that of yeast grown aerobically on other carbon sources. There is only a slight "contre-Pasteur" effect [see Fig. 4(ii)] and this, together with the nucleotide data, shows that the yeast is only able to compensate to a limited extent for its loss of oxidative ability by increasing ethanol production, suggesting that the control culture is exhibiting a fermentative ability very close to that which is maximally available to the yeast. The continued maintenance of a high [NADH] / [total pyridine nucleotide] ratio after glucose is exhausted reflects the ability of these cells to continue generation of this reduced nucleotide. The consequent decrease in alcohol production which ensues fixes the route of this synthesis to the citric acid cycle. Thus although the cells now have virtually none of the characteristics of

respiratory competent yeast, the citric acid cycle is still functional. This indicates the maintenance of a compartmentalized function despite the structural disorganization acruing from the suppression of respiratory chain enzyme activity[13]. The lower nucleotide levels observed at 22 and 24 hours are similar to the respiratory phase equilibrium values observed in the control. This probably reflects a Type III negative feed back control, in that the functioning turnover rate of the citric acid cycle has equilibrated to the rate of oxidation of its products; the respiratory rate remains relatively constant during these phases in both cultures [Fig. 4(iii) B].

The control culture shows similar kinetics. The initial increase in NADH levels preceeds the respiratory de-repression. As the rate of oxidation continues to increase the level of NADH falls and the low point in the [NADH] / [total nucleotide] ratio coincides with the peak of oxidative ability. The higher ratios noted in the late stages of growth reflect the establishment of an integrated interplay between the citric acid cycle and the respiratory chain when ethanol is being metabolized. It is plausible that the de-repression of the citric acid cycle, which produces the elevated [NADH] level, initiates the metabolic de-repression which preceeds de-repression of enzyme synthesis. The dynamic kinetics of pyruvate metabolism are more profound. The accumulation of pyruvate in the inhibited system is difficult to explain. Immediately after this elevation both curves, control and inhibited, manifest the same diphasic shape and similar rates of oxidation. It is possible that the elevated pyruvate con-

centration reflects an adaptive mobilization of stored carbohydrates in response to glucose exhaustion, and that the accumulation is halted by a feed back mechanism coupled to the oxidative removal of the released pyruvate. In order to maintain the observed levels of pyruvate the control cells must produce as well as oxidize pyruvate. The inflection point in the control curve coincides with the point at which de-repression maximizes [compare Fig. 4(iii) A and 4(vii)]. The secondary rate of pyruvate utilization probably reflects an approach to equilibrium conditions, whereas the primary rate indicates a rapid utilization of pyruvate which would generate the energy necessary for the internal rearrangements inherent in the secondary adaptive lag phase. These observations reinforce the concept that all the normal control mechanisms are operative in the presence of D(-) chloramphenicol.

The major difference between control and drug inhibited cultures is the ability to generate ATP. The inhibited system demonstrates a very restricted ability to generate ATP once glucose is exhausted [Fig. 4(viii) A]. A dramatic fall in ATP concentration concomitant with glucose disappearance is complemented by a sequential increase in AMP and then ADP. During this period the change in ADP levels relative to the total adenosine phosphate concentration in this culture is more significant than that observed in the control. Since chloramphenicol had no effect on the coupling mechanism in isolated mitochondria, the impaired phosphorylation must be attributed to a reduced or suppressed level of respiratory chain proteins. The control culture not only provides a contrast to the drug inhibited

cells but also illustrates the changes in adenosine phosphate ratios during de-repression. Polakis and Bartley[60] monitored changes in these nucleotides but only to where the fermentable carbon source was consumed. The relative concentrations of these nucleotides at this stage as reported by them are similar to those reported here [Fig 4(viii) A, B, and C]. The interconversion of adenosine phosphates appears to be a complex process under our experimental conditions. From equilibrium data of this nature it should be possible to deduce the control sequence by applying the crossover theorem[45]. During this experiment there is a rapid uptake of inorganic phosphate and a subsequent increase in the total adenosine phosphates which precludes the direct application of this theorem. The ATP concentration in the control culture alters markedly but it is not possible to suggest whether this increase is at the expense of preformed ADP and AMP or from nascent ADP and AMP synthesized in response to the altered conditions resulting from glucose exhaustion.

Immediately after glucose is consumed in the control experiment the ATP and ADP levels increased slightly while AMP decreased. These changes precede the rapid inorganic phosphate uptake and must be associated with the metabolic de-repression of respiration. The addition of an excess of ADP to isolated mitochondria causes an immediate increase in oxygen uptake and in electron transport. This is reflected in a shift from "state 4" to "state 3" according to Chance and Williams[10]. The conditions necessary for this change (excess NADH and a limiting oxidative phosphorylating enzyme system) are fulfilled at the time when de-repression is first manifested,

Such a change in state would successfully relate the changes in respiratory rate and the pyridine and adenine nucleotide levels. As the inorganic phosphate is taken up, the level of ADP is maintained whereas the AMP level drops slightly and the ATP level increases rapidly. This can be explained by several mechanisms. Pyrophosphate formed from inorganic phosphate reacts with AMP to form ATP or else inorganic phosphate reacts with AMP to form ADP which in turn reacts with another molecule of inorganic phosphate to finally form ATP. This latter sequence may be a minor pathway or else the relative rates of this reaction are such that ADP is in an equilibrium undergoing synthesis and conversion, resulting in an apparent constant concentration. The rapid formation of pyrophosphate has been described in yeast metabolizing ethanol[71]. The formation of ATP from this compound is conceivable, and an enzyme has been found recently which catalyzes this reaction[72]. The alternate reaction AMP → ADP → ATP would allow ADP to increase before AMP [cf. Fig. 4(viii) B, Fig. 4(viii) C, control curves] and would also permit the obverse change, AMP to increase prior to ADP [Fig. 4(viii) A and C, inhibited curves]. The pyrophosphate pathway would not allow this transition. Maitra and Estabrook[71] who observed a complementarity in the kinetics of AMP and ATP [Fig. 4 (viii) A and C] together with a constant ADP concentration in yeast oxidizing ethanol suggested that this "buffered" ADP level was the result of an active adenylate kinase. This third explanation is also consistent with the observed results. It seems probable that the similar levels of ADP in these cultures are, considering the very different response profiles observed for ATP, AMP and inorganic phosphate,

coincidental and do not reflect any real similarity between the cultures. Assuming that the pathways for generating adenosine phosphates are similar in both cultures the second hypothesis explains the data more completely than the others.

The increased level of NADH which provides excess substrate to the limiting functional enzymes of the electron transport system is a necessary condition for the "state 4" → "state 3" transition. Thus the primary event in the de-repression phenomenon would be the release of the citric acid cycle from an inhibition due to fermentative intermediates, which in turn would facilitate an increased level of electron transport. The ADP necessary for the "state 4" → "state 3" transition begins to accumulate as a result of a slower genesis of ATP from fermentation. These changes would then initiate further alterations in the levels of the various metabolic intermediates leading, via Type III control, to the various synthetic responses associated with the de-repression phenomenon [cf. Chapter 5]. Polakis and Bartley[60], observing the changes in nucleotides associated with fermentative growth, concluded that ADP was the key nucleotide in the control of the respiration/fermentation balance. Our data indicate that although ADP can exercise a control function, it is not the substance involved in the primary trigger mechanism of de-repression.

CHAPTER 5

The Effects of D(-) Chloramphenicol on Enzyme Synthesis

The experiments described in this chapter were performed in order to study the kinetics of enzyme synthesis during aerobic galactose fermentation, and the transition from galactose fermentation to ethanol respiration. The control culture should provide insight into normal synthetic patterns for aerobic yeast, and the kinetics should reveal the sequence in which enzyme synthetic rates are altered during de-repression [see Chapter 4, Fig. 4(iv)]. The chloramphenicol experiment was performed in order to test the effect of inhibiting the synthesis of mitochondrial proteins. It is felt that the evidence provided by Linnane *et al.* (see Introduction) together with that reported in Chapter 4, justify the claim that D(-) CAP is inhibiting mitochondrial protein synthesis, without directly altering any other facets of cellular metabolism.

Fig. 5(i) shows the aerobic control growth curve, an anaerobic growth curve, and an aerobic + 3.0 mg/ml D(-) CAP growth curve; the carbon source was 2.7% galactose in each case. The yield at 9.0 hrs reflects the relative amounts of respiration occurring in the cultures, as respiration provides for a more efficient utilization of the primary carbon source. D(-) CAP, at a concentration of 3.0 mg/ml causes a very great inhibition of respiration and [Table 5

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Fig. 5(I) and Table 5(I).

Yeast were grown aerobically in 2.7% galactose Y.An. medium to mid-exponential phase before harvesting and transfer to a second growth condition. Fig. 5(I) shows the growth rates under various conditions; Table 5(I) shows the changes in the specific activity of cytochrome c oxidase and malic dehydrogenase in the same experiments.

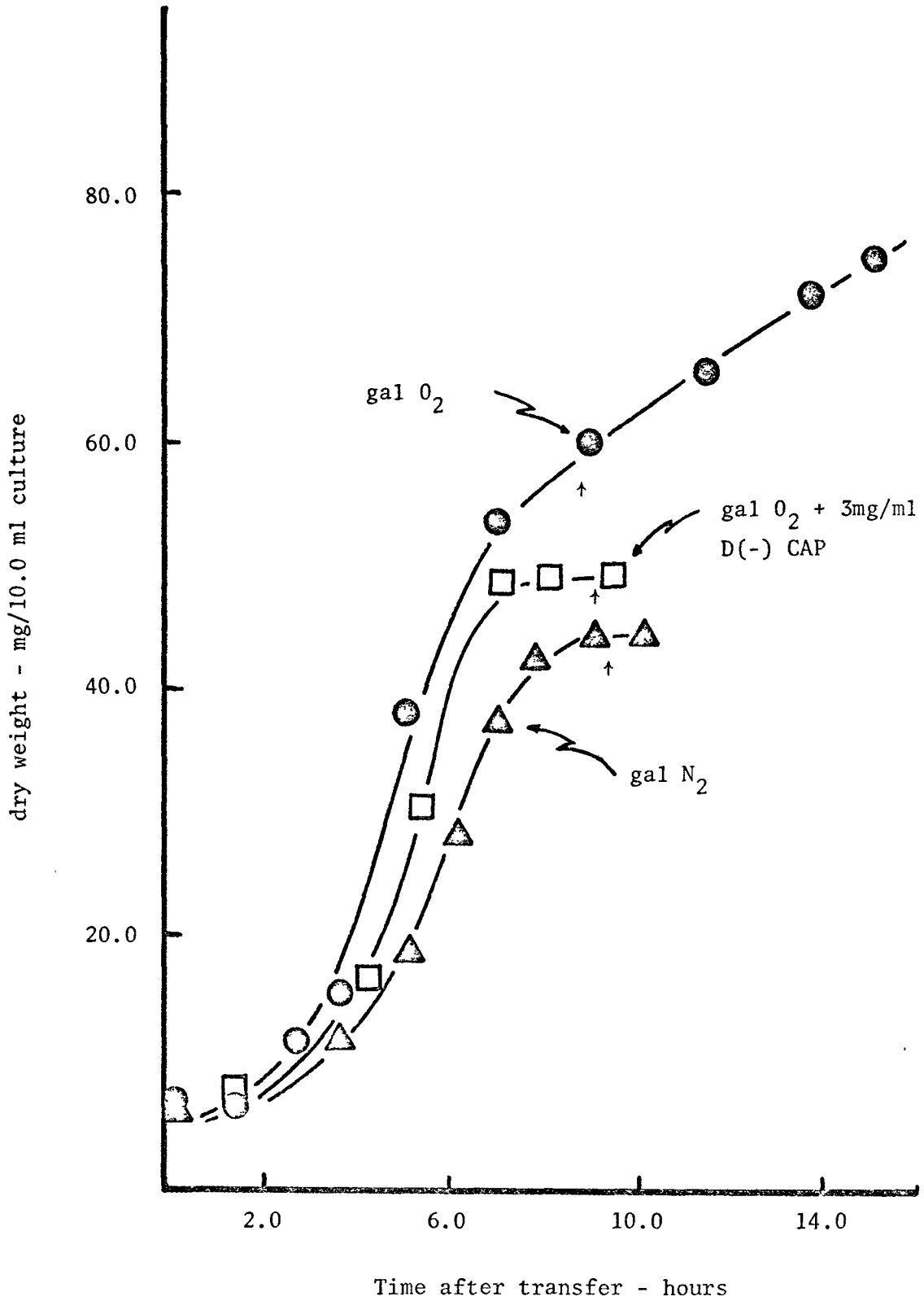

Fig. 5(i) Growth curves: galactose → galactose transfers

↑ indicates galactose completely utilized in all Fig 5 diagrams

TABLE 5 (I)

A Comparison Between the Effects of Chloramphenicol and Anaerobiosis

Time of Sample	Gal (O_2) control	Gal (O_2) 3.0 mg/ml D(-) CAP	Gal (O_2) 3.0 mg/ml L(+) CAP	Gal (O_2) to Gal (N_2)
	Cytochrome c Oxidase (1st order rate constant/mg protein)			
Zero	1.720	1.760	1.490	1.380
Early Log	0.509	0.941	0.580	0.930
Mid-Exponential	0.581	0.207	0.880	0.530
Early Stationary	1.360	0.055	1.600	0.260
	Malic Dehydrogenase (μmoles/min/mg protein)			
Zero	5.7	4.6	5.15	4.15
Early Exponential	5.3	4.9	4.55	2.85
Mid-Exponential	4.8	3.6	3.6	2.3
Early Stationary	7.3	4.5	6.35	2.1

LEGEND

Fig. 5(II) and (III).

The parameters shown in these diagrams were measured on yeast grown aerobically to mid-exponential phase in 2.7% galactose Y.An. medium before transfer to the second condition. The control condition was 2.7% galactose Y.An.; D (-) chloramphenicol (CAP) was added to a concentration of 1.0 mg/ml. Growth was aerobic throughout the experiment.

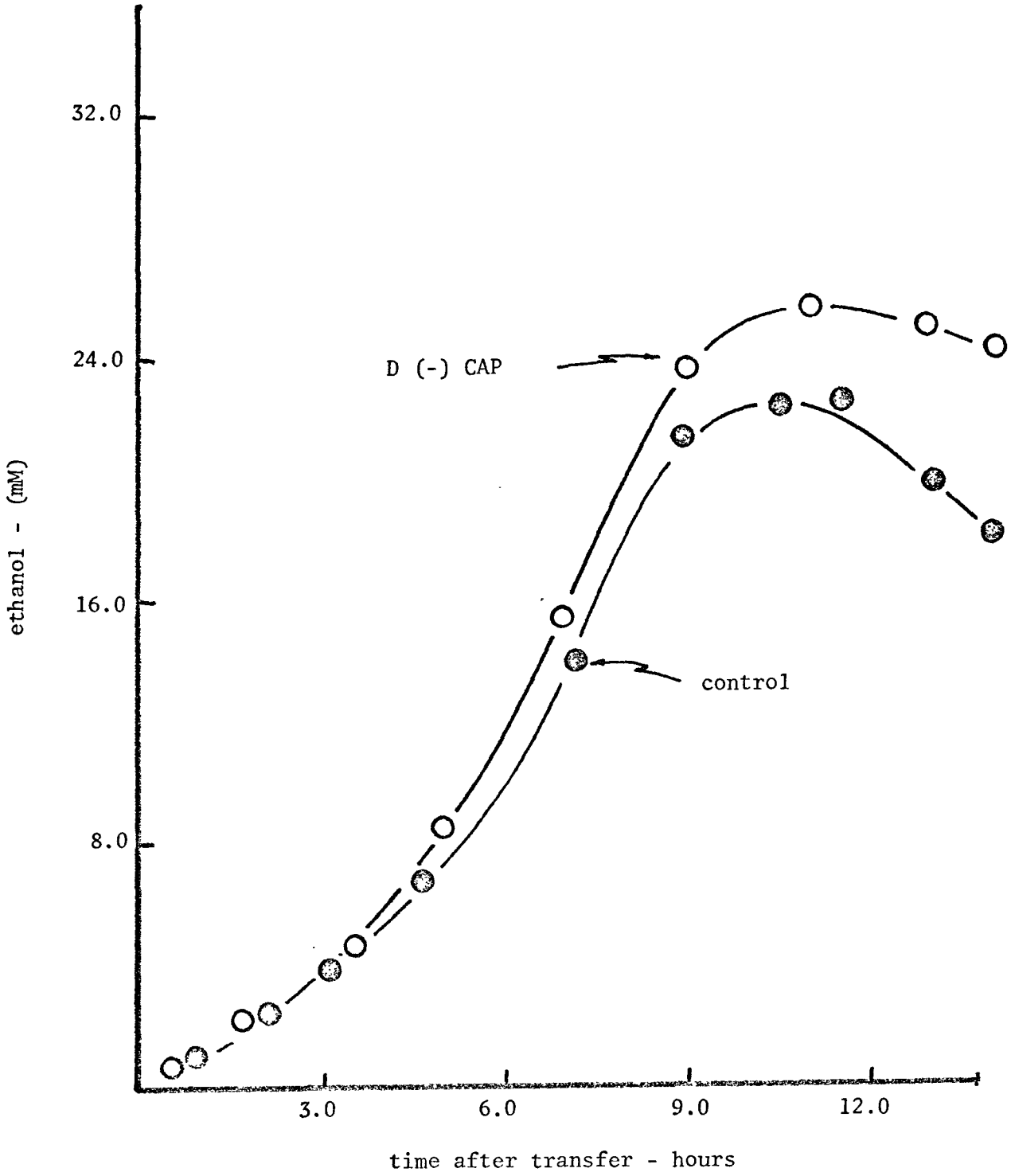

Fig 5(ii) Ethanol metabolism: 2.7% galactose Y.An.O_2 transfer

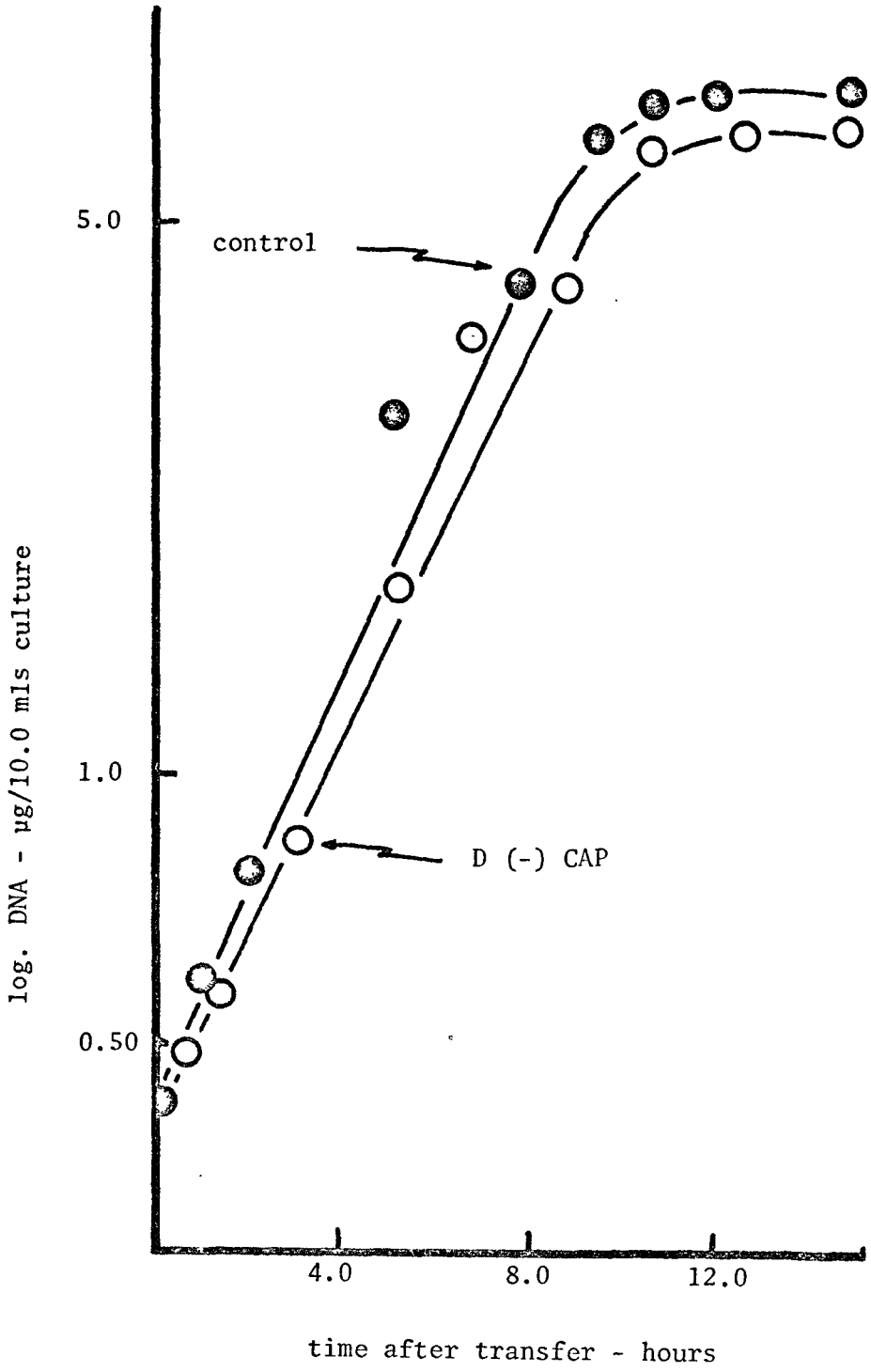

Fig 5(iii) Deoxyribonucleic acid (DNA) synthesis:
2.7% galactose Y.An.O_2 transfer

(I)] in the synthesis of cytochrome-c oxidase (cyt. c. oxid)

It was thought that too drastic an inhibition of mitochondrial protein synthesis might disrupt the cellular control systems to an extent which would rob the kinetic results of any physiological significance which they might have. Preliminary studies had shown that the effect on growth yield and respiration of 1.0 mg/ml of D(-) CAP was intermediary between that observed for the aerobic control and the 3.0 mg/ml D(-) CAP inhibited culture. This more gradual loss of respiratory ability should be equivalent to a slow transition from aerobic to anaerobic growth conditions. For these several reasons the experiments described below were performed with 1.0 mg/ml of D(-) CAP in the inhibited culture. The experiments were performed as transfers (see Methods) and the growth conditions were aerobic, on 2.7% galactose complete medium (2.7% galactose Y.An.O_2.)

Fig. 5(ii) shows the rate of production and utilization of ethanol under these contrasting conditions. For the first four hours after transfer the rate of ethanol production is the same in both cultures; after this time the rate of production in the inhibited culture begins to increase and this leads to an increased final yield. Galactose becomes exhausted at 9.5 hrs in the control and 9.75 hrs in the inhibited culture. Ethanol production continues for a short time after this point, and then utilization begins. The utilization rate is much reduced in the inhibited culture, probably as a result of the low Q_{O_2} [Fig. 5(iv)].

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Fig. 5(iv) and (v).

The respiration data shown here were estimated under experimental conditions similar to those of Fig. 5(ii) and (ii). The transfer conditions were 2.7% galactose Y.An.O_2. D (-) CAP was added to a concentration of 1.0 mg/ml.

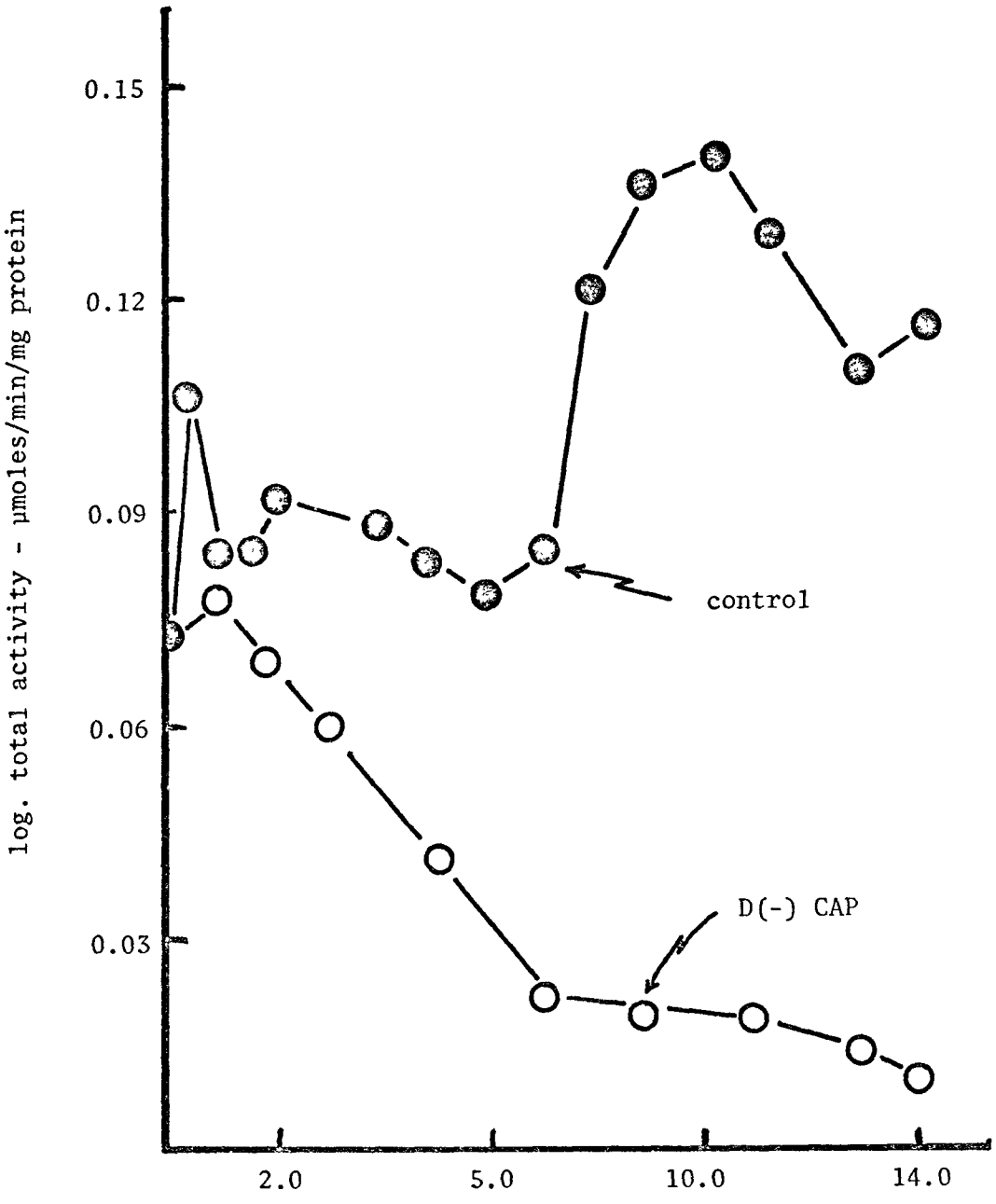

Fig 5 (iv) Respiratory ability (QO_2): 2.7% gal. Y.An.O_2

Although the control growth curve shows two phases [Fig. 5(i)], both of which are logarithmic, DNA synthesis, [Fig. 5(iii)] exhibits only one logarithmic phase before synthesis ceases altogether. The inhibited and control cultures are identical in this respect.

As expected D(-) CAP inhibits the synthesis of respiratory ability [Fig. 5(v)] and causes the Q_{O_2} to be gradually decreased [Fig. 5(iv)]. In the control culture the doubling rate for respiratory synthesis is 2.0 hrs, and this is the same as that observed for other growth conditions [Fig. 2(v)]. The inhibited culture shows an initial doubling time of 3.0 hrs which continually increases as growth continues until no further increase in total respiration is observed.

The total activity curve (log-linear plot) for malic dehydrogenase (MDH) [Fig. 5(vi)] shows diphasic logarithmic kinetics in the control culture, and the inhibited culture duplicates this result up until the advent of galactose exhaustion. The secondary synthetic phase is inhibited by D(-) CAP. Holzer *et al*[52 73] have characterized two isozymes of MDH in yeast and the ratios of the cytoplasmic:mitochondrial isozymes are shown in Table 5 (II). It can be seen from these results that CAP represses the induction or de-repression of mitochondrial isozyme synthesis.

The values in Table 5 (II) were determined by column chromatography as described in the Methods section. Fig. 5(vii) A shows the distribution of the isozymes in the column eluents. The polarity

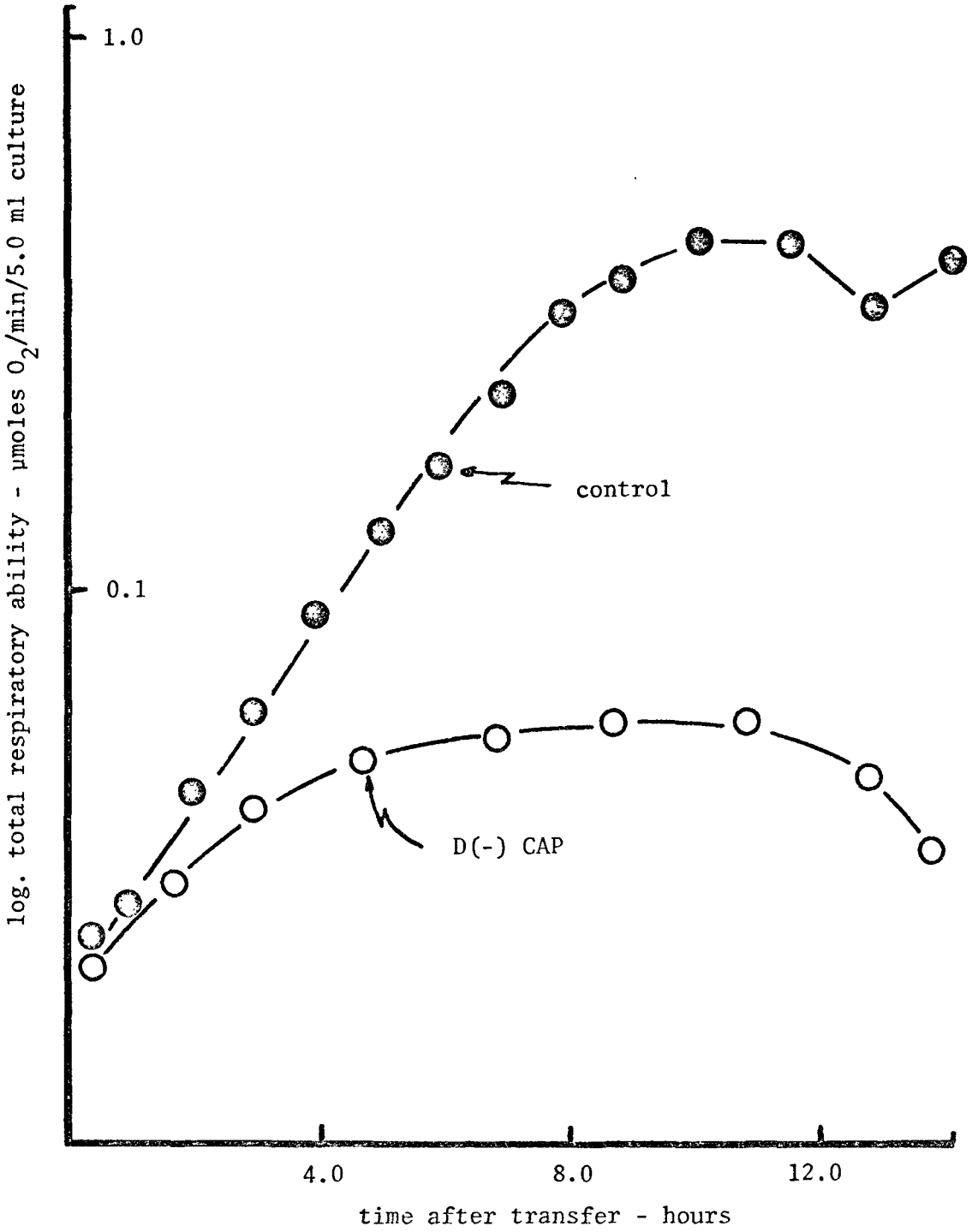

Fig 5(v) Total respiratory ability 2.7% gal. Y.An.O_2

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Fig. 5(vi).

Malic dehydrogenase was determined on samples taken from a 2.7% galactose Y.An.O_2 → 2.7% galactose Y.An.O_2 transfer. D (-) CAP was 1.0 mg/ml.

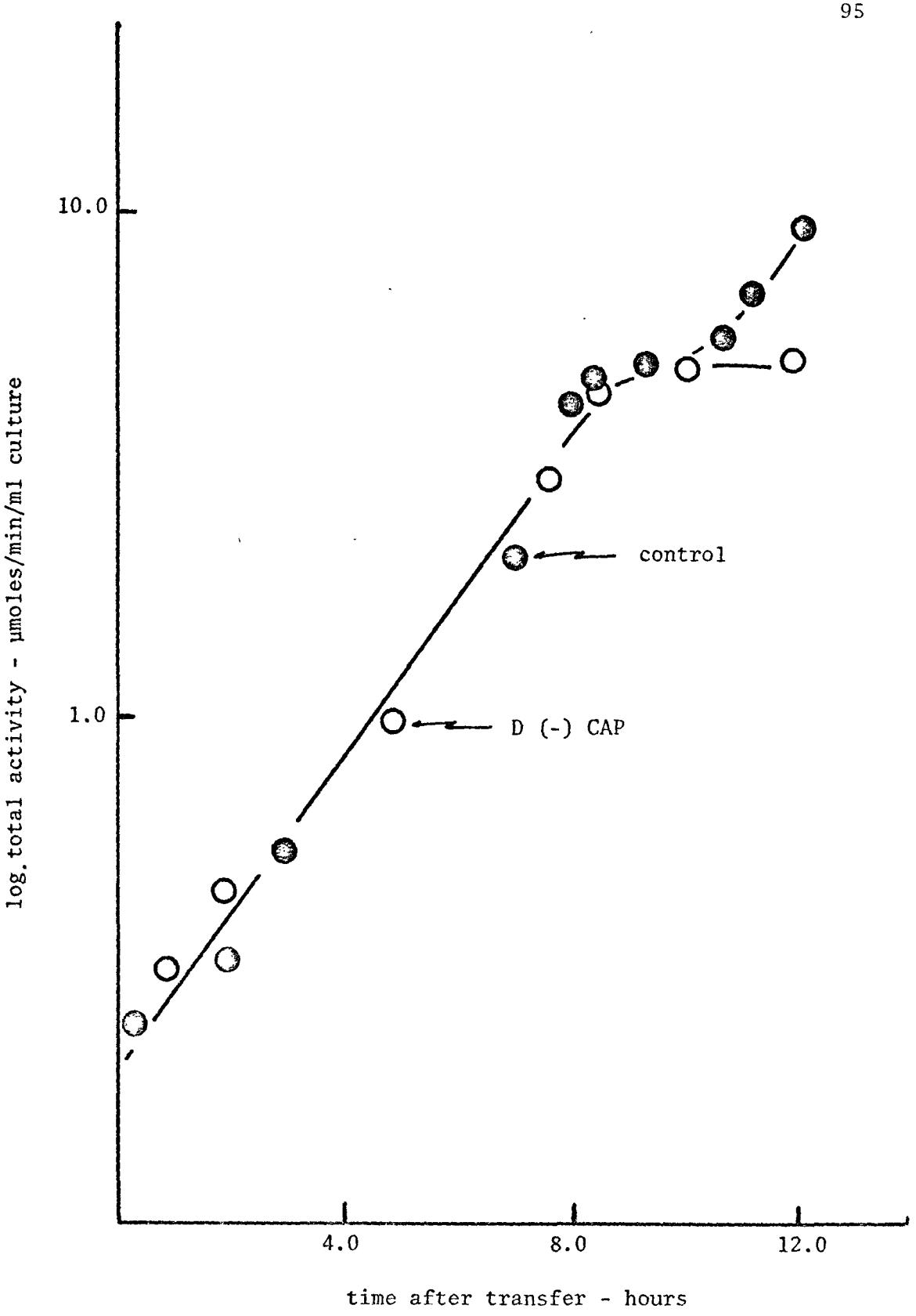

Fig 5(vi) Malic dehydrogenase, total activity:
2.7% galactose Y.An.O_2 transfer

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Table (II)

Shows the ratio of the ratio, cytoplasmic:mitochondrial for the isozymes of malic dehydrogenase. Ratios were determined chromatographically (cf. Fig. 5(vi)A). Growth conditions were as described in Fig. 5(vi).

TABLE 5 (II)

Changes in the Isozymes of MDH During Aerobic Growth: 2.7% Galactose Y.An.O_2.

	Time (hours)			
	0.0	4.0	10.0	14.0
Control	1:4.73*	-	1:4.76	1:10.75
D(-) CAP	-	1:5.5	-	1:5.56

* ratio cytoplasmic:mitochondrial

of the yeast isozymes is reversed compared to that observed by Schmidt *et al.*[58], the mitochondrial enzyme appears in fraction II.

The rat liver isozymes give a normal distribution and this validates the methodology. The oxaloacetic acid sensitivity of these isozymes is also reversed when compared to those described by Holzer[74] [cf. Fig. 5(vii) B]. Growth on acetate favours the synthesis of the cytoplasmic isozymes[74]; under our growth conditions the yeast do not begin metabolizing acetate until 24.0 hrs after inoculation (Polakis and Bartley[75]) and the results of prolonged aerobic growth on the isozyme distribution are shown in Fig. 5(viii) #5. When compared to the disc electrophoresis pattern of 24.0 hr grown yeast the 36.0 hr culture exhibits a much increased proportion of the cytoplasmic isozyme. As predicted by the column polarities, the cytoplasmic isozyme does not migrate in this system,

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Fig. 5(vii) A.

Shows the elution profile for the malic dehydrogenase iso-enzymes in the eluent from D.E.A.E. Sephadex A ion-exchange chromatography column. Elution was stepwise as indicated.

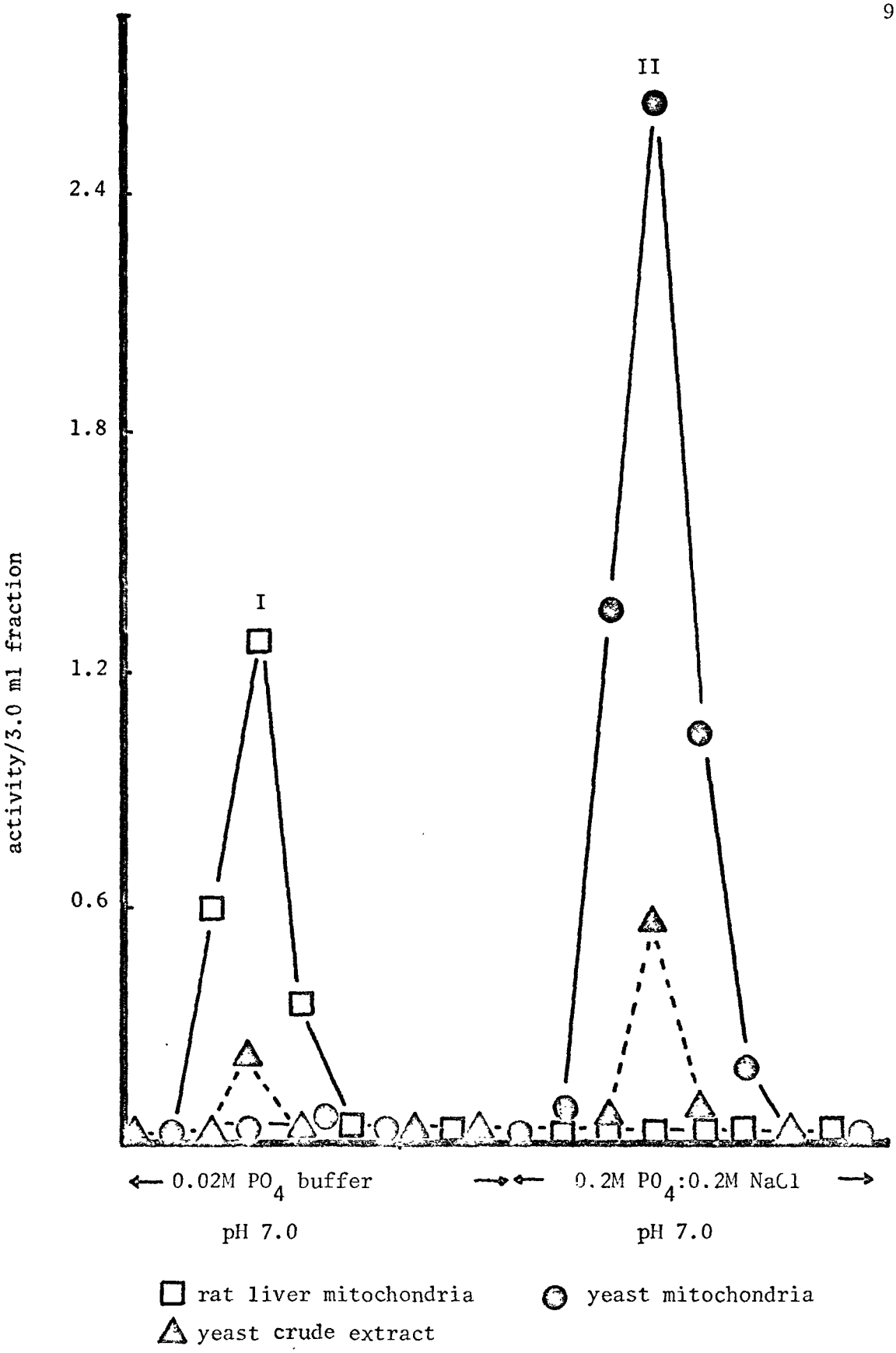

Fig. 5(vii)A Malic dehydrogenase iso-enzymes: column chromatography

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Fig. 5(vii)B

The fraction showing the highest activity for each peak in Fig. 5 (vii)A was assayed for sensitivity of oxaloacetic acid inhibition. Assay conditions were as described in the Method's section: oxaloacetic acid concentration was varied as indicated.

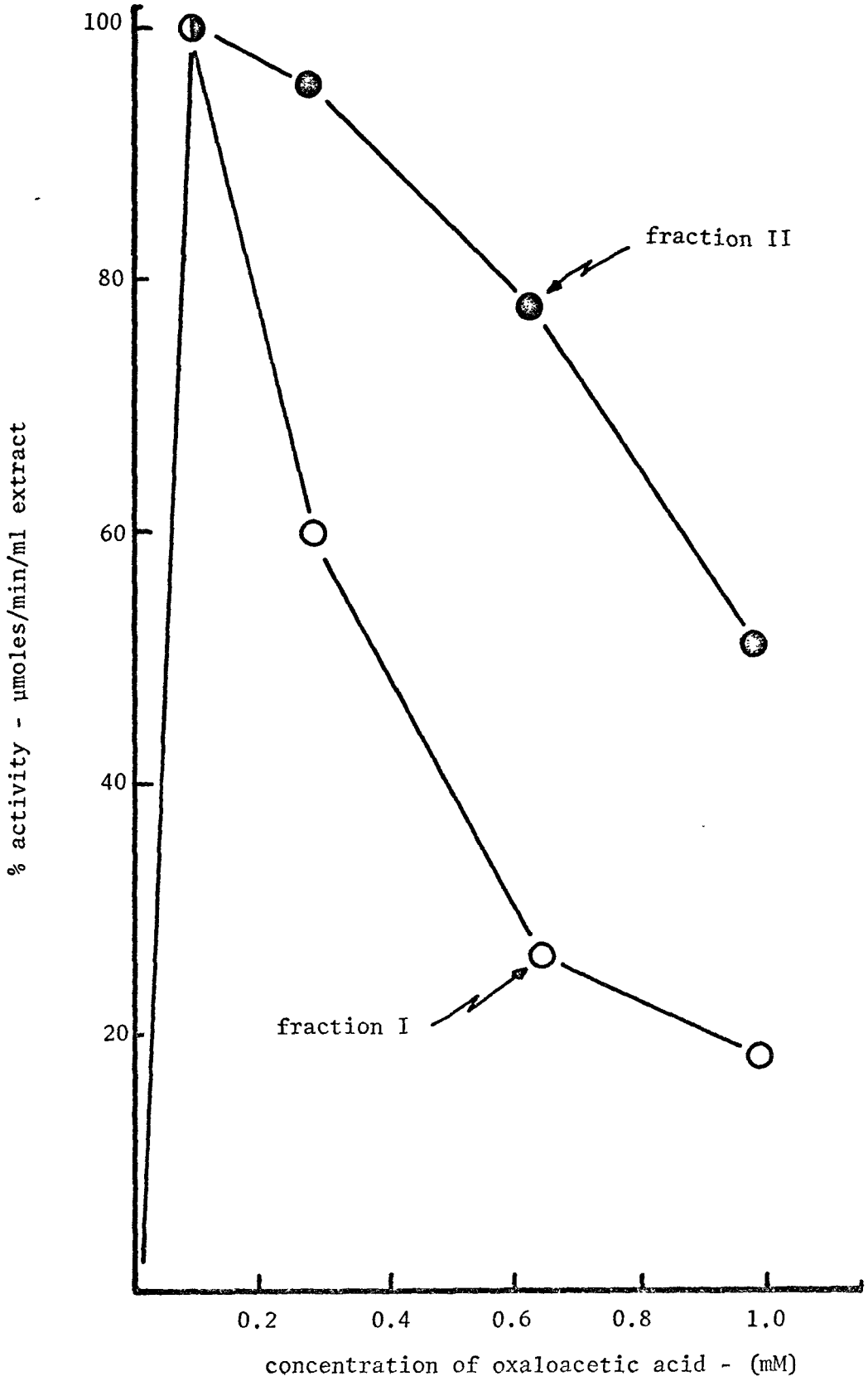

Fig. 5(vii)B The Effect of Oxaloacetic Acid on the Activity of MDH Isozymes.

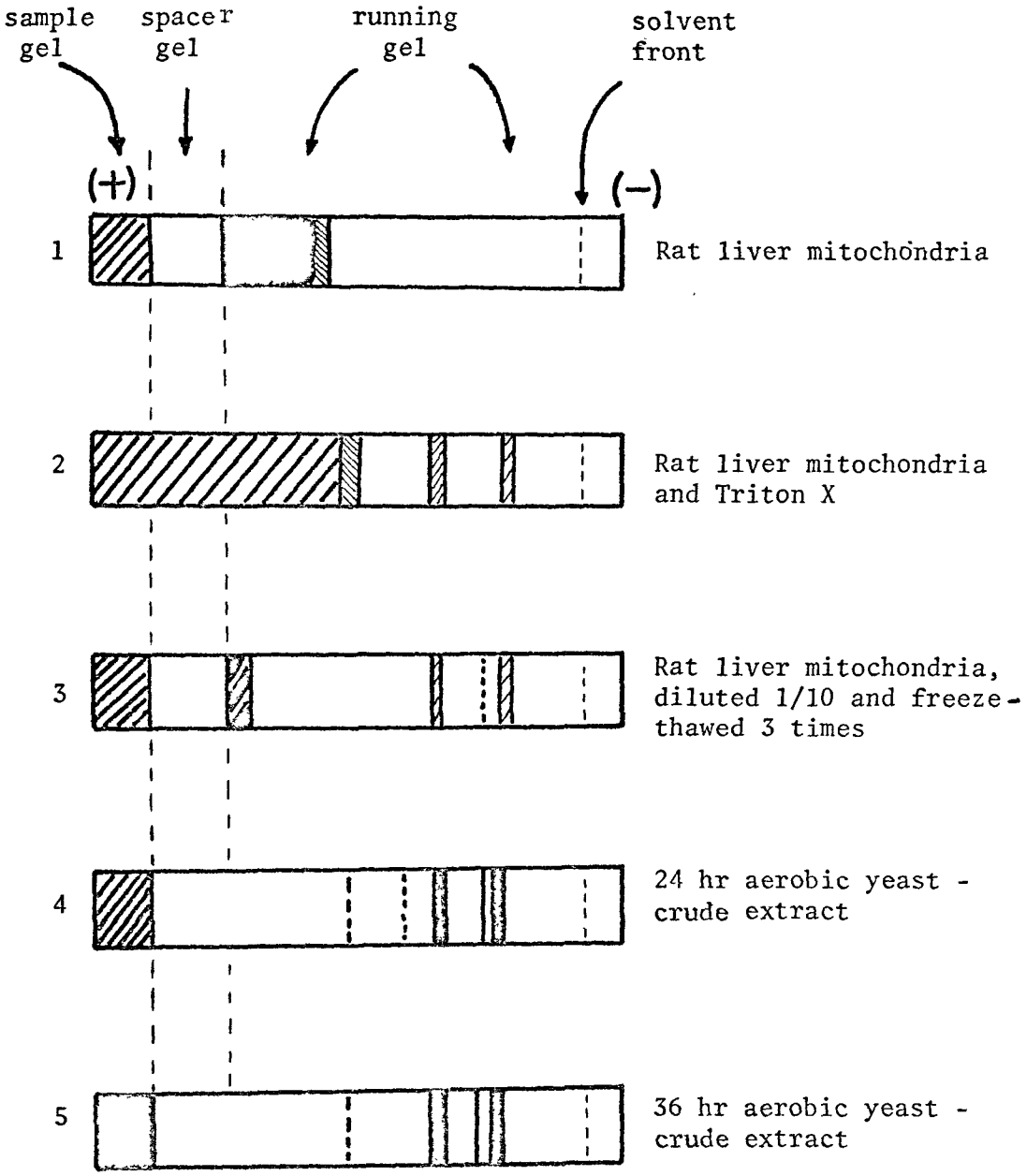

Fig 5(viii) Malic dehydrogenase iso-enzymes:disc electrophoresis.

but is retained in the spacer gel. In the region in which one would expect to find the mitochondrial enzyme there are four or possibly five bands, [Fig. 5(viii) #4 and #5]. The crude extracts are the result of french press breakage, and do not contain intact mitochondria. In this respect the results obtained with rat liver mitochondria, run as a systems control, are extremely interesting. The MDH of intact, carefully isolated rat liver mitochondria migrates slowly into the running gel and is not appreciably "stacked". When rat liver mitochondria are treated with triton "X" (5% solution) two more bands appear and the initial distribution is blurred throughout the sample and spacer gels. Treatment with osmotic shock (1/10 dilution) and freeze-thawing liberates a large portion of the "bound" MDH and the resulting pattern is similar to that observed for the yeast crude extracts. Throughout these experiments with the rat liver system a small amount of enzyme is retained in the sample gel, and this is probably a small amount of cytoplasmic isozyme.

Alcohol dehydrogenase (ADH) [Fig. 5(ix)] is another enzyme which is reported to exist as isozymes in yeast[76]. These are most probably the soluble ADH, traditionally associated with glycolysis, and the alcohol oxidase associated with the mitochondrion [see Chapter 4, Table 4(I)]. By analogy with MDH the diphasic synthetic curve observed in the control is probably the result of a preferential synthesis of the alcohol oxidase once galactose is exhausted. The upturn, or resumption of synthesis co-incides with the initiation of ethanol utilization [Fig. 5(ii)]. The kinetics of ADH synthesis are parabolic

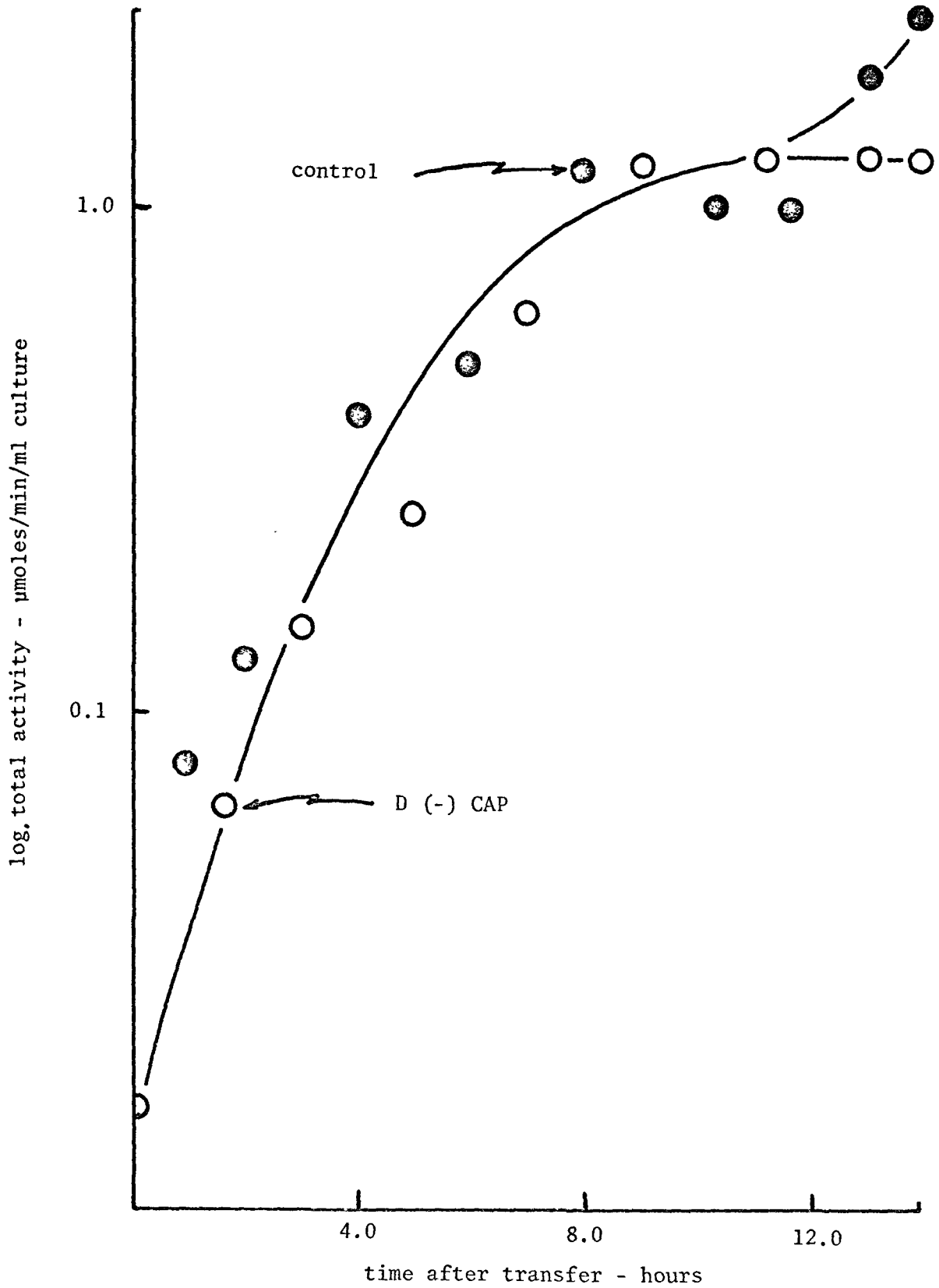

Fig 5(ix) Alcohol dehydrogenase, total activity:
2.7% galactose Y.An.O_2

and D(-) CAP does not alter these kinetics until after galactose is exhausted, when the induction or de-repression synthesis of the secondary growth phase is inhibited.

Parabolic kinetic curves for enzyme synthesis (see Chapter I) are also observed for hexokinase (HK) [Fig. 5(x)], isocitric dehydrogenase (IDH) [Fig. 5(xv)] and pyruvate decarboxylase [Fig. 5(xiv)]. A parabolic curve is characterized by a straight line on a log-log plot [Figs 5(xi) and 5 (xiii)] and a parabolic curve on a log-linear plot [Figs. 5(x) and 5(xii)]. The log-linear plot was retained for the majority of these enzymes to provide a more easily recognizable contrast to cyt. c. oxid. and NADH-cytochrome-c-reductase (cyt, c, red.), [Figs. 5(xvi) and 5(xvii)] which demonstrate logarithmic rather than parabolic synthetic curves.

Chloramphenicol does not affect the synthesis of HK or IDH until galactose is exhausted, when further synthesis of IDH is inhibited. IDH provides the third real case of induction or de-repression. In both the control and inhibited cultures IDH synthesis is turned off for the first 2.5 hrs when it resumes at a normal rate. The doubling times for HK and IDH, calculated from the log-log plots [Figs 5(xi) and 5(xiii)] are identical and probably reflect the general synthetic rate for proteins synthesized in the nucleo-cytoplasmic system, (nuclear genes plus cytoplasmic ribosomes). Synthesis of both pyruvate decarboxylase and succinic dehydrogenase (SDH) is partially inhibited by the drug. Pyruvate decarboxylase retains the same kinetics of synthesis, but the final yield of total activity is

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Fig. 5(x) A to 5(xviii).

All the parameters shown in these figures were determined on the same experiments. The control culture was a 2.7% galactose Y.An.O_2 → 2.7% galactose Y.An.O_2 transfer. The test culture was identical in every respect except that D (-) CAP was added to a concentration of 1.0 mg/ml [cf. Fig. 5(ii), (iii) and (vi)]. Activities were measured as described in the Method's.

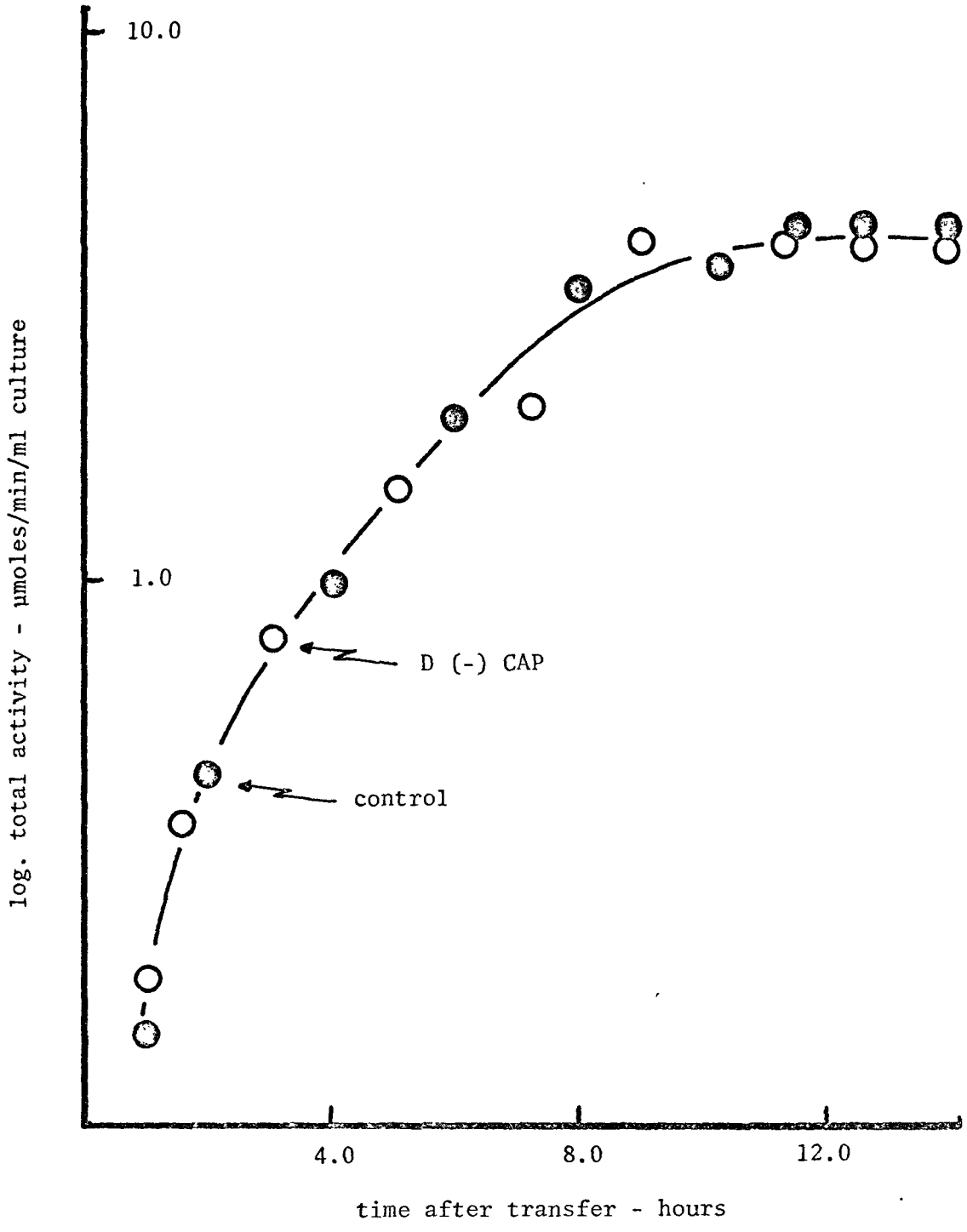

Fig 5 (x) A Total activity - hexokinase: 2.7% gal. Y.An.0_2

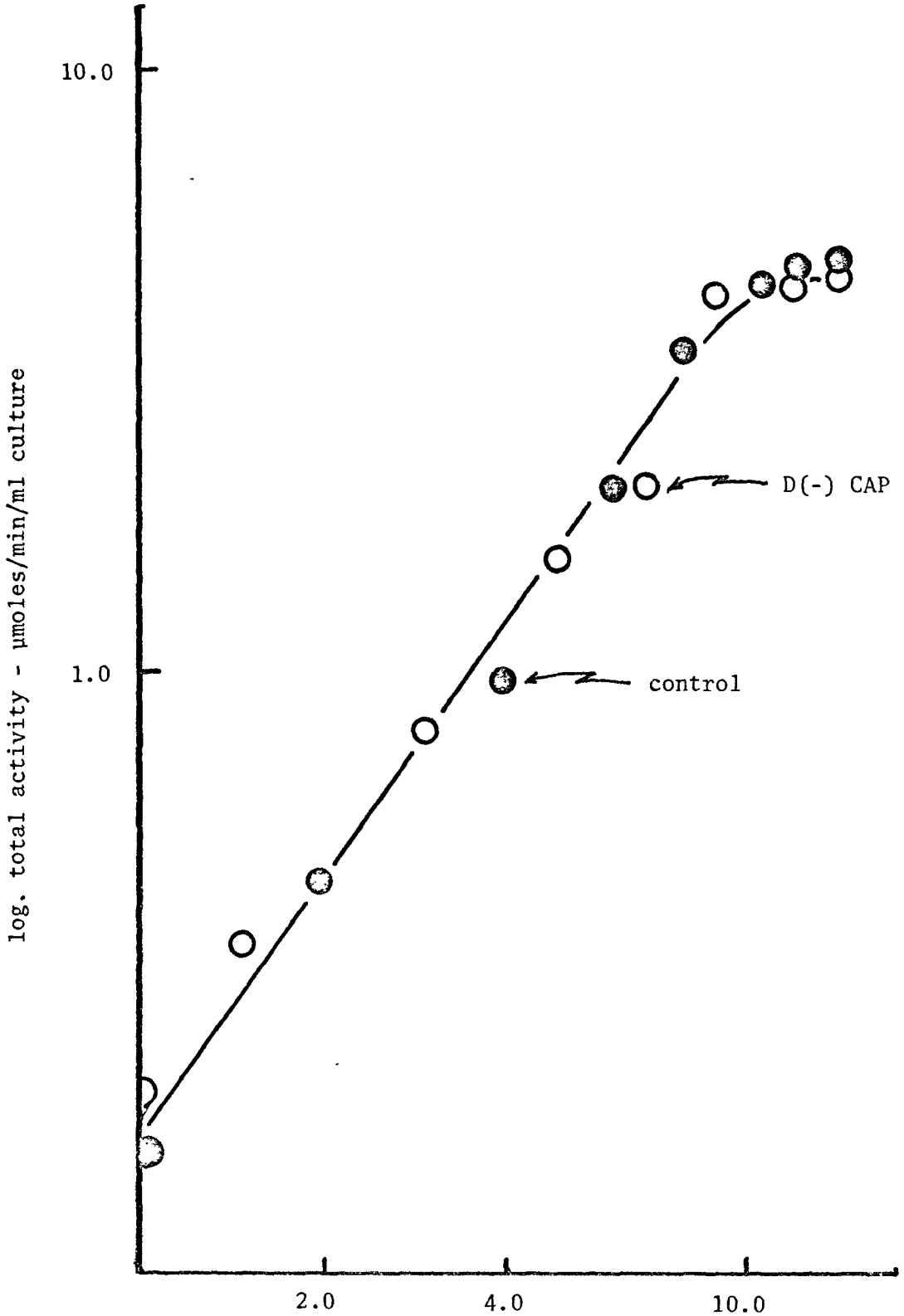

Fig 5(xi) Total Activity - hexokinase: 2.7% gal. Y.An.O_2

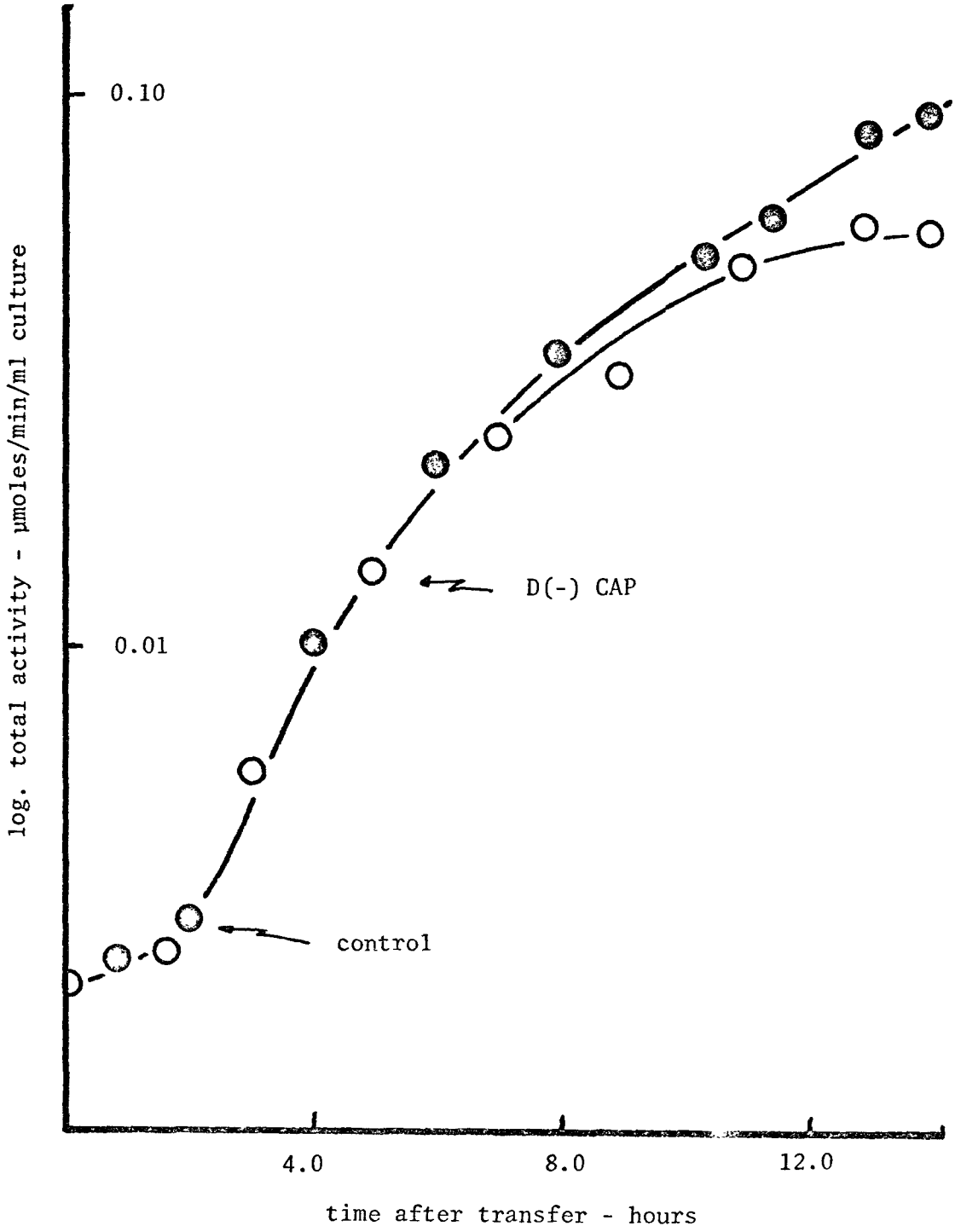

Fig 5 (xii) Total activity - isocitric dehydrogenase:2.7% gal. Y.An.O_2

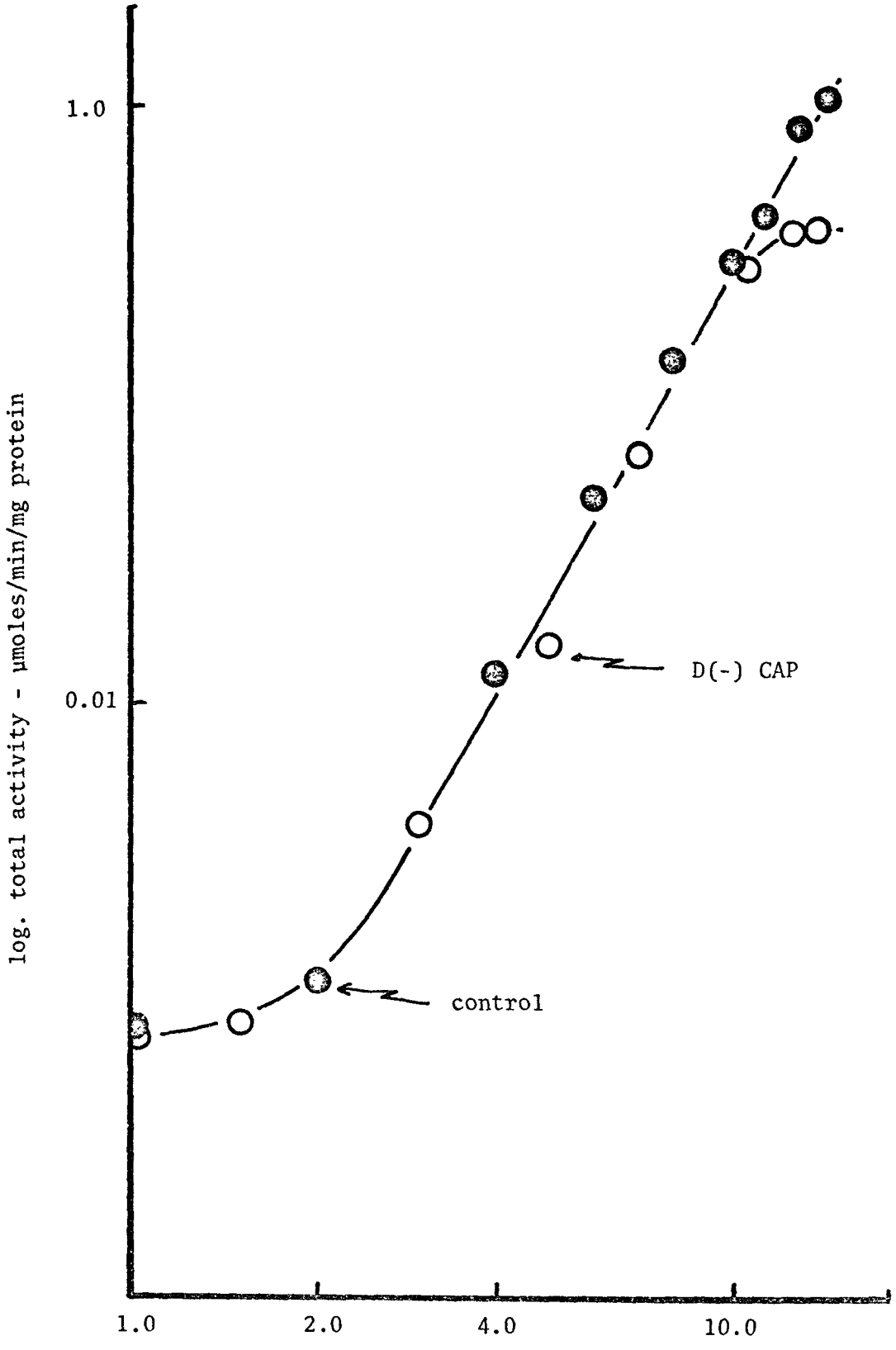

Fig 5(xiii) Total activity - isocitric dehydrogenase 2.7% gal. Y.An.O_2

reduced by 25%. In the case of SDH the kinetics approximate to a logarithmic curve and the final total activity is reduced by 40%. In the control culture, only the synthesis of HK and pyruvate decarboxylase are turned off at the end of the fermentative phase. However, growth will continue for a further 12.0 hrs under these conditions, and the pyruvate decarboxylase which is a component of the pyruvate oxidase system may enter a further secondary synthetic phase.

In contrast to all the above enzymes, cyt. c. oxid., cyt. c. red. activities, and total respiration increase logarithmically. The synthetic rate for these two enzymes is constant throughout the experiment, and is not affected by the galactose → ethanol transition. The doubling time for cyt. c. oxid. is 2.25 hrs and that for cyt. c, red. is 2.0 hrs. These agree well with the figures for the total respiratory ability reported in Fig. 5(v) and Fig. 2(v). In the presence of D(-) CAP the synthetic rate is reduced but the kinetics are still logarithmic. The doubling time for cyt. c. oxid. under inhibited conditions is 6.0 hrs and that for cyt. c. red. is 3.0 hrs. The de-repression phenomenon reported by other workers [Mahler *et al.*[22], Utter *et al.*[21]] is absent in the case of cyt. c. oxid. and cyt. c. red. if one considers the rates of synthesis derived from the total activities. Only IDH exhibits a de-repression profile during fermentative growth, and the de-repression or induction exhibited by MDH and ADH occurs only after the galactose is completely utilized. The apparent de-repression observed by these workers probably results from the use of specific activity as a parameter of cellular synthesis.

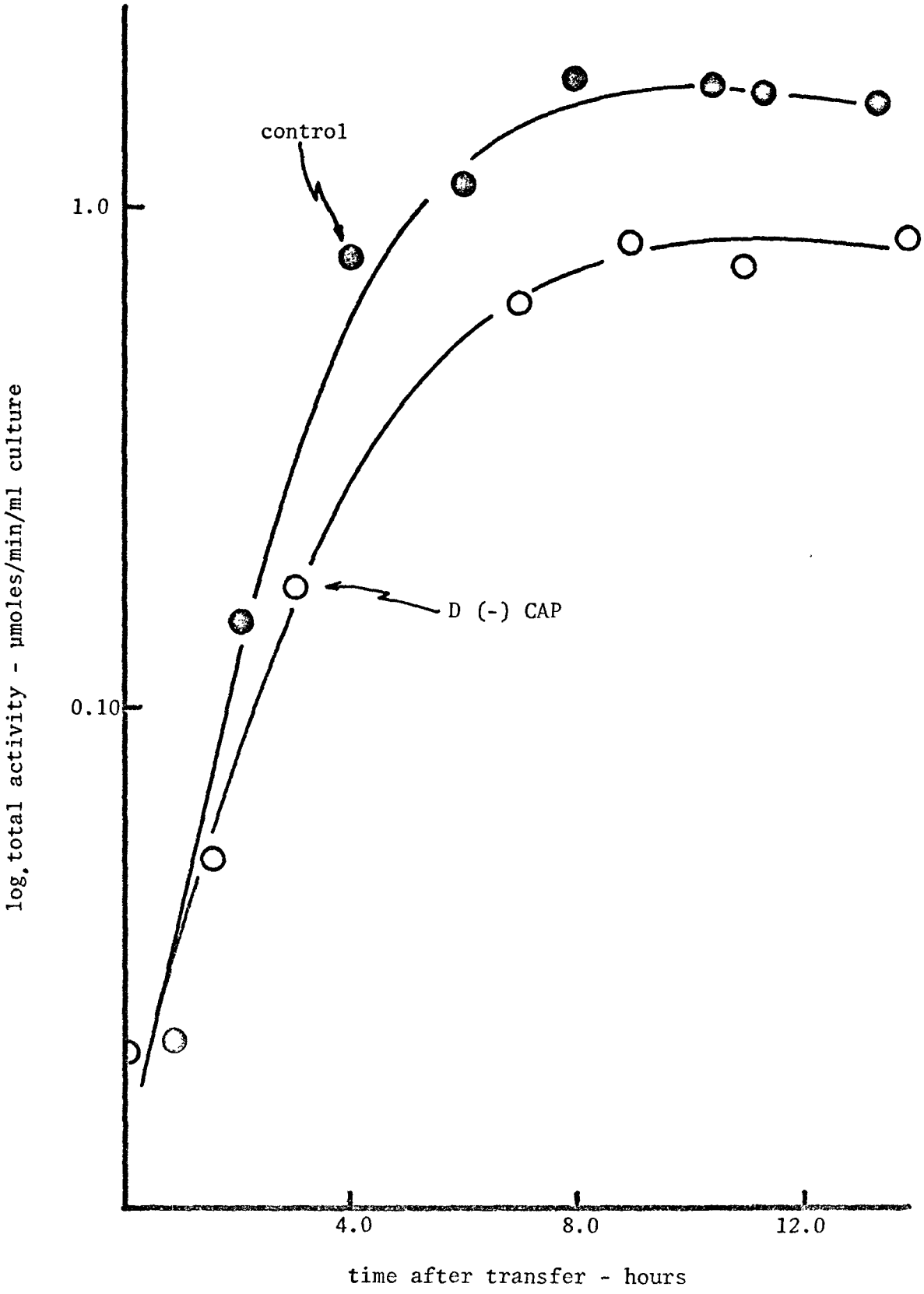

Fig 5(xiv) Pyruvate decarboxylase, total activity: 2.7% glucose transfer

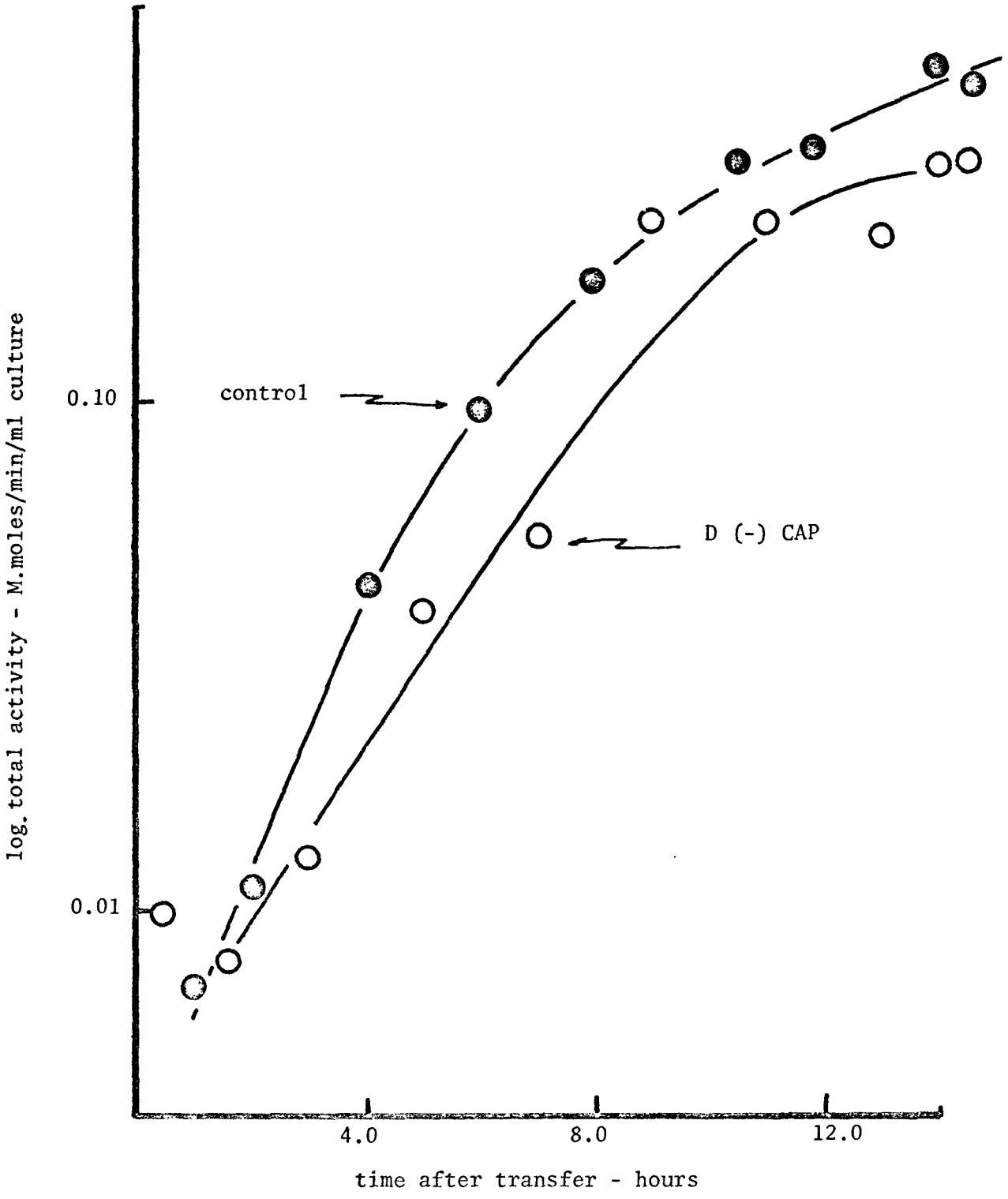

Fig 5(xv) Succinic dehydrogenase, total activity: 2.7% galactose Y.An.O_2 transfer

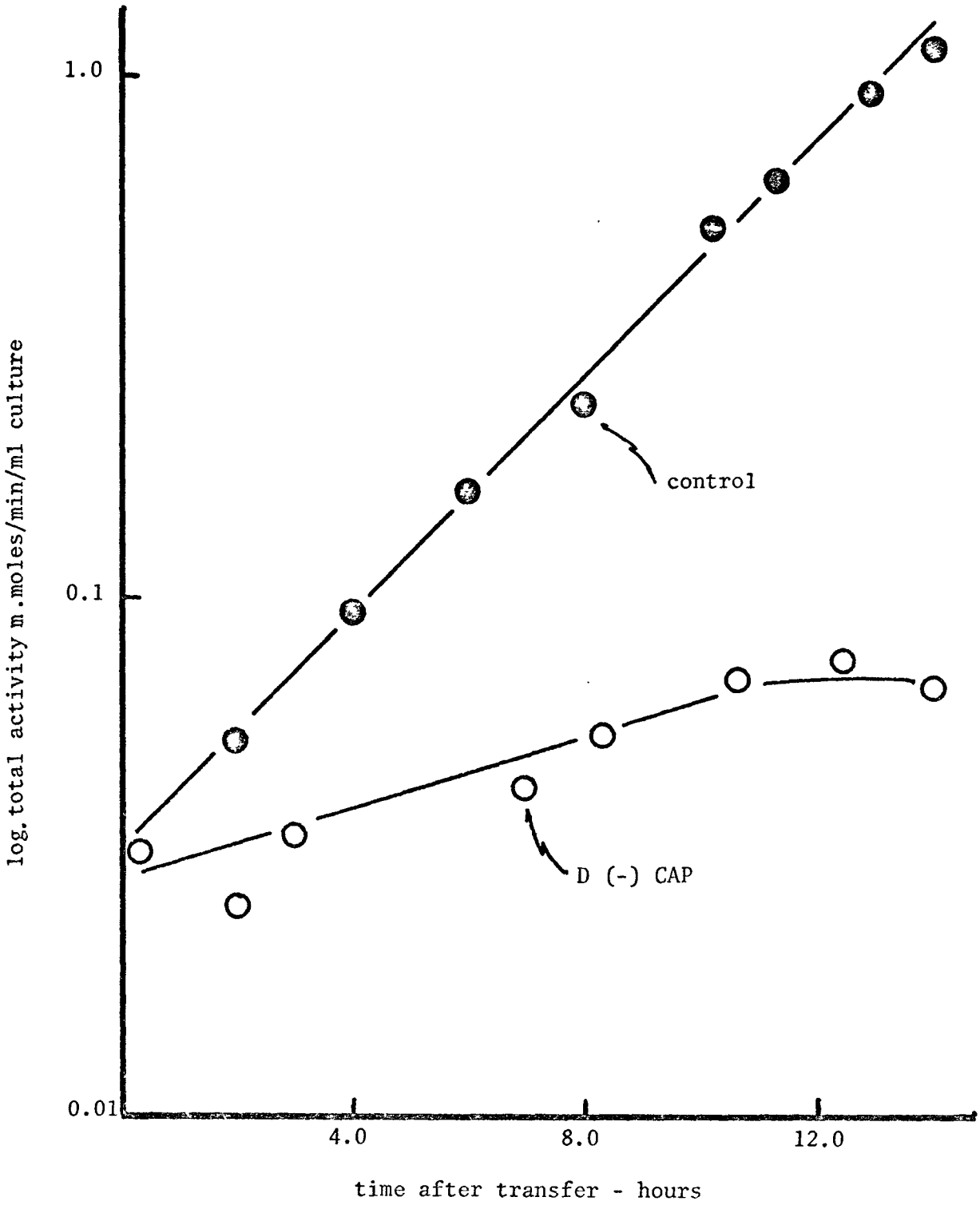

Fig 5(xvi) Cytochrome c oxidase, total activity:
2.7% galactose Y.An.O_2

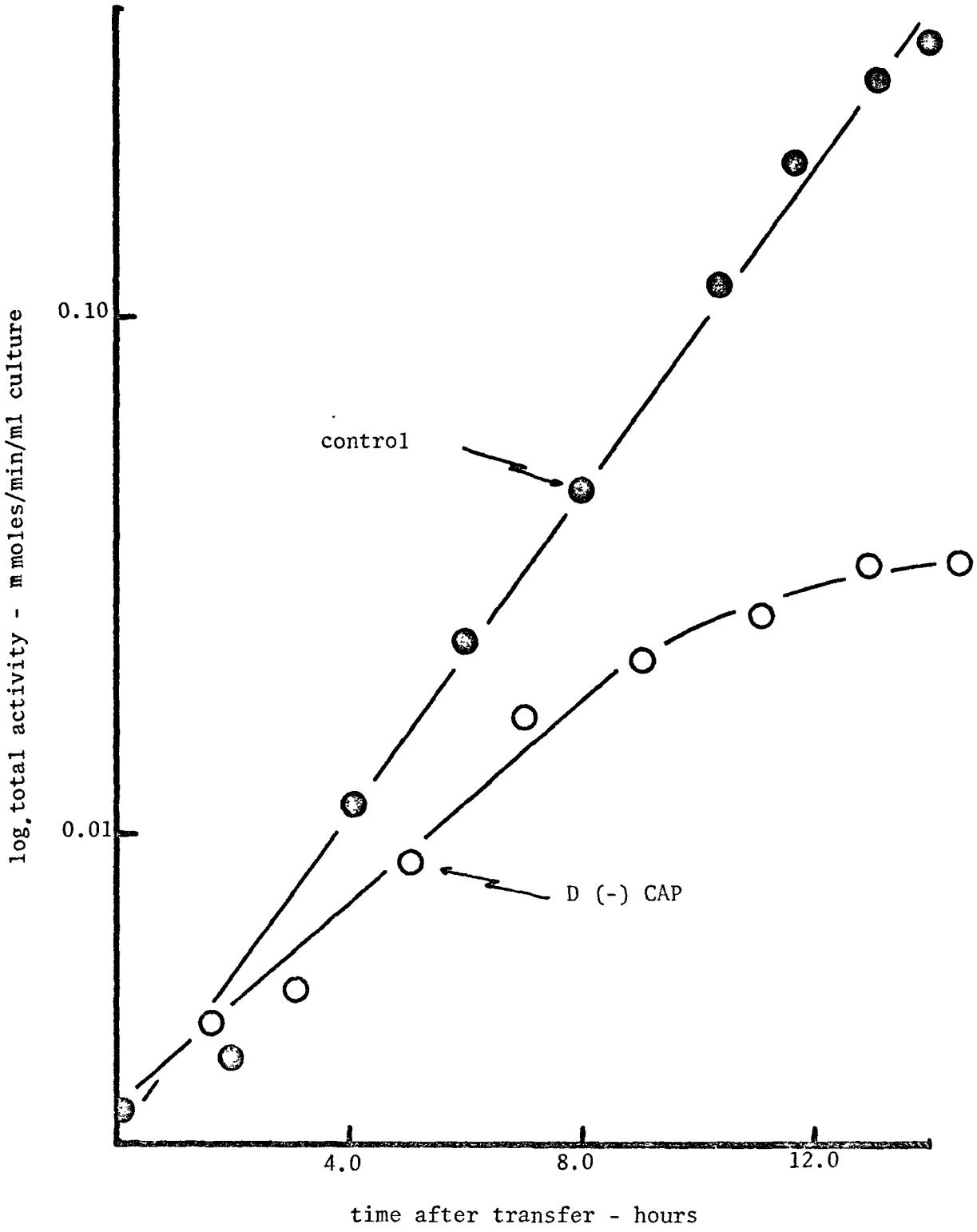

Fig 5(xvii) NADH-cytochrome-c reductase, total activity: 2.7% galactose Y.An.O_2

This is amply illustrated in Fig. 5(xviii) which shows the specific activity of cyt. c. red. as compared to total activity, Fig. 5(xvii) control curve. It is obvious that in this case the increase in specific activity is due to a decrease in the rate of synthesis of other cellular proteins rather than to a de-repression of cyt. c. red. synthesis. Similar results have been observed for cyt. c. oxidase [cf. Fig. 3(iii) and Fig. 5(xvi), control curve] and MDH [cf. Fig. 3(iv) and Fig. 5(viii), control curves].

Discussion

As the respiratory ability of the cells decreases [Fig. 5 (iv)] the glycolytic pathway should be released from respiratory inhibition, (Crabtree effect) and glycolysis should be stimulated. This is in fact observed in the rate of ethanol production [Fig. 5 (ii)]. The reduced rate of ethanol utilization in the inhibited culture is attributed to the reduced respiratory ability.

The DNA data is of interest in that both dry weight increase [Fig. 5(i)] and enzyme synthesis [Figs. 5(viii), (ix), (Xii), (xiv) and (xv)] occur in the absence of DNA synthesis. The parabolic synthetic curves exhibited by most of the enzymes studied, which are not affected by the "petite" mutation and therefore presummed to be coded for by the nuclear DNA, are probably generated by the transition from a fast initial growth rate to the second slower growth rate. Through Type II controls (general RNA → DNA feedback), one would expect the rate of translation of m-RNA to be gradually decreased. Also, the rate of m RNA production will be affected by

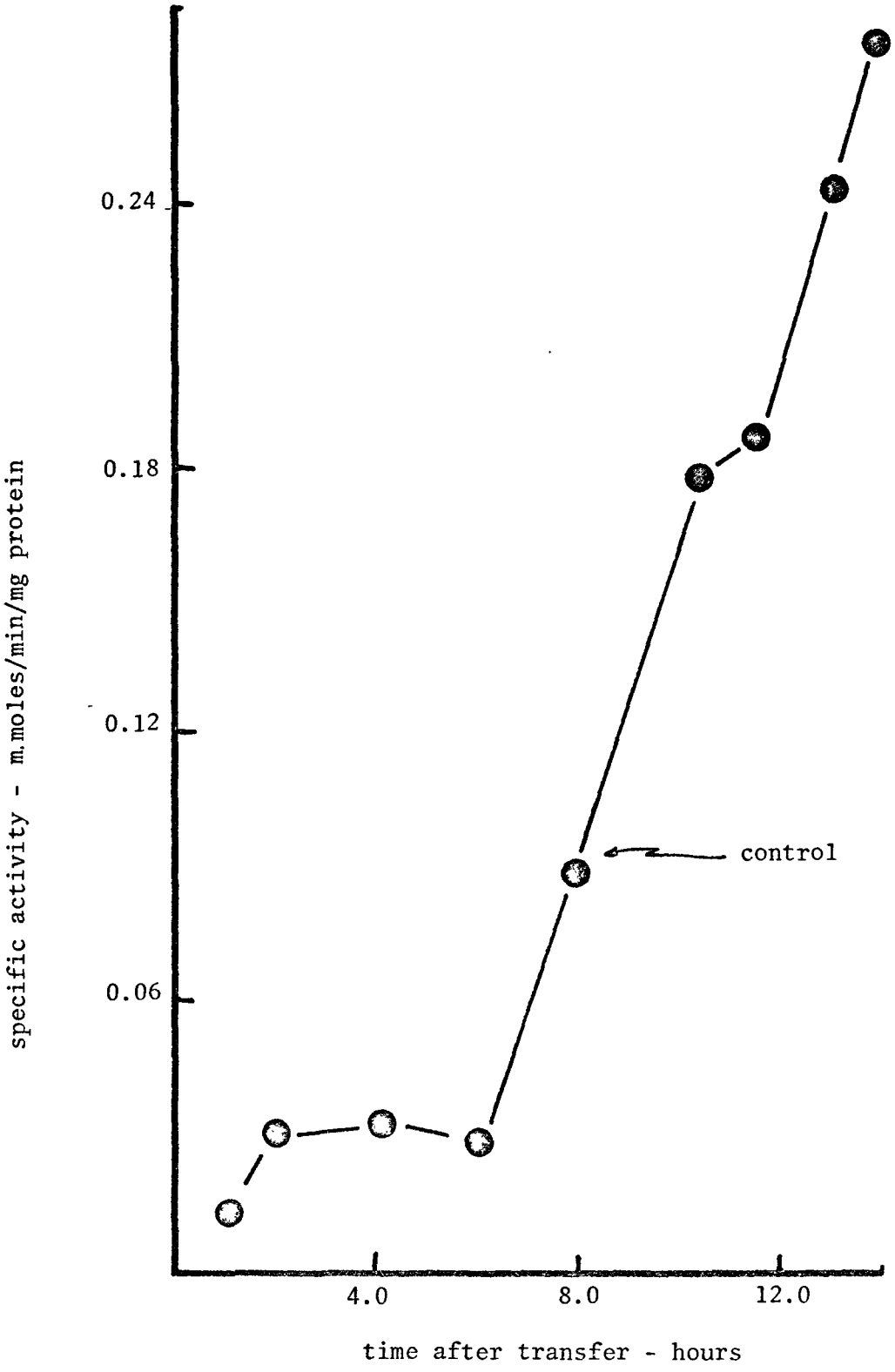

Fig 5(xviii) NADH-cytochrome c reductase, specific activity: 2.7% galactose Y.An.O_2 transfer

the number of genomes present in the culture, and the cessation of DNA synthesis would remove one log factor from the kinetics of synthesis [cf. Fig. 5(iii)]. It therefore seems very probable that the parabolic kinetics are a reflection of the gross cellular physiology. The kinetics exhibited by the control curves of IDH and SDH [Figs. 5(xii) and (xv)] fit this hypothesis very well. Hexokinase and pyruvate decarboxylase deviate from these kinetics once the galactose is exhausted, and this lack of synthesis probably represents a cessation of gene translation, associated with the fermentation → respiration transition. The gross kinetics exhibited by ADH and MDH are complicated by the presence of the isozymes, and although the MDH kinetics more closely resemble a logarithmic rather than a parabolic curve, the synthetic rate is very different from that of cyt. c. oxid. and cyt. c. red. Another reason for including MDH in this group is that Munkres[38] working with *N. crassa* has demonstrated that the structural genes for the MDH isozymes are located in the nucleus. The secondary synthetic phases are obviously a response to the carbon source transition, and this also sets these two enzymes (ADH and MDH) apart from those of the respiratory chain. In the inhibited culture, the synthesis of all the enzymes stops once galactose is exhausted. This probably reflects a metabolic "shut down" in response to the energy starvation enforced by the removal of the yeast's oxidative capacity. Prior to galactose exhaustion the kinetics of HK, IDH, ADH and MDH synthesis remain unaltered, so that one may conclude that D(-) CAP is not affecting the synthesis of these enzymes, and that the loss of respiratory ability is not conditioning the synthetic

rate in any way. It seems probable that the synthetic rate of these enzymes is controlled by the fermentative capacity of the yeast [cf. Fig. 3(iv)]. That the fermentative capacity is already maximal can be seen by comparing the growth curves in Fig. 5(i) and also from the fact that galactose is present in the inhibited culture for at least half an hour longer than in the control. Thus one would expect the results observed.

Pyruvate decarboxylase and succinic dehydrogenase are enzymes which exhibit kinetic parameters similar to those of the nuclear-system group, yet their synthesis is affected by D(-) CAP. Pyruvate decarboxylase, as measured in this experiment, represents the soluble pyruvate decarboxylase[77] and the pyruvate decarboxylase which is part of the pyruvate oxidase system[78]. This latter multi-enzyme complex is mitochondrial in location, and depends upon an integrated structure for maximal activity. In the presence of D(-) CAP the kinetics of synthesis are gradually altered and the final yield is decreased by 25%.

This inhibition may well be due to structural disorientation, resulting from the primary inhibition of mitochondrial protein synthesis. In the case of SDH the changes are a little more drastic; the kinetics more closely approach those expected for logarithmic synthesis, and the final total activity is reduced by 40%. SDH is a respiratory chain enzyme, structurally linked to complex II[79], and any disruption of the mitochondrial membrane structures would be expected to affect the activity of this enzyme. The logarithmic kinetics suggest that SDH synthesis in the inhibited culture is limited

by some protein, or other factor of mitochondrial origin, and that the decreased total activity is due to a disruption of mitochondrial structures. The fact that cyt. c. red. synthesis is inhibited by approximately 50% adds further credence to this conclusion. [As assayed in these experiments cyt. c. red. consists of complex I and III as described by Green[79]].

The kinetics of cyt. c. oxid., cyt. c. red. and the total respiratory ability are obviously related; the doubling time in the control culture is approximately 2.0 hrs, D(-) CAP affects this doubling rate throughout growth, and the kinetics are logarithmic in form. It seems highly probable that these two enzymic activities are produced by the "rate limiting substances" alluded to in the discussion of Chapter 2. D(-) CAP inhibits the synthesis of cyt. c. oxid. to twice the extent that it inhibits the synthesis of cyt. c. red. This difference probably explains the continuously decreasing rate of synthesis of total respiratory ability observed in the inhibited culture, [Fig. 5(v)]; although this provides a nice illustration of the interaction of two logarithmic synthetic rates to produce parabolic kinetics, it is still puzzling[80]. A similar differential inhibition was observed by Mahler *et al.*[81] and these authors were unable to find a rational explanation for this phenomenon. The rates of synthesis observed in the control culture suggest that under normal conditions the rate of gene transcription and subsequent translation is the same for both enzymes. D(-) CAP does not affect transcription, therefore the effect must be manifested either at translation or in the subsequent assembly of the mitochondrial complexes

[cyt. c. oxid. = complex IV[79]]. If cyt. c. oxid. activity is dependent upon more than one protein of mitochondrial origin, perhaps the "elusive" structural protein, then the inhibition of translation would result in a greater accumulative inhibition of an activity dependent upon two proteins (cyt. c. oxid.) than it would have on an activity dependent upon only one (cyt. c. red.).

That D(-) CAP inhibition of cyt. c. oxid. and cyt. c. red. synthesis causes an alternation in the activity of other enzymes dependent upon the strucutral integrity of mitochondrion for maximum activity, correlates well with the cytological studies performed on yeast treated with CAP [Linnane *et al.*[65]] and with the other conditions (anaerobiosis, glucose respiration, "petite" mutation) which cause the yeast to lose cytochromes $a+a_3$, b and c, and produce degenerate mitochondrial profiles[12,13]. The results recorded above illustrate the metabolic events leading to the production of degenerate mitochondrial profiles. One can conclude from this, as from the previous studies of other workers (see introduction) that the production of specific proteins from mitochondrially inherited factors is a necessary condition for the maturation of mitochondria. The Q_{O_2} and total respiration data show that mature mitochondria do not degenerate under repressive conditions [see also Fig. 3(ii)] and this, together with the data presented above, implies that degenerate mitochondrial profiles may result from the synthesis of mitochondrial components controlled by the nucleus in the absence of a simultaneous synthesis of proteins produced and controlled in-

tramitochondrially. The kinetic data show that the mitochondrial protein synthesizing system does not respond to changes in the cellular growth rate, or to substrate and energy source transitions [Figs. 5(xvi) and (xvii) control curves] and that it is independent of any direct nuclear control.

These experiments indicate that cellular control of mitochondrial proliferation is limited to preventing mitochondrial proliferation under conditions where the cell itself is unable to grow. This is managed by starving the mitochondrial system of necessary building materials e.g. amino acids, lipids, nucleosides etc., and control of this kind is exhibited by the inhibited culture. Cellular metabolism is shut down due to energy starvation, and this in turn causes the inhibited mitochondrial system to shut down completely. Under conditions of rapid cellular growth (see Chapter 2) the mitochondria are diluted out of the yeast. Both the nuclear-cytoplasmic DNA-RNA-protein system and the mitochondrial DNA-RNA-protein system are completely independent with respect to genetic and metabolic control mechanisms during rapid cellular proliferation. However, like many other symbionts, there are occasions e.g. growth on non-fermentable substrates, when these two systems become mutually dependent.

CHAPTER 6

General Discussion and Conclusions

The evidence derived from the experiments reported in Chapters 2, 3, and 5 provide information about the general control of energy metabolism in yeast. Detailed analysis, at the level of specific enzyme control is at present scanty, but some general conclusions may be extracted from the data.

The experiments in Chapter 4 illustrate the effects of the fermentation:respiration transition on intermediary energy metabolism, and the effect of a lowered respiratory capacity on these changes. A detailed discussion of these Type III control interactions is contained in Chapter 4 and this will not be elaborated below. Suffice it to say that, as indicated by the *in vivo* and *in vitro* studies referred to in the Introduction and in Chapter 4 (discussion), the three major energy generating systems of yeast are linked by Type III controls (allosteric control of enzyme action), and these mechanisms are continuously operative throughout the growth cycle.

The data of Chapter 2 demonstrate that under conditions where cell growth is not limited by respiration the rates of proliferation of the respiratory system and of cellular mass are entirely independent. This conclusion implies that during growth

on non-fermentable substrates the cellular proliferation will be limited by the rate of synthesis of the respiratory apparatus, and that the rates of synthesis will be co-ordinated. This is actually observed [Fig. 2(vi)] in the early stages of aerobic growth before the onset of fermentation, and probably accounts for the rate change observed in the latter part of the aerobic growth curve [Fig. 2(v)]. This conclusion is also reinforced by the data on cyt. c. oxid. and cyt. c. red. synthesis obtained in Chapter 5 [Fig 5(xvi) and Fig. 5(xvii). The logarithmic doubling time for the synthesis of these enzymes is the same as that for the synthesis of respiratory ability under the various growth conditions reported in Chapter 2.

A large portion of the data discussed in the latter part of the introduction implies that the yeast mitochondrion possesses all the biochemical equipment necessary for the replication of its own DNA. So far very little evidence has accumulated with respect to the *in vivo* functioning of this system. The recent work of Mahler *et al.*[81] demonstrates that there are two separate protein synthesizing systems operative in yeast *in vivo* under conditions where the yeast is undergoing a fermentation → respiration transition. One system, sensitive to D(-) CAP and insensitive to cycloxheximide is characterized by a rapid uptake of radioactive amino acids into an "insoluble" fraction of the mitochondrion, and then a transport of this activity to other more soluble fractions of the mitochondrion. The second system, sensitive to cycloxheximide inhibition, but in-

sensitive to D(-) CAP is characterized by a rapid incorporation of radioactive amino acids into the microsomes, followed by a rapid, and proportionally much larger, transport of this activity to all fractions of the mitochondrion. The characteristics of these two systems fit well with what one would predict from the synthetic data observed in Chapter 5. In addition to the separation of these two synthetic systems, Mahler was also able to demonstrate that prolonged inhibition of the cytoplasmic synthesizing system resulted in an inhibition of the mitochondrial system. This is equivalent to the energy-starvation "shut-down" observed in Chapter 5. Mahler *et al.*[81] were also able to demonstrate that when the mitochondrion is deprived of respiratory energy, (Antimycin A used as an uncoupler of phosphorylation) synthesis of proteins by the D(-) CAP sensitive system is inhibited. This is analagous to the loss of cyt. c. oxid. activity observed in Chapter 3, Fig. 3(iii) which is conditioned by the removal of oxygen. Mattoon[82] has reported that oligomycin which inhibits electron transfer inhibits the synthesis of cytochromes $a+a_3$, b and c, in aerobically grown yeast, and preliminary results from this laboratory indicate that 2,4 dinitrophenol (an uncoupler) also causes the same type of inhibition. The conclusion drawn in Chapters 2 and 5 that the mitochondrial protein synthesizing system is independent of nuclear control, and that the synthesis of proteins in this system is related to its oxidative function is well corroborated by the experimental results of other workers in this field. (See also Chapman and

Bartley[83]).

The data on MDH presented in Chapter 3, and that for the other nuclear derived enzymes (Chapter 5) strongly suggests that these enzymes are synthesized in response to the macro-environment outside the yeast cell. These enzymes, which belong to the glycolytic pathway and the Kreb's cycle are not directly related to the respiratory chain either functionally or structurally, in the same way that the constituent parts of the respiratory chain complexes are. One would expect the synthesis of these enzymes to be subject to Type II control (general RNA → DNA feedback), and this is observed in the experiments of Chapter 5. Also, the various and diverse responses of these enzymes to the changing conditions of the aerobic growth cycle would suggest that these enzymes are also subject to Type I control (specific induction of DNA transcription). From a consideration of the substrate/product relationships of these enzymes the Type I control mechanism of induction/repression by specific substances seems entirely plausible. This type of control has been demonstrated for the enzymes which metabolize galactose → glucose-6-PO_4 and it seems reasonable to suppose that similar mechanisms are controlling the synthesis of the glycolytic and Kreb's cycle enzymes. The turning off of HK, ADH, MDH, and pyruvate decarboxylase synthesis at the end of the fermentative phase and the subsequent resumption of MDH and ADH synthesis with the onset of ethanol oxidation, emphasize this point.

The data so far discussed, including those from other labor-

atories, suggest that there is an independent DNA-RNA-protein, information processing system in the yeast mitochondrion. The synthetic data in Chapter 5 suggest that the mitochondrial proteins (cyt. c. oxid., cyt. c. red.) share a common translation-transcription rate and that synthesis of these enzymes is coordinated. This system should be amenable to Type II control, but it is doubtful that this system is affected by Type I controls. The proteins concerned are large molecules which are integrated into a large lipid-protein complex. The substrates with which these enzymes (cyt. c. oxid., cyt. c. red. : complexes III and IV) interact are macromolecules, and the products generated, hydrogen ions, hydroxyl ions, ATP, ADP and Pi, are general consituents of the cellular metabolism which are obviously equilibrated with the cellular metabolic pools (see also introduction, page 4). Thus there are no specific substances available from which Type I control mechanisms could be derived. In contrast, any disruption in the components necessary for complex formation e.g. inhibition of cellular metabolism, would result in an accumulation of free proteins in the mitochondrion and Type II feedback control should become operative. This is probably the mechanism by which the inhibition of *petite* "sensitive" protein synthesis by anaerobiosis, antimycin A, oligomycin, and di-nitrophenol, and fermentation is mediated.

A discussion of the control of mitochondrial protein synthesis leads directly to the problem of mitochondriogenesis in yeast. By

analogy with the systems observed in bacteria, plants, fungi, and higher animals, there must be a relationship between the rate of mitochondrial DNA replication and the rate of synthesis of the products coded for by this DNA. From the foregoing, one concludes that some form of integrated structure, which would prevent Type II feedback by "free" mitochondrial proteins must be necessary to the continued proliferation of the mitochondrial DNA. That this DNA is proliferated under conditions where mitochondrial function is inhibited seems certain from the nature of the organism. Prolonged anaerobic or fermentative growth does not cause the majority of cells to become "petite", which implies that the information necessary for the synthesis of cytochromes $a + a_3$, b and c, i.e. the mitochondrial DNA, is retained in all the cells, and this can only be attained by DNA replication. Inhibition of mitochondrial DNA replication by acriflavin causes the cells to become "petite", and interestingly enough, this mutagen is inoperative in the absence of cell growth[84]. The independent proliferation of mitochondrial DNA envisioned [see Fig. 6(i)] would also explain the very high normal frequency of "petite" formation common to facultative yeast. Compared to the mutational frequencies common to bacteria, mutation rates in excess of 1% are very large. The curing of wild type yeasts by the loss of mitochondrial DNA could easily be explained by the independent proliferation theory.

The scheme outlined in Fig. 6(i) is a tentative and highly schematic attempt to correlate the factors which affect the mani-

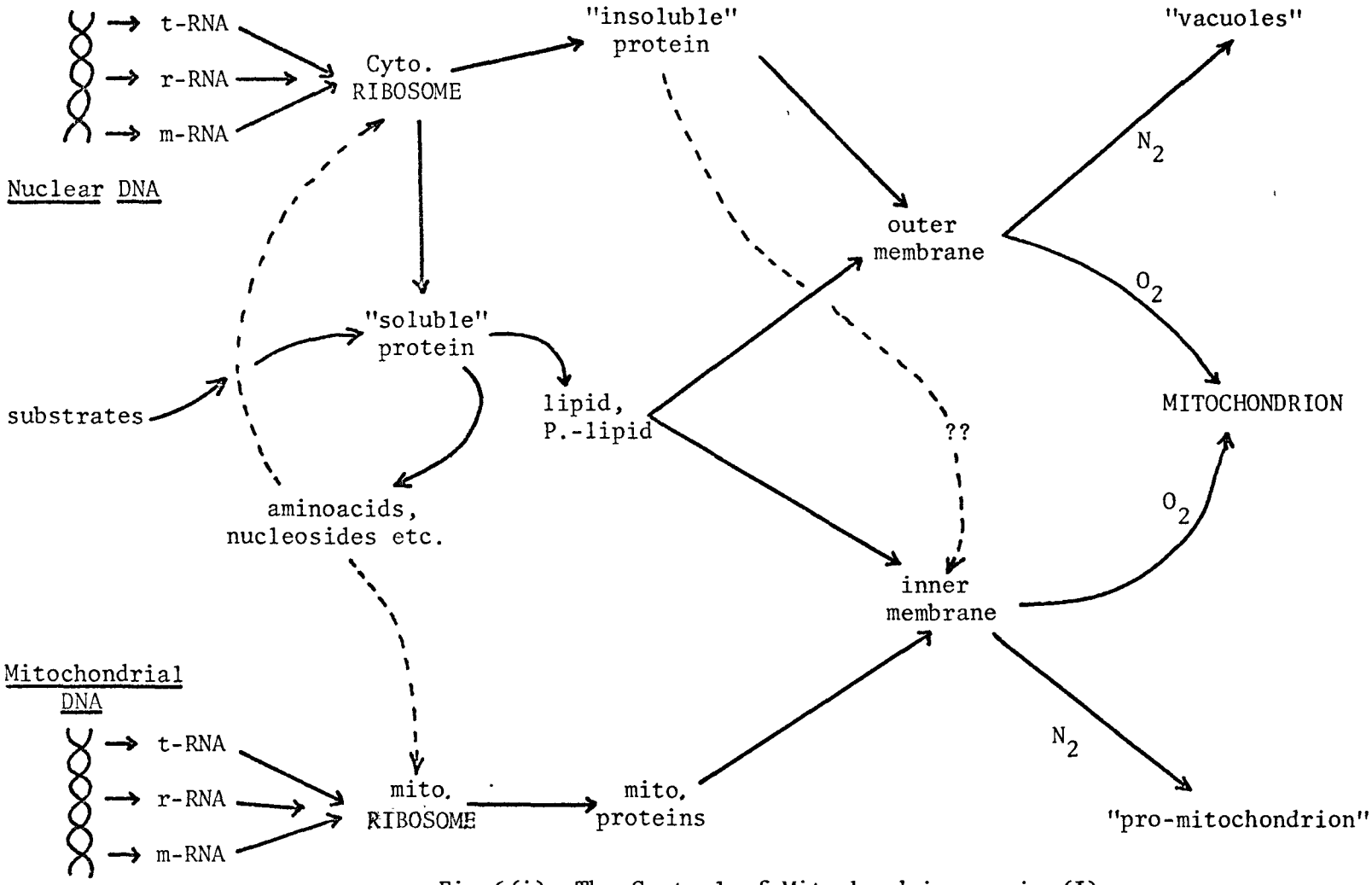

Fig 6(i) The Control of Mitochondriogenesis (I)

festation of respiratory function in yeast. Cytological studies of anaerobically grown yeast have revealed degenerate mitochondrial profiles and depending upon the anaerobic growth conditions these profiles vary in number and degree of degeneration[12 25]. Criddle *et al.*[42] have recently examined these degenerate profiles, both cytologically and biochemically and have dubbed these structures pro-mitochondria. There is DNA associated with these structures as well as SDH and cyt. c. red. activity. Biochemical confirmation of these results appeared previously in the work of Soumonalien[85], who isolated "mitochondrial fractions" from anaerobic, glucose repressed, and ethanol metabolizing yeast. His data show that although the amounts of the mitochondrial enzymes/cell are altered to a very considerable extent, cyt. c. red., cyt. c. oxid. and NADH oxidase activities are associated with the same fractions under all conditions. It seems likely that Soumonalien observed the transition from the pro-mitochondrion → mature mitochondrion, by the addition of outer membrane structures derived from nuclear information, which was proposed by Criddle *et al.*[42].

Although this data[42,85] suggests the existence of a pro-mitochondrion, some anomalies become apparent when the data are scrutenized more carefully. Soumanalien used the gentle spheroplast technique (cf. Chapter I) to isolate his "mitochondrial fractions" and obtained particles which were larger, and possessed a greater range of respiratory activities than those of Criddle *et al.* The digestion was carried out in tightly stoppered flasks

so that post-harvesting exposure to oxygen would be minimized. In contrast, Criddle *et al.*[42] took excessive pre-cautions against post-harvesting oxygen exposure and protein synthesis, but used a mechanical homogenization technique to liberate the pro-mitochondria. This resulted in a much smaller particle (harvested at 105,000 x g. v. 20,000 x g. for mature mitochondria) which did not possess cyt. c. oxid. or NADH oxidase activity (cf. Soumanalien[85]). From a theoretical view point one would expect the pro-mitochondrion to be even more fragile than the mature mitochondrion which favours the data of Soumanalien. Also from the discussion of Type II control (see above) one would expect to find an integrated respiratory chain in the pro-mitochondrion. It seems therefore that the experimental approach to the isolation of the pro-mitochondrion must be improved if its isolation and characterization are to be completely credible.

Also under anaerobic situations other workers [Polakis, Bartley and Meek[20], Linnane *et al.*[25]] have reported the formation of "vacuole like" membraneous structures. It is possible that under some conditions where mitochondrial proliferation is repressed, the structural integrity of those parts of the mitochondrion not essential to the respiratory chain are in fact essential to cellular proliferation. Preliminary studies on the re-assembly of the mitochondrion (Green *et al.*[79]) indicate an auto-assembly like method of manufacture. It is therefore possible that these membraneous "vacuoles" are formed as a result of the auto-assembly

of outer membrane consitutents. The location of the various mitochondrial enzymes within the mitochondrion is still in dispute, but it seems probable that the respiratory chain enzymes are located in the inner membrane, and the other mitochondrial enzymes are largely located in the outer membrane. In Fig. 6(i), N_2 represents conditions which limit the rate of synthesis of mitochondrial components derived from either synthetic system. The protein → DNA (Type II control) and substrate/product → DNA (Type I control) feedbacks are omitted for the sake of clarity. This diagram also emphasizes the indirect (amino acids, lipids etc.) but definite, dependency of mitochondrial proliferation upon cellular metabolism.

Altmanns original theory characterized the mitochondrion as a degenerate bacterium. The role of the mitochondrion and its method of proliferation described above fit well with this concept. Perhaps the mitochondrion is not a true degenerate bacterium, but as suggested by Sagan[86], a symbiotic relationship evolved between two pre-protist organisms at a time when the earthly environment was changing from one of a reducing atmosphere to that of an oxidizing atmosphere. The system envisaged for the facultative yeast is what one would expect to find in the half way stage between parisitology and obligate symbiosis. Perhaps the mitochondria of higher organisms, which appear to contain less DNA than do yeast mitochondria[32], represent a further degeneration to the stage of obligate symbiosis.

The data described in this thesis is consistent with the

hypothesis that the proliferation of mitochondrial DNA, and the translation of the information encoded in this DNA, is independent of direct nuclear control. Also, the data indicate that the nucleargenome does not respond to any direct feedback controls from the mitochondrial system. The general body of literature relevant to these studies support this conclusion, and in general supports the schematic diagram for mitochondriogenesis illustrated in Fig. 6(i).

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