"Oxidative Phosphorylation in Micro-organisms"

A Thesis

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by

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PREFACE

The work described in this thesis was undertaken as part of a general programme of an investigation into the mechanism of oxidative phosphorylation. It was carried out in the Department of Biochemistry, University of Adelaide, and was supported in part by Research Grants from the National Health and Medical Research Council of Australia.

My grateful thanks are due to Professor Sir Mark L. Mitchell for permission to work in his Department; to Dr. P.M. Nossal, Reader in Biochemistry, for much helpful discussion and advice; and to Dr. M.F. Utter (Associate Professor, Department of Biochemistry, Western Reserve University, Cleveland, Ohio) for his keen interest and many helpful suggestions during the progress of this work.

The experiments described here were carried out solely by the author and have not been submitted for any other degree.

PREFACE

CONTENTS

ABBREVIATIONS

AMP, ADP and ATP; adenosine-5'-mono-, di-, and tri-phosphates, respectively.

DPN and DPNH; oxidised and reduced forms, respectively, of diphosphopyridine nucleotide.

CDP; cytosine-diphosphate.

GDP; guanosine-diphosphate.

ITP; inosine-triphosphate.

UDP; uridine-diphosphate.

2.4-DNP; 2.4-dinitro-phenol.

2.4.6-TNP; 2.4.6-trinitro-phenol.

p-cmba; p-chloro-mercuri-benzoate.

CEE; cysteine ethyl ester.

CoA; coenzyme A.

EDTA; ethylene diamine tetra-acetic acid.

Chapter 1

INTRODUCTION

The first realisation that phosphorylation mechanisms were involved in the production of biologically useful energy from oxidations, involving oxygen as the acceptor, came from the observations of Kalckar (1937, 1939). Earlier, other investigators had obtained unmistakable evidence of oxidative phosphorylation without fully realising its significance. In homogenates or "extracts" of liver and kidney showing high rates of oxygen uptake, Kalckar observed a simultaneous disappearance of inorganic phosphate from the medium. The phosphate was subsequently recovered in the form of organic phosphates such as hexose-phosphates, glycero-phosphate and phosphopyruvate. This so-called "aerobic" phosphorylation was related to respiration; it did not occur anaerobically or in cyanide-poisoned systems; it could not be ascribed to glycolysis since it occurred in the presence of fluoride.

Since the role of ATP in energy production during glycolysis was just becoming clear at that time, Kalckar suggested the possibility that phosphorylation served a similar energy-producing role in oxidative metabolism.

Valuable as were these studies of Kalckar in pointing to phosphorylation as a mechanism of energy storage during biological oxidation, it was the work of Belitzer (1939) and Belitzer and Tsibakowa (1939) which furnished the first quantitative assessment of the process

and defined clearly certain important questions. Using minced muscle suspensions and tricarboxylic acid cycle intermediaries, Belitzer and Tsibakowa measured the relation between the amount of phosphate esterified and the amount of oxygen taken up. It was found that for each atom of oxygen taken up, nearly two molecules of orthophosphate disappeared from the medium and could be recovered as newly formed phosphocreatine.

Unknowingly, these workers were using a crude phosphate "trap" since their preparations contained ADP, creatine and creatine-kinase. Thus ATP formed was removed enzymically and ADP regenerated. More recent work with mitochondria has necessitated addition of an artificial trapping mechanism; the system generally used is yeast hexokinase and glucose. The reactions involved are:

$$ADP + Pi + \text{energy} \longrightarrow ATP$$

$$ATP + \text{creatine} \xrightarrow{\text{creatine-kinase}} \text{creatine-phosphate} + ADP$$

or

$$ATP + \text{glucose} \xrightarrow{\text{hexokinase}} ADP + \text{glucose-6-phosphate}.$$

The "P/O ratio" of 2.0 was considered by Belitzer to be highly significant. If each dehydrogenation step in the tricarboxylic acid cycle is accompanied by a phosphorylation of the substrate itself and followed by an enzymic transfer of the group to ADP, as could be visualised by analogy with the oxidative reaction of glycolysis, then a P/O ratio of only 1.0 would be expected. However, the P/O ratio observed indicated that two phosphorylations took place for each pair of hydrogen atoms (electrons) transferred to oxygen. Clearly, if a "substrate-linked" phosphorylation took place, another phosphorylation must occur during electron transport. Belitzer pointed out that the actual P/O ratios might be higher than 2.0, since the muscle suspensions

he studied were capable of hydrolysing ATP and other organic phosphate compounds such as creatine-phosphate.

From a consideration of the thermodynamic relationships between the oxidation-reduction potentials of various substrate systems and the O_2 - H_2O system, Belitzer pointed out that enough energy could be theoretically liberated in the process of electron transport to cause as many as four to six phosphorylations, depending on the nature of the substrate oxidised. He therefore postulated that oxidative phosphorylations occurred along the respiratory carrier chain, and not primarily at the substrate level.

This hypothesis envisaged that the oxidation of the reduced forms of certain carriers, - (such as DPN, flavoprotein, or the cytochromes by the next oxidised carrier at a higher potential) - represented energy-yielding reactions coupled with phosphorylation. Certain of these respiratory carrier systems therefore could be looked upon as loci of energy "transformers". Experimental proof of this hypothesis has only recently been provided by Lehninger and Smith (1949) and Lehninger (1952) using isolated rat liver mitochondria.

Earlier work had revealed that oxidative phosphorylation, - the process by which ADP and inorganic phosphate are converted to ATP by the energy, liberated as electrons pass from substrate to molecular oxygen via the respiratory carriers, - was associated with the particulate elements of tissue homogenates and extracts. Often, preparations designated as "washed particles", "washed residues", or "cyclophorase" were employed. With the finding that the fatty acid oxidase system

and the complex of tricarboxylic acid cycle enzymes were associated with mitochondria (Green, Loomis and Auerbach, 1948), it was thought likely that phosphorylation might accompany such oxidations. This was found to be the case in qualitative experiments performed with P^{32} as a tracer by Kennedy and Lehninger (1948, 1949). Since then, mitochondria isolated from sucrose homogenates of liver (Kielley and Kielley, 1951; Copenhaver and Lardy, 1952), kidney (Copenhaver et al., 1952), brain (Brody and Bain, 1952), and tumours (Williams-Ashman and Kennedy, 1952; Lindberg, Ljunggren, Ernster and Revesz, 1953) have been found to possess high oxidative phosphorylation activity. Similarly, the so-called sarcosomes or mitochondria of heart and skeletal muscle have been studied (Slater and Holton, 1954; Chappell and Perry, 1953). "Mitochondria" isolated from plant cells also exhibit the phenomenon (Bonner and Millerd, 1953).

For the demonstration of oxidative phosphorylation in suspensions of isolated mitochondria, it is necessary to add only the oxidisable substrate (e.g. Krebs' cycle intermediates, glutamate or α-hydroxybutyrate), Mg^{++}, orthophosphate, ADP and sufficient KCl or sucrose (or certain other solutes) to maintain a minimum level of osmolarity. Freshly prepared mitochondria already contain a full complement of enzymes and co-enzymes for oxidation and for the coupled phosphorylations. The phosphorylation of ADP to ATP, occurring during oxidation, is most effectively studied if purified yeast hexokinase and glucose are added. This high energy "trap" ensures a constant supply of a phosphate acceptor, hence only catalytic amounts of ADP are necessary. Large amounts of hexose-phosphates may accumulate with little or no dephosphorylation

"leak", since hexose-phosphates are not readily attacked by the phosphatases of mitochondria.

An important characteristic of the oxidative phosphorylation process is the fact that phosphorylation may be "uncoupled" from oxidation by certain compounds which permit oxidation to proceed but which inhibit phosphorylation. A large number of uncoupling agents have been described and, of these, 2.4-DNP has received most attention.

The numerous studies of oxidative phosphorylation by liver mitochondria and heart muscle sarcosomes have all indicated that phosphorylation is dependent on the integrity of these cytoplasmic particles. This places limitations on the study of the mechanisms of phosphorylation. The fact that any extensive structural alteration of the mitochondria results in a loss of the coupled phosphorylation has up to date prevented solubilisation and purification of the constituent enzymes. Purified enzyme studies have been the main technique whereby the actual mechanisms of complex metabolic sequences have been elucidated. Therefore, in search for a simpler, less sensitive system, several investigators have turned their attention to the use of bacterial preparations. Phosphorylation coupled to the oxidation of substrates has been reported for crude extracts, of a few bacterial species (Hyndman, Burris and Wilson, 1953; Brodie and Gray, 1955).

Pinchot (1953) has prepared from Alcaligines faecalis, a soluble preparation capable of coupling the oxidation of DPNH to the phosphorylation of ADP. Phosphorylation coupled with the oxidation of molecular hydrogen, succinate, DPNH and TPNH by Azotobacter vinelandii

has been described separately by Tissieres and Slater (1955), Hyndmann et al. (1953), and Rose and Ochoa (1956).

In this laboratory, Nossal (1953) developed a very rapid reciprocal shaker in which micro-organisms, including yeast, can be disintegrated rapidly and at low temperature. Preliminary studies (Nossal, 1954) had shown that a particulate fraction obtained by differential centrifugation resembled animal mitochondria in several biochemical characteristics. Lindegren (1949), Mudd, Brodie, Winterscheid, Hartman, Beutner and McLean (1951), Sarachek and Townsend (1953) and Mundkur (1953) using purely histological techniques, have also referred to certain particles in the yeast cell as mitochondria.

In view of these facts, an attempt has been made in this work to correlate the previous histological and biochemical evidence by demonstrating the existence of a mechanism of oxidative phosphorylation in the yeast particles, since this biochemical phenomenon is almost invariably associated with mitochondria. Although the evidence presented here is mainly concerned with oxidative phosphorylation, it does add further weight to the idea that micro-organisms, particularly yeast, have a certain similarity to animal and plant cells in the intracellular distribution of enzymes.

The project reported here was concerned with the demonstration of the existence of an oxidative phosphorylation mechanism in yeast particles. First, the conditions necessary for the uptake of inorganic phosphate, coincident with substrate oxidation, were established. Then, the properties of the particles with regard to the phosphorylating mechanism were investigated in detail.

Chapter 2

MATERIALS AND METHODS

Materials.

Yeast. The yeast used for this work was a commercial strain of bakers' yeast (Saccharomyces cerevisiae), air-freighted twice weekly from the Effront Yeast Co., South Yarra, Victoria. The yeast samples were stored in a humidor at $0^{\circ}C$.

Hexokinase. Prepared from bakers' yeast by the procedure of Berger, Slein, Colowick and Cori (1946). For most purposes, step 3 provided a sufficiently purified enzyme, but, for balance experiments, the hexokinase was purified further to step 5 to remove contaminating enzymes. The steps involved are the preparation of a yeast autolysate; precipitation with ethanol; isoelectric precipitation to remove inert protein; fractionation with ethanol and adsorption onto $Al(OH)_3$ gel followed by elution. Finally, hexokinase is isolated by a further fractionation with ethanol.

Glycolytic enzymes. These enzymes were prepared according to the method of Slater (1953) and used for the estimation of hexose mono- and diphosphates. The method involved the use of two rabbit muscle fractions, - one, the 30 - 50 per cent $(NH_4)_2SO_4$ saturation (fraction A) from a 0.003N KOH muscle extract, and the second, the 50 - 70 per cent saturation (fraction B) of the same extract. The former, after refractionation, contained chiefly phosphohexokinase, hexose phosphate isomerase and mutase. Fraction B contained aldolase, glycerophosphate dehydrogenase and lactic dehydrogenase.

Coenzymes. DPN was prepared from bakers' yeast by an unpublished method of Kornberg and Pricer. The essential steps were precipitation of the silver salt from a boiled aqueous yeast extract, decomposition of the salt by H_2S, and precipitation of free DPN by the addition of acetone in the cold. The resultant DPN was 45 per cent pure and contained no TPN. Sigma "65" was also used.

DPNH was prepared by hydrosulphite reduction of DPN and subsequent precipitation with ice-cold ethanol (Gutcho and Stewart, 1948).

The nucleotides, AMP, ADP, ATP, UDP, GDP, CDP, ITP were all commercial preparations. The barium salts were converted to the sodium salt by passage of the solutions through the ion-exchange resin, ZeoKarb 225 (Na form).

CoA was prepared from pig liver (Buyske, Handschumacher, Higgins, King and Strong, 1951). The purity of the final product was 1 per cent as assayed by acetylation of amino-azo-benzene (Handschumacher, Mueller and Strong, 1951).

Celite. The material used throughout this work was Johns Manville Celite 535 or Hyflo Supercel, treated according to Zbinowsky (unpublished). The treatment consisted of heating to 80°C with 8N HCl for 10 minutes and then allowing to stand overnight. Next day the Celite was washed free of chlorides with water, and the water removed by washing with 95 per cent ethanol. After 20 - 30 minutes equilibration of the celite with a solution containing 1 per cent 10N H_2SO_4 in absolute ethanol, all traces of ethanol were removed with ethyl ether and the celite dried at 100°C.

Silica gel. Silica gel for partition column chromatography was prepared as described by Isherwood (1946). Sodium silicate was acidified with HCl and the silica gel formed allowed to age for three weeks. After this period, the gel was washed further with conc. HCl and then with water to remove all traces of chloride, equilibrated overnight with H_2SO_4 in ethanol, washed with ether and dried.

Solvents for extraction and chromatography. The ethyl ether used was of anaesthetic grade. Technical n-butanol was redistilled. Technical grades of chloroform, n-amyl alcohol, iso-butanol and tert-amyl alcohol were used without further purification.

Ion-exchange resins. The polystyrene sulphonic acid, ZeoKarb 225, was treated as described by Partridge and Brimley (1952) for ZeoKarb 215, i.e. treatment with 5N HCl overnight, followed by recycling through the Na and H forms using 5 per cent Na_2CO_3 and 2N HCl. Ultimately the resin was converted to the Na form and washed with water. This resin was used mainly to remove heavy metals from various chemicals and replace them with sodium or hydrogen ions.

Dowex 1, 4 per cent cross-linked, 200 - 400 mesh was converted to the chloride form by treating the resin with 5 vols. 2N HCl for 15 minutes and centrifuging at 1,000 x g. The washing with 2N HCl was continued up to fifteen times. The HCl treatment was considered complete when an aliquot of the supernatant gave a value of less than 0.020 when read in a spectrophotometer at 250 mμ. The resin was then washed with distilled water to remove the excess

chloride. The washing was continued until an aliquot of the washings no longer gave a precipitate with silver nitrate. This resin was used in the separation of the adenine nucleotides from various reaction mixtures.

Methods.

Preparation of granules. A mixture consisting of 2 g. wet weight yeast, 10 g. Ballotini glass beads No. 12, 9 ml. 1 per cent NaCl and 1 ml. 0.1M EDTA (pH 6.8) was shaken at 6,000 cycles per minute for 10 seconds at 2°C in the high-speed mechanical disintegrator described by Nossal (1953). Such treatment resulted in the disruption of 20 - 40 per cent of the cells.

The resulting mixture was centrifuged for 10 minutes at 1,000 x g to remove glass and cell debris. The first supernatant, i.e. the cell-free whole extract, was centrifuged in a refrigerated angle head for 20 minutes at 20,000 x g and the second supernatant discarded. The residue (referred to hereafter as either yeast granules or yeast particles) was washed (to the original volume) with a 1 per cent NaCl suspending solution containing 0.015M sodium phosphate (pH 6.8) and 0.001M EDTA (pH 6.8). After centrifuging at 20,000 x g for 20 minutes, the granules were finally suspended to one-third the original whole extract volume in the above suspending solution.

All operations were carried out at 2°C either in a cold-room or in refrigerated centrifuge.

Routine study of the mitochondrial activity required the daily preparation of fresh material from yeast described in the previous section. It was realized very early in the project that the Q_{O_2} and QPO_4 of preparations obtained varied from day to day. As a consequence, the general practice adopted was to refer all experimental results to a daily standard.

Manometric methods. Most of the experiments were conducted in Warburg vessels of approximately 15 ml. volume with the following assay mixture: 50 micromoles sodium succinate, 50 micromoles glycylglycine (pH 6.8), 2 micromoles ATP, 25 micromoles glucose, 20 micromoles sodium fluoride, 10 micromoles magnesium chloride, 0.5 units of hexokinase and granules equivalent to 5 - 8 mg. of protein, in a total volume of 2.25 ml. The suspension of granules contained 15 micromoles inorganic phosphate and 1 micromole EDTA. All experiments were conducted at 22°C.

All the components of the assay mixture were added to the main chamber of the flask and pre-cooled in ice before addition of the hexokinase and granules. The centre walls of the experimental vessels contained 20 per cent NaOH and filter paper. After the addition of the granules, the vessels were attached to the manometers as quickly as possible and equilibrated for 10 minutes at 22°C. At the end of this period, the reaction was terminated in the control vessels, by the addition of 0.1 ml. 10N H_2SO_4, to provide zero-time control values for inorganic phosphate and other determinations. Manometric measurements were then initiated with the experimental vessels and terminated as above after the desired period.

Trichloroacetic acid extract. 1 ml. aliquots of the Warburg vessel contents were added to 3 ml. 10 per cent trichloroacetic acid and the mixture diluted to 10 ml. with glass-distilled water. The protein precipitate was removed either by centrifugation or by filtration through Whatman No. 42 filter paper.

Protein estimation. The protein precipitate described in the previous paragraph was collected by centrifugation and assayed colorimetrically by a modification of the biuret reaction (Robinson and Hogden, 1940).

Inorganic phosphate estimation. The method of Fiske and Subbarrow (1925) was used for inorganic phosphate estimation of 1 ml. aliquots of the trichloroacetic acid extract, described previously.

Hexose phosphate assay. Hexose esters were estimated spectrophotometrically using the glycolytic enzymes described under MATERIALS. Fraction A contained the necessary enzymes for converting hexose monophosphates to fructose-1-6 diphosphate in the presence of ATP. Fraction B contained the enzymes for converting triose phosphates or hexose diphosphates to α-glycerophosphate at the expense of added DPNH.

Diphosphate esters were assayed by using Fraction B and following the change in optical density at 340 mμ. Estimation of monophosphate esters required the use of both fractions and the

addition of ATP to the reaction mixture.

A change in optical density of 0.414 at 340 mμ is equivalent to 0.2 micromoles DPNH oxidised, or 0.1 micromoles hexose mono- or diphosphate converted to α-glycerophosphate.

Preparation of organic acids for chromatography. Approximately 7 g. prepared Celite was placed in a mortar. 2 - 5 ml. of reaction mixture from the Warburg vessels were added after being made 2N with respect to H_2SO_4, and intimately mixed with the Celite. The mixture was transferred quantitatively to a Soxhlet thimble and extracted for 3 hours with ethyl ether.

The resulting extract was evaporated to dryness below 40°C in a stream of air in a 6" x 1" test-tube. Immediately prior to chromatography, the residue was dissolved in a known volume of 10 per cent n-butanol in chloroform, and an aliquot of 1.0 ml. or less applied to the top of the silica gel column.

Silica gel chromatography. 1.1 g. Silica gel was added to 0.8 ml. 0.05N H_2SO_4, mixed thoroughly and sieved through 85 mesh copper wire.

Approximately 10 ml. chloroform was added and the slurry poured into a 30 cm. x 0.6 cm. chromatographic tube having a filter paper disc at its constricted end. The column was allowed to settle under gravity. The sample was added to the column and allowed to pass into the gel. After rinsing with about 1 ml. chloroform to ensure complete absorption of the sample, a further 2 ml. chloroform was added

to the top of the column and the tube attached to the solvent reservoir. The latter was constructed to the design of Donaldson, Tulane and Marshall (1952) to deliver a continuously changing solvent. The reservoir contained 50 ml. of 30 per cent (v/v) n-amyl alcohol in chloroform and 50 ml. of 30 per cent (v/v) tert-amyl alcohol in chloroform and led into a mixing chamber containing 50 ml. chloroform. The rate of flow was adjusted to 0.3 ml. per minute by applying 2-4 lb./sq.in. pressure from a nitrogen cylinder.

This procedure has previously been described (Ladd and Nossal, 1952) and further modified (Nossal, Hansen and Ladd, 1957).

Ca determination. Calcium was assayed using the method of Debruey (1952) modified by Slater and Cleland (1953). The procedure involves ashing the material, dissolving the residue in HCl and neutralising with KOH. The solution is brought to pH 12 with KOH and a drop of saturated ammonium purpurate is added. The solution is immediately titrated with 10^{-3}M EDTA until the colour matches that of a control prepared simultaneously with glass-distilled water.

Separation and assay of adenine nucleotides. Aliquots containing from 1.5 to 2.0 micromoles adenine nucleotides were added to Dowex -1 (4 per cent cross-linked, Cl^- form, 200-400 mesh) column 40 mm. long and 0.7 mm. in diameter. The solutions were at the phenolphthalein end-point prior to addition.

Elution of the nucleotides:-

AMP - 50 ml. 0.01N HCl was added to the column and 10 ml. portions thereafter until the optical density reading was 0.02 or lower (per ml.). All fractions were pooled and assayed as described below.

ADP - 50 ml. 0.01N HCl containing 0.05M NaCl was added followed by 10 ml. portions, as above.

ATP - 50 ml. 0.01N HCl containing 0.2M NaCl followed by 10 ml. portions, as above.

Assay of the eluate:-

The eluate from the column was collected in 10 ml. fractions. 1.0 ml. aliquots from each sample were introduced into cuvettes and 0.1 ml. 3N HCl and 1.9 ml. water were added. From the reading observed in the spectrophotometer at 260 mμ, the optical density units per ml. were determined. The optical density units were converted to micromoles by dividing by 14.7, i.e., the coefficient for adenine compounds in 0.1N acids.

Granule volume changes. These were studied spectrophotometrically at room temperature (20°C) by following the optical density of suspensions at 520 mμ, as described by Cleland (1952). Sufficient yeast granule suspension was added to cuvettes to give an initial optical density of approximately 0.300.

Phosphatase activity. Phosphatase activity was determined by the liberation of inorganic phosphate from the substrate in 10 minutes at 22°C.

Chapter 3

DEMONSTRATION OF OXIDATIVE PHOSPHORYLATION

Introduction.

The presence in yeast cells of sub-cellular particles having staining properties similar to those of animal mitochondria has been reported (Mudd, Brodie, Wintersoheid, Hartmann, Bautner and McLean, 1951; Hartmann and Liu, 1954). Further evidence that yeast cells contain organised respiratory particles has been provided by studies which have shown that particulate fractions obtained by the disruption of cells contain many of the enzymes associated with terminal oxidation (Slonimsky and Hirsch, 1952; Nossal, 1954 (a); Linnane and Still, 1955; Nossal, Keech and Morton, 1956).

In carefully prepared animal mitochondria, respiration is accompanied by esterification of inorganic phosphate. Similar findings have been reported for plant tissues (Bonner and Millerd, 1952). The knowledge of respiratory and especially phosphorylative processes in microorganisms has lagged behind. One of the reasons for this could be the greater resistance of microorganisms to mechanical disintegration.

Using a rapid high-speed mechanical disintegrator (Nossal, 1953) which disintegrates microorganisms far more rapidly than older type machines, Nossal (1953, 1954, 1956) has isolated from cell-free yeast extracts, a particulate fraction which is capable of oxidising a variety of substrates. Attempts were therefore made to study further the respiration and phosphorylation of yeast cytoplasmic

particles and to determine whether these are the equivalent of animal mitochondria.

Results.

The granules obtained by differential centrifugation from yeast cells disrupted as described in Chapter 2, couple phosphorylation with the oxidation of succinate (Table 1). Phosphorylation was either greatly depressed or abolished by the omission of ATP, hexokinase, or magnesium chloride, although oxidation was unaffected or even increased. Therefore, yeast particles can oxidise substrates under conditions when phosphorylation does not occur, i.e., their respiration is not obligatorily coupled to phosphorylation as in the case of well-prepared animal mitochondria.

The washed granules exhibit no measurable oxidation or phosphorylation in the absence of added substrate. The presence or absence of sodium fluoride had little effect on the reaction, but sodium fluoride was routinely included since early experiments had indicated the presence of an ATPase.

Over a very large number of experiments, the P/O ratios observed for the oxidation of succinate have varied from 0.4 to 1.1 with the majority of experiments falling between 0.6 and 0.9. In occasional experiments there was failure to observe phosphorylation or even oxidation. The negative experiments were traced mainly to improper disintegration or pH control procedures. The variables

Table 1. Oxidative Phosphorylation with Yeast Granules.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 2 micromoles ATP, 20 micromoles sodium fluoride, 25 micromoles glucose, 10 micromoles magnesium chloride yeast hexokinase, 1 ml. granules and water to a final volume of 2.25 ml.

Temperature 22°C. Time 30 minutes.

Components	Phosphate Uptake /umoles	Oxygen Uptake /uatoms	P/O
Complete	3.93	6.35	0.62
- ATP	1.01	6.8	0.15
- $MgCl_2$	1.2	8.2	0.15
- Hexokinase	-0.88	7.3	—
- NaF	4.1	6.8	0.61
- Succinate	0.8	0.6	0.8

controlling the yield of granules and particularly the P/O ratio are only partially understood but include disintegration time, pH during centrifugation and assay, maintenance of an optimum level of ADP during assay, and the age of the yeast. These factors are considered in later experiments.

Variation in preparation of Granules.

The effect of variations in pH upon oxidation and phosphorylation is shown in Table 2. Oxygen uptake increased markedly as the pH was increased from 6.3 to 7.5 while phosphorylation showed a rather broad maximum between 6.6 and 7.5. On the basis of P/O values, the pH range of 6.6 to 6.9 seemed to be the most satisfactory and has been used in these studies.

The preparation of a particulate system with a high degree of organisation by rapid and drastic disintegration is easily affected by a number of variables. For example, the duration of the disintegration period has a marked effect on the activities of the granules subsequently obtained (Table 3); the second column of which shows the yield of protein present in the whole cell-free extract. It will be noted that with increasing disintegration periods, there is an almost linear relationship between the disintegration period and the protein content of the whole extract which falls off rather rapidly. In the first fifteen seconds disintegration, the granular protein (third column) in the extract remains constant at approximately one-fifth of the total protein. Longer shaking periods considerably

Table 2. Effect of pH on oxidative phosphorylation.

Experimental conditions were the same as in Table 1., except that a series of glycyl-glycine buffers were used at the pH indicated.

Expt. No.	pH	Phosphate Uptake /μmoles	Oxygen Uptake /μatoms	P/O
1	6.3	1.8	2.9	0.62
	6.6	3.0	4.35	0.69
	6.9	3.0	5.0	0.60
2	6.6	2.8	3.6	0.78
	7.2	3.5	6.65	0.52
	7.5	3.1	7.45	0.41

Table 3. Effect of Disintegration Period.

Experimental conditions were the same as described in Table 1.

Disintegration time seconds	Yield of protein		Phosphate Uptake	Oxygen Uptake	P/O
	Total mg.	Granules mg.	µmoles	µatoms	
5	6.0	1.2	2.9	3.2	0.91
10	10.4	2.2	2.0	3.5	0.57
15	13.6	2.8	0.8	4.0	0.20
20	18.2	2.8	0.3	5.1	0.06
30	20.0	2.6	0.2	6.3	0.03
40	21.6	2.6	0	6.3	----

"Total protein" refers to the protein contained in 1 ml. whole cell-free extract while "granule protein" refers to the amount of sedimentable protein contained in 1 ml. whole cell-free extract.

reduced this ratio. The interpretation placed upon these results is that the percentage of cells disintegrated up to 20 seconds remains constant but after this period the quantity of cells being disintegrated decreases. Furthermore, it appears that after 15 seconds disintegration, appreciable amounts of granules become disrupted or fragmented and these were no longer sedimentable at 20,000 x g.

The fifth column shows the oxidative activity of approximately equal amounts of granules obtained after different shaking periods. There is some variation in oxidative activity, but the most notable effect is a constant decrease in the P/O ratio as the period of shaking is increased. The loss of phosphorylative activity (column four) may be due to further disruption of the granules since Nossal (1954, a) has found that with longer shaking periods, several enzymes including fumarase, aconitase and alcohol dehydrogenase, tend to move from the particulate to the supernatant fractions.

The composition of the medium in which the yeast cells are disrupted and in which the resultant granules are washed and suspended has considerable influence upon the properties of the granules. Granules prepared by disintegration in slightly alkaline phosphate medium (Nossal et al., 1956) are able to oxidise a number of substrates in addition to succinate, but in view of the known effects of phosphate on mitochondrial integrity (Hunter and Ford, 1955), this procedure seemed unsuitable for the present study. Preliminary studies using 0.9% KCl yielded granules with considerable endogenous activity and

with lower P/O values than similar preparations obtained with NaCl. EDTA aids greatly in the stabilisation of the granules, as has been reported for animal mitochondria (Slater and Cleland, 1952). In these experiments, 1% NaCl containing 0.01M EDTA has been employed routinely as the medium for disintegration. The effect of tonicity of the NaCl used in disintegration and washing media is shown in Table 4. It will be noted that there is a sharp peak in the phosphorylative activity with 0.75% NaCl and a slight depression in the rate of oxidation with 1% NaCl.

Sucrose was also investigated as a possible medium in which to conduct the disintegration, washing and assay procedures, since sucrose solutions are used almost exclusively in oxidative phosphorylation studies with animal mitochondria. A series of solutions were used varying from 0.125M to 0.5M. The results (Table 5) show that in increasing sucrose concentrations optimum activity was reached at 0.375M.

A comparison was also made between 1% NaCl and 0.375M sucrose to determine the medium which would provide the best P/O values. From the results shown in Table 6, it can be seen that the granules prepared in sucrose had slightly better activity with regard to both respiration and phosphorylation, although there was virtually no difference between the two media on a P/O basis.

Most of the experiments reported in this work were conducted with yeast granules prepared as described in Chapter 2. unless

Table 4. Effect of tonicity on oxidative phosphorylation.

Experimental conditions were the same as described in Table 1, except that the yeast cells were disintegrated and washed in media with varying NaCl concentrations as shown in table.

NaCl concentration of disintegrating and washing media %	Phosphate Uptake µmoles	Oxygen Uptake µatoms	P/O
0.25	1.1	7.1	0.11
0.50	2.0	6.8	0.25
0.75	2.5	6.8	0.40
1.00	1.8	5.5	0.34
1.25	0.7	7.0	0.09

Table 5. Varying sucrose concentrations.

Experimental conditions were the same as in Table 1. The granules were prepared and assayed in the sucrose solutions as shown.

Experiment No.	Sucrose Concentration M	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O
1	0.125	1.1	5.8	0.19
	0.187	1.4	6.1	0.23
	0.25	2.3	6.7	0.35
2	0.25	3.0	6.05	0.50
	0.375	3.4	7.1	0.48
	0.5	2.8	7.1	0.40

Table 6. Comparison between 1% NaCl and 0.375M sucrose.

Experimental conditions were the same as in Table 1. The disintegration, washing and suspending media were as shown. Each solution also contained EDTA and inorganic phosphate as described in Chapter 2.

Experiment No.	Medium	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O
1	NaCl	2.5	5.1	0.49
	Sucrose	3.2	7.1	0.51
2	NaCl	4.9	6.5	0.75
	Sucrose	5.9	8.2	0.72

specifically stated otherwise. That is, the granules were disintegrated in a 1% NaCl solution containing 0.01M EDTA, washed and finally suspended in a 1% NaCl solution containing 0.001M EDTA and 0.015M phosphate (pH 6.7). The washing procedure was necessary to remove contaminating soluble proteins. Table 7 shows the effect of repeated washings and it will be noted that further washing did not affect the amount of phosphorylation taking place. However, a decrease in the rate of oxidation was observed resulting in an improved P/O ratio.

Although this experiment showed that increased ratios could be obtained by further washing of the particles, it was not practical to do this routinely and all future experiments were carried out using granules prepared by the standard procedure.

Stability of the yeast granules.

Disruption of mitochondrial structure by freezing and thawing has been found to result in a loss of the phosphorylating activity, although the enzymes involved in electron transport may survive such treatment. However, it has been found that yeast granules can be stored for several days at -15°C. Table 8 shows that there was no change in phosphorylative activity in the first four days of storage and only a 20 per cent reduction in activity after eleven days storage. The greatest change observed was the 33 per cent loss in the rate of succinate oxidation in the first four days. After this initial loss, there was no alteration for the remainder of the storage period. The yeast granules had lost 75 per cent of their phosphorylating activity after 25 days.

Table 7. Effect of washing the yeast granules.

The experimental conditions were the same as in Table 1. The granules were prepared as described in Chapter 2 except that certain fractions were washed a varying number of times.

No. of washings	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O
2	2.7	5.2	0.52
3	2.6	4.75	0.55
4	2.7	4.3	0.63
5	3.0	3.8	0.79

Table 8. Effect of storage at -15°C.

Experimental conditions were the same as in Table 1.

The granules were prepared, washed and suspended in 0.375M sucrose.

No. of days	Phosphate Uptake /umoles	Oxygen Uptake /umoles	P/O
1	2.5	7.6	0.33
2	2.6	7.3	0.36
3	2.7	7.1	0.38
4	2.5	5.1	0.49
11	2.0	5.1	0.39
25	0.6	5.8	0.10

Centrifugal Behaviour.

In the foregoing experiments, the particulate fractions were obtained from the centrifugal interval between 1,000 x g and a much higher value ranging from 12,000 to 23,000 x g. Probably the fractions used contained sedimentable material of greatly varying size and activity. To determine whether the oxidative and phosphorylative activities were correlated in any regular fashion with centrifugal behaviour, fractionation studies were attempted. Sedimentable material was collected by centrifuging successively at 5,000, 10,000 and 23,000 x g. Each centrifugation was carried out in a refrigerated angle head for 15 minutes. After washing, each fraction was tested on succinate along with another aliquot obtained by centrifuging directly at 23,000 x g (Table 9).

Fraction A, representing material thrown down over the entire range from 1,000 to 23,000 x g gave essentially the same results as the 5,000 x g material (Fraction B). The material sedimented between 5,000 and 10,000 x g (Fraction C) gave slightly lower oxidative activity and a slightly lower P/O value. The last fraction (D), presumably representing the smallest particles, showed practically no activity at all. The lack of activity in this fraction was due to the inability to concentrate, in this experiment, sufficient material to enable reliable measurements to be made. It has been shown (Utter, Keech and Nossal, in press) that provided a sufficiently high protein content can be obtained in this fraction, comparable P/O ratios are observed although the activity of the granules is considerably reduced. From

Table 9. Centrifugal separation of yeast particles.

The experimental conditions were the same as those described in Table 1.

Fraction	Centrifugal Interval 10^{-3} x g.	Yield of protein mg.	Phosphate Uptake µmoles	Oxygen Uptake µatoms	P/O
A	1 - 23	1.37	2.7	3.5	0.78
B	1 - 5	1.11	2.35	2.9	0.81
C	5 - 10	0.31	1.6	2.5	0.64
D	10 - 23	0.18	0	0	-

Yield of protein refers to the protein sedimented from 1 ml. of cell-free extracts. Each Warburg vessel contained approximately equal quantities of granular protein except for fraction D.

the second column (Table 9), in which the yield of granules is given, it can be seen that about 70% of the total sedimentable protein is obtained in fraction B, about 20% in fraction C, and considerably less in fraction D.

The experiment shown in Table 9 and several other attempted fractionations all indicated that the active material is distributed over a considerable range of particle size and that half or more of the sedimentable protein can be obtained at surprisingly low centrifugal forces. The "respiratory granules" as identified by microscopic observation have been reported to be from 0.3 to 1.0 micron in size (Mudd et al., 1951). Linnane and Still (1955) prepared granules from yeast by a method similar to the one used here except that the disintegration period was longer and the process was carried out in sucrose. They attributed the oxidative activity observed, to granules estimated to be about 0.3 microns in diameter, although the granules were sedimented between 3,000 and 23,000 x g and no fractionation was reported. The present results could be explained if the active material is relatively small in size but is associated to a varying degree with larger structural elements of the cell. A second possible explanation is that partial aggregation of small particles occurs under the conditions used. Whole yeast cells can be virtually eliminated from consideration since parallel experiments with yeast suspensions which have been treated in the usual way (except for disintegration) show that at the most 1 - 2 per cent of the granule protein is in the form of unbroken cells. Direct examination by phase contrast microscopy also indicates that whole

cells are very few in number.

Microscopy.

Photographs 1 and 2 substantiate the view that the active material is relatively small in size but is associated to a varying degree with larger structural elements of the cells. Photograph 1 shows a whole yeast cell which contains a medium sized vacuole. Attached to the external surface of the vacuole membrane are small, refractile granules. After disruption of the cell membrane (and presumably the vacuole membrane also), and isolation of the particulate fraction by centrifugation, the granules can be seen under phase contrast. Frequently, many granules appear to be associated and fixed on to a background material which shows as a shaded area in photograph 2 and is assumed to be a fragment of the vacuole membrane. The number of granules and the size of the aggregates varies considerably. Frequently, single isolated granules can be observed. The material used in photograph 2 was obtained by fixation with osmium tetroxide in acetate buffer. After fixation, the material was dehydrated by repeated washing with increasing concentrations of aqueous alcohol. Microscopic examination was performed with Leitz phase contrast equipment.

Examination of sections in the electron microscope gave further information on the morphology of the granules isolated under the conditions outlined in "Methods". Photograph 3 shows a section of yeast granules fixed in osmium tetroxide and magnified 20,000 x.

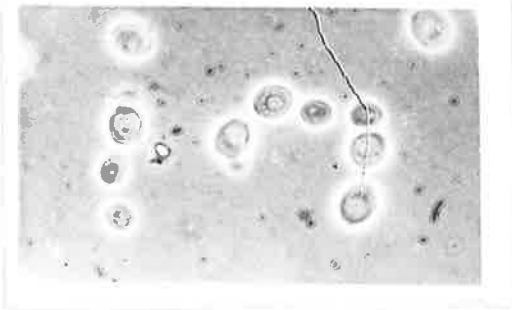


Plate 1. Whole yeast cells.

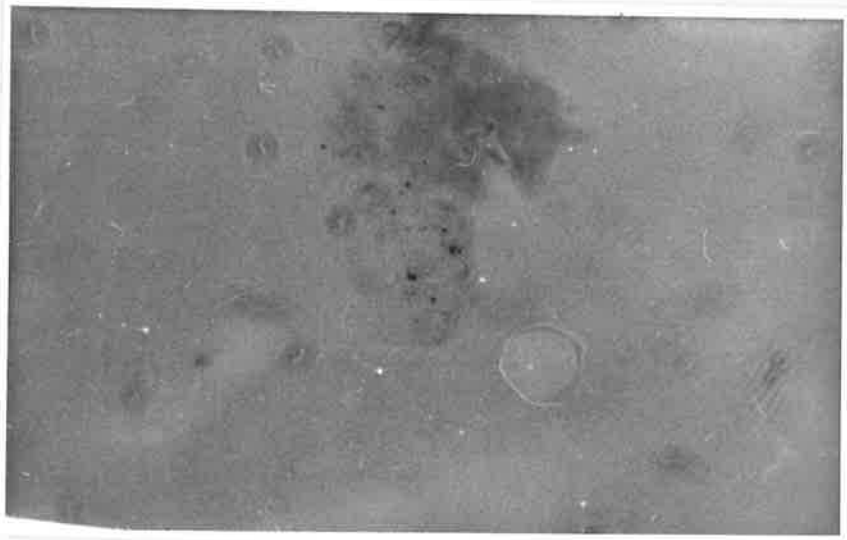
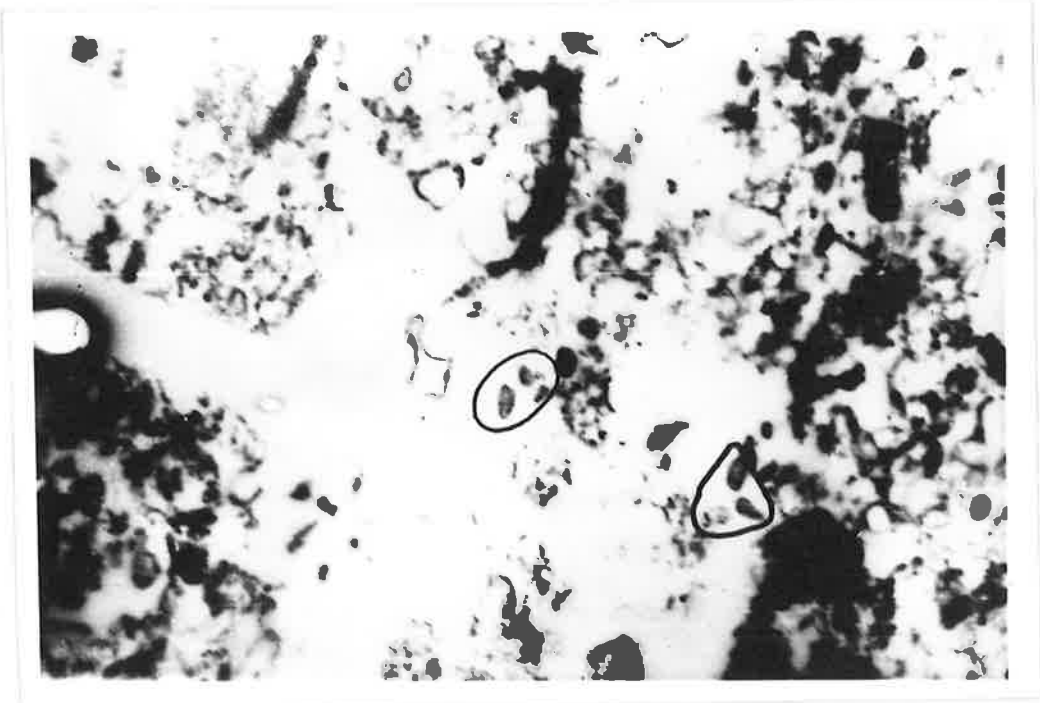


Plate 2. Photograph of osmium fixed isolated yeast particles, viewed through a Leitz phase-contrast microscope.



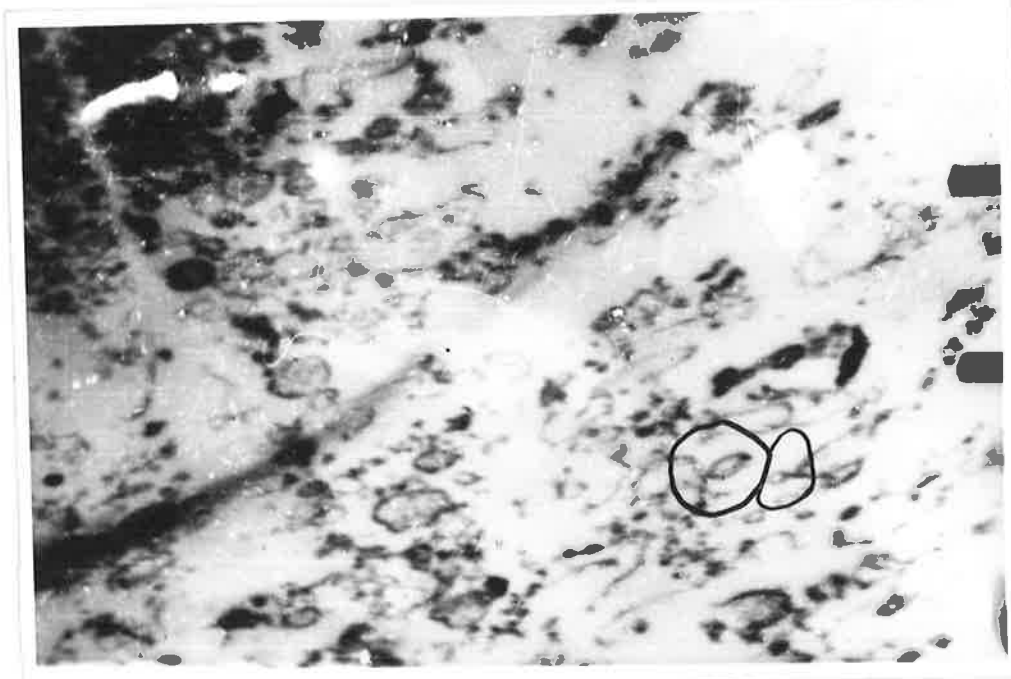


Plate 3. Photographs of osmium fixed yeast granules viewed through the electron microscope.

Examination shows the granules to be ovoid and not, as suspected under phase contrast, spherical. Their size varies, the average being approximately 0.5 microns in length and 0.3 microns across. Some particles appear to be well preserved and even show a suggestion of internal structure, whereas others appear to be swollen and damaged, probably due to the drastic procedures used in disintegrating the yeast cell. Ample evidence was also obtained to support the hypothesis that the granular material is attached to a supporting membrane.

Effect of inhibitors.

The effect of various inhibitors on both oxidative and phosphorylative mechanisms in yeast granules is shown in Table 10. With succinate as substrate the addition of sodium azide to the reaction mixture drastically reduces the phosphorylative activity and at the same time, the rate of oxidation is partially inhibited. At 1.8×10^{-4}M., oxidation is inhibited by 28 per cent, whereas phosphorylation is reduced by 70 per cent. At a higher concentration (3.6×10^{-4}M.) the rate of phosphorylation is almost completely inhibited while the rate of oxidation is reduced to approximately a half the rate of the control.

With 4.4×10^{-4}M. cyanide, oxidation and phosphorylation are both strongly inhibited. The drop in P/O could suggest that cyanide uncouples, but the results are not sufficiently definite for such a conclusion.

0.2 microgram antimycin A is sufficient to completely

Table 10. Effect of inhibitors on succinate oxidation and phosphorylation.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 20 micromoles sodium fluoride, 2 micromoles ATP, 25 micromoles glucose, 10 magnesium chloride, yeast hexokinase, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.

Inhibitor	Concentration	Phosphate Uptake /umoles	Oxygen Uptake /uatoms	P/O
Sod. azide	None	3.8	6.0	0.63
" "	1.8×10^{-4}M	1.1	4.3	0.26
" "	3.6×10^{-4}M	0.1	2.8	0.04
Pot. cyanide	None	4.6	6.8	0.67
" "	4.4×10^{-4}M	0.4	1.7	0.23
2.4-DNP	None	4.9	6.4	0.77
" "	3.6×10^{-4}M	0.9	5.6	0.16

inhibit the oxidation of succinate for one hour. At the end of this period, respiration begins but proceeds at a slower rate than the control.

2.4-DNP at 3.6×10^{-4}M concentration uncouples oxidative phosphorylation by 79 per cent. Unlike sodium azide, 2.4-DNP only reduces the oxygen uptake slightly while phosphorylation is inhibited 80 per cent.

The use of these inhibitors suggests that the mechanism of succinate oxidation and the concurrent phosphorylation in yeast granules is, in many respects, similar to that occurring in animal mitochondria. Cyanide is known to inhibit the cytochrome system by forming chelate complexes with the cytochrome iron, while antimycin A is believed to produce a metabolic block at cytochrome b. Evidence that a flavoprotein is also involved in succinate oxidation was indicated by the fact that the granules stain with Janus Green B. At a final concentration of 1 part per 100,000 in the presence of succinate, the granules stain pink while in the absence of any added substrate, the granules take up the dye, but it remains blue.

Chapter 4

UNCOUPLING

Introduction.

As stated previously, one of the important characteristics of the oxidative phosphorylation process in animal mitochondria is that phosphorylation may be "uncoupled" from the oxidative process. The term "uncoupling" has its experimental basis in the simple observation that electron transport can proceed in the respiratory chain, without the esterification of phosphate, and without the need for any inorganic phosphate. Incubation of the mitochondria at room temperature for short periods is sufficient to abolish phosphorylation without affecting oxidation. Early workers in this field also realised that both arsenate and arsenite uncoupled, - by competing with inorganic phosphate in the primary phosphorylating reaction. Various oxidation-reduction dyes, particularly methylene blue and brilliant cresyl blue also uncoupled. It is now agreed that such substances act as artificial electron carriers. They thus bypass the biological electron transport system and the normal sites of phosphorylation.

Another uncoupling agent in intact mitochondria, is calcium (Slater and Cleland, 1953; Lindberg and Ernster, 1954). Although the precise function of this metal ion is not known, Slater et al. (1953) advanced several theories whereby calcium could exert its inhibitory effect, all of which suggested that calcium acted indirectly on the phosphorylating mechanism.

The antibiotic, gramicidin, has been effectively used as an uncoupling agent in animal mitochondria but the inhibitory mechanism of this compound remains unknown.

The activity of 2.4-DNP as an uncoupling compound is well known and it is frequently used to determine whether phosphorylation is due to oxidation via the electron transport chain or to substrate level oxidation. Only electron transport phosphorylations are affected by 2.4-DNP. Lehninger (1954) considers that investigation of the action of 2.4-DNP provides one avenue of studying the primary energy recovery mechanisms associated with the action of the electron carriers.

The similarity in structure between thyroxine and 2.4-DNP has been recognised for some time and attempts to implicate thyroxine as a biological uncoupler both in vivo and in vitro have been partially successful. However, the concentrations of the hormone required for activity leave some doubt whether this is its effect in the living cell.

Various theories have been advanced to explain the uncoupling mechanism of these compounds, but all require further experimental proof.

Pinchot (1953), and later Tissieres and Slater (1955), using cell-free extracts prepared from bacterial suspensions, were able to observe an inorganic phosphate uptake which was dependent upon concurrent oxidation. These apparent oxidative phosphorylations, however, were insensitive to 2.4-DNP. Their evidence could mean

either that the mechanism of oxidative phosphorylation in microorganisms is different from that in animal mitochondria, with respect to 2.4-DNP, or that the morphology of the mitochondria is in some way concerned in the uncoupling phenomenon.

A survey was therefore conducted to determine the effects in yeast granules of some of the various compounds which had shown uncoupling activity in animal mitochondria.

Results.

Aging.

Lehninger (1949) showed that even brief treatment at temperatures above 0°C was sufficient to inactivate the enzyme systems involved in oxidative phosphorylation and he found that mitochondria were more stable in the presence of substrates (and the cofactors necessary for measuring oxidative phosphorylation). However, even under these conditions, inactivation of the phosphorylating system during the course of the experiment could be considerable. This led to the practice of using short reaction times and also prompted several investigators to carry out their experiments at low temperatures.

Since Slater and Holton (1954) were able to show that by the addition of EDTA, a constant P/O ratio could be maintained by rat-heart sarcosomes for 45 minutes at 25°C, the use of EDTA became an accepted standard practice. Preliminary experiments show that EDTA also stabilises liver mitochondria. Similarly EDTA stabilises the

phosphorylating mechanism in yeast granules.

The results in Table 11 were obtained by preparing the granules in the manner described in Chapter 2. except that half the granules were finally suspended in 1% NaCl-0.015M sodium phosphate and the other half were suspended in the normal suspending medium, i.e. 1% NaCl containing 0.015M sodium phosphate and 0.001M EDTA. Each of the two suspensions was again divided into two equal parts, one part being held in an ice bath at 0°C for 15 minutes and the other half incubated at 22°C for 15 minutes. After this preincubation, the granules were added to the complete reaction mixture and incubated again. A loss of 50% activity is shown by the granules preincubated in the absence of EDTA; and even holding at 0°C for 15 minutes without EDTA caused a loss in activity. However, no inactivation of the granules incubated in the presence of EDTA could be observed.

Further work on the protective action of EDTA is described elsewhere.

Effect of nitro-phenols.

It has been known for some time that 2.4-DNP (and other nitro-phenols) stimulates the respiration of intact cells, while at the same time the energy functions of the cell are impaired.

The nature of the effect on the energy functions became clearer with the discovery by Loomis and Lipmann (1949), and Cross, Taggart, Covo and Green (1949), that dinitro-phenol "uncoupled" oxidative phosphorylation, i.e., it inhibited the synthesis of ATP

Table 11. Effect of EDTA on aging.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 2 micromoles ATP, 20 micromoles sodium fluoride, 25 micromoles glucose, 10 micromoles magnesium chloride, yeast hexokinase, 1 ml. granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.

Pretreatment of granules			Phosphate Uptake	Oxygen Uptake	P/O
EDTA present /μ moles/ml.	Incubation period mins.	Temp. °C.	/μmoles	/μatoms	
0	15	0	2.0	3.9	0.52
0	15	22	1.0	3.9	0.26
1.0	15	0	3.2	5.1	0.63
1.0	15	22	3.5	5.5	0.64

from ADP without inhibiting respiration. However, the mechanism of the stimulation of respiration is still obscure.

Potter and Reckmagel (1953) and Lardy and Wellman (1952, 1953) have defined the conditions necessary for demonstrating the 2.4-DNP stimulation of respiration in isolated liver mitochondria. Carefully prepared liver mitochondria low in ATP-ase activity respire only slowly in the absence of a phosphate acceptor system, but the respiration can be greatly stimulated by adding 2.4-DNP. Lardy's results (1952, 1953) showed that the addition of 2.4-DNP to mitochondria already supplied with hexokinase and glucose has little effect on the respiration rate. On the other hand, Slater and Lewis (1954) showed that the respiration of muscle sarcosomes - isolated from the thoracic muscle of the blowfly - is markedly increased by 2.4-DNP, even in the presence of hexokinase and glucose.

Table 12 shows that, in yeast granules, oxidation of succinate was not stimulated by the addition of 2.4-DNP but in fact was slightly inhibited. However, the addition of the phosphate acceptor system (ATP, glucose and hexokinase), stimulated oxidation. Inhibition of oxygen uptake by 2.4-DNP was also observed in the presence of the phosphate acceptor system. The amount of 2.4-DNP employed in the experiment shown in Table 12 was sufficient to inhibit phosphorylation completely.

Since the uncoupling action of 2.4-DNP on oxidative phosphorylation was described (Loomis and Lipmann, 1949; Cross et al., 1949), this compound has become recognised as the classical uncoupling

Table 12. Effect of 2.4-DNP on the respiration of yeast granules.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 20 micromoles sodium fluoride, 10 micromoles magnesium chloride, 1 ml. yeast granules and water to a final volume of 2.25 ml.

Temperature 22°C. Time 30 minutes.

Additions: 2.4-DNP, 5.3×10^{-4}M.

Phosphate acceptor system: 2 micromoles ATP.
25 micromoles glucose.
yeast hexokinase.

Additions	Oxygen Uptake / μ moles
None	5.7
2.4-DNP	5.0
Phosphate acceptor	7.9
Phosphate acceptor, 2.4-DNP	6.6

reagent. Only electron transport phosphorylations are affected by the addition of the nitro-phenol; substrate level phosphorylations are unaffected. The uncoupling activity of this compound upon oxidative phosphorylation in yeast granules oxidising succinate is shown in Table 13.

The reversibility of 2.4-DNP uncoupling is shown in Table 14. The data shown here was obtained by preparing a batch of yeast granules and suspending one-half in a 1% NaCl solution containing 6×10^{-4}M 2.4-DNP. The granules were removed from the nitrophenol by centrifuging in the cold and then resuspended in the normal NaCl suspending medium. The results show that removal of the yeast particles from the uncoupling reagent completely restores the P/O ratio although oxidative and phosphorylative activities were lowered. The lowered activities, particularly the oxidation, can be explained by the additional washing procedure and agrees with the data given in Table 7.

However, the activity of the granules, both oxidative and phosphorylative, was somewhat reduced by this treatment.

Cross et al. (1949) believe that a high degree of structural specificity underlies the action of 2.4-DNP and only certain changes are permissible in its structural configuration for it to retain its uncoupling effect. From their limited data, they concluded that the phenolic group is indispensable for inhibitory activity. Saturated and unsaturated cyclic constituents tend to enhance inhibitory activity, whereas the introduction of a carboxyl or third nitro group yields compounds which are completely inactive at 2×10^{-4}M. Table 15 shows

Table 13. Effect of 2.4-DNP on phosphorylation.

Experimental conditions were the same as in Table 11. 2.4-DNP added as shown.

2.4-DNP added	Phosphate Uptake /nmoles	Oxygen Uptake /µatoms	P/O	Inhibition %
None	4.9	6.4	0.77	
0.4×10^{-4}M	3.4	5.6	0.61	21
1.8×10^{-4}M	1.8	6.4	0.28	62
3.6×10^{-4}M	0.9	5.6	0.16	79
5.3×10^{-4}M	0	5.1	-	100

Table 14. Reversibility of 2.4-DNP uncoupling.

Experimental conditions were the same as in Table 11.

The "treated" granules had been suspended in a 1% NaCl solution containing 6×10^{-4}M 2.4-DNP prior to incubation. The granules were then removed by centrifugating and finally suspended in a normal suspending medium.

Type of granules	2.4-DNP added	Phosphate Uptake /µ moles	Oxygen Uptake /µ atoms	P/O
Treated	0	2.4	4.4	0.55
"	5.3×10^{-4}M	0.3	4.6	0.07
Normal	0	3.2	7.3	0.44
"	5.3×10^{-4}M	0	6.2	-

Table 15. Effect of nitrophenols and related compounds.

Experimental conditions were the same as in Table 11.

The compounds tested were used at a final concentration of 5.3×10^{-4} M

Expt. No.	Position of the substituent in benzene ring						Phosphate Uptake	Oxygen Uptake	P/O
	1	2	3	4	5	6	/umoles	/uatoms	
1	Control, no addition						2.6	5.8	0.45
	OH	NO_2					2.6	6.4	0.41
	OH		NO_2				0.7	4.7	0.15
	OH			NO_2			0	4.6	-
	OH	NO_2		NO_2			0	5.2	-
2	Control, no addition						3.6	5.8	0.63
	OH	NO_2		NO_2		NO_2	3.7	6.4	0.58
3	Control, no addition						2.9	6.2	0.47
	OH	NO_2		NO_2			0	5.0	-
	NH_2	NO_2		NO_2			2.6	6.1	0.43

the effect of various nitro compounds on the phosphorylation of yeast granules oxidising succinate.

Using rat liver or kidney cyclophorase preparations, Cross et al. (1949) did not observe any uncoupling effects with mono-nitrophenols, or with 2.4.6-TNP. The data in Table 15 shows that yeast granules vary somewhat from the cyclophorase preparation in that they exhibited considerable uncoupling with both m- and p-nitrophenols. The lack of uncoupling by 2.4.6-TNP, o-nitrophenol and 2.4-dinitroaniline observed in the yeast granules was similar to that described by Cross et al.

The effect of 2.4-DNP on phosphatase activity is discussed in a later section.

Thyroxine uncoupling

Thyroxine has been shown to uncouple oxidation from the accompanying phosphorylations in the respiratory chain of liver mitochondria in vitro. In order to achieve this effect, rather special conditions proved to be necessary. Martius and Hess (1955), Klemperer (1955) and Hoch and Lipmann (1954) found that uncoupling took place only after preincubation of thyroxine with rat liver mitochondria. It was concluded that the hormone first had to pass through the mitochondrial membrane before acting on the phosphorylating enzymes, and that the rate of penetration was very slow with "intact" mitochondria. Hoch et al. (1954) also studied mitochondria isolated from hamster liver. For thyroxine to act as an uncoupling agent, preincubation

was not necessary with these mitochondria, which were considered relatively "leaky" in comparison with rat liver mitochondria.

From such experiments it has been suggested that this uncoupling action, together with compensatory changes in respiration, provides a possible explanation for the physiological effect of the thyroid hormone. Since the metabolic processes in yeast are presumably not under hormonal control, it was of interest to test the effect of thyroxine upon oxidative phosphorylation in yeast granules.

As was the case with 2.4-DNP, a higher concentration of thyroxine was required for uncoupling in yeast particles than was the case with animal mitochondria. From Table 16, it can be seen that at a concentration of 5.3×10^{-4}M, 2.4-DNP completely uncouples phosphorylation from oxidation, while thyroxine, at the same concentration, exhibited only a 46 per cent inhibition of phosphorylation. Tapley and Cooper (1956) obtained a 35 per cent inhibition of phosphorylation in rat liver mitochondria at 3×10^{-5}M thyroxine in the presence of 5×10^{-3}M magnesium chloride. Complete inhibition at the same concentration of thyroxine could be observed in the absence of added magnesium, suggesting that magnesium exerted a protective effect against the hormonal action. The magnesium antagonism towards thyroxine could also be demonstrated with yeast granules but not as dramatically as was the case with rat liver mitochondria (Table 17). It is also of interest to note that triiodothyronine also uncouples in this system.

The yeast granules used in the experiments described above were not subjected to any special pretreatment intended to eliminate a possible structural barrier and therefore it is concluded that the

Table 16. Uncoupling by iodine containing compounds.

Experimental conditions were the same as in Table 11.

Additions as shown.

Expt. No.	Additions	Phosphate Uptake μ moles	Oxygen Uptake μ atoms	P/O
1	None	2.6	5.8	0.45
	5.3×10^{-4}M Thyroxine	1.4	5.2	0.27
	5.3×10^{-4}M 2.4-DNP	0	4.3	0
2	None	2.2	6.1	0.36
	5.3×10^{-4}M Triiodothyronine	0.5	5.4	0.10
3	None	2.0	7.6	0.26
	4.4×10^{-4}M MgI_2	2.0	7.3	0.27
	2.2×10^{-3}M MgI_2	1.9	5.3	0.36
	4.4×10^{-3}M MgI_2	0.8	4.7	0.18

Table 17. Magnesium antagonism towards thyroxine uncoupling.

Experimental conditions were the same as in Table 11 except that the magnesium ion concentration was varied, and thyroxine added, as indicated.

Final magnesium ion concentration x 10^{-4}M	No thyroxine			1.7×10^{-4}M thyroxine		
	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O
4.4	2.6	6.8	0.39	2.3	6.6	0.35
2.2	1.8	6.3	0.29	1.2	4.5	0.27
1.1	1.6	6.5	0.25	0.2	5.2	0.04

yeast particles resemble more closely the hamster liver mitochondria described by Hoch and Lipmann (1954) rather than intact rat liver mitochondria.

The ability to suppress oxidative phosphorylation is believed, by Middlebrook and Szent-Gyorgyi (1955), to be a specific function and if 2.4-DNP and thyroxine share it, it is likely that they share the same mechanism. These workers were able to demonstrate that iodine would also uncouple and concluded that thyroxine might be looked upon as being essentially iodide linked to an organic radical which provided it with a specific reactivity and affinity, enabling it thus to exert its action in low concentration at specific points.

The action of iodide, on this basis, has to be explained by some specific quality which is also common to thyroxine and 2.4-DNP. Middlebrook *et al.* (1955) point out that one specific quality of iodide is its ability to quench fluorescence, that is, quench the excited state of certain molecules by taking over and dissipating their energy of excitation. It follows that if iodide inhibits oxidative phosphorylation by quenching, 2.4-DNP and thyroxine also should be found to be quenchers. McLaughlin *et al.* (1955) have actually shown that 2.4-DNP, other nitrophenols and thyroxine act as quenchers.

Because the theory outlined above suggests that iodide is the component of thyroxine which is functional in uncoupling, iodide was also tested to determine its effects on phosphorylation. The iodine was added to the reaction mixture as magnesium iodide and in order to maintain a constant concentration of magnesium, the concen-

tration of magnesium chloride was progressively reduced as that of magnesium iodide was increased.

At the same concentration that thyroxine exhibited a 46 per cent inhibition of phosphorylation (4.4×10^{-4}M), magnesium iodide did not inhibit at all, but in fact required a tenfold increase in concentration to effect a 60 per cent inhibition of phosphorylation. This inhibition is even less convincing when it is considered that nearly a 40 per cent reduction in amount of respiration occurred at the same time.

Gramicidin

Of the antibiotic substances described in the literature which are capable of uncoupling phosphate esterification, gramicidin has probably received the most attention. At a concentration of 1.6×10^{-5}M, almost complete uncoupling was observed in yeast granules (Table 18). Essentially the same results were obtained with sucrose-EDTA suspended granules. Complete uncoupling in both sucrose-EDTA and NaCl-EDTA prepared granules was obtained at a concentration of 3.2×10^{-5}M. At this concentration (the approximate limit of solubility of gramicidin), Cross et al. (1949) obtained a 77% inhibition of phosphorylation and an appreciable stimulation of respiration. No stimulation of respiration was observed in yeast granules with succinate as substrate, when treated with this concentration of gramicidin.

Table 18. Uncoupling by Gramicidin.

Experimental conditions were the same as in Table 11.

Granules suspended in two types of media were used: (a) the normal NaCl-EDTA solution, and (b) a 10% sucrose-EDTA solution.

Gramicidin added	Sucrose-EDTA granules			NaCl-EDTA granules		
	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O
None	3.2	7.1	0.46	2.5	5.1	0.49
1.6×10^{-5} M	0.8	7.5	0.11	0.8	5.3	0.15
3.2×10^{-5} M	0.1	7.3	0.01	-0.1	4.3	0.0

Effect of calcium

For a long time calcium has been recognised as an inhibitor of oxidative phosphorylation. Potter first showed this (1947) with rat kidney 'homogenates' and later it was confirmed by Lehninger (1949) using rat liver mitochondria. Lehninger believed that the inhibition of phosphorylation might be due to the activation of ATP-ase by calcium. Potter had evidence that this could not be the only reason for the inhibition of the esterification of inorganic phosphate.

Slater et al. (1953), using a heart muscle preparation, showed that the instability of the oxidative phosphorylation mechanism was due to the calcium already in the preparations. By incorporating EDTA into the media used to isolate and wash the sarcosomes, Slater achieved a stability better than anything previously reported. The increased stability was attributed to the removal of calcium by EDTA. This hypothesis was further strengthened by the fact that the sarcosomes still retained their improved stability after the EDTA had been removed.

The addition of calcium to yeast granules prepared in NaCl-EDTA does not uncouple, but, in fact, supports phosphorylation to some extent (Chapter 7). Table 25 shows that optimum conditions for supporting phosphorylation are reached at a final concentration of 3.3×10^{-3}M. However, when the granules are suspended in 10% sucrose containing 0.001M EDTA and 0.015M sodium phosphate, uncoupling can be observed. Table 19 shows that very little inorganic phosphate esterification occurs when calcium is the sole divalent metal ion added to the assay system. The addition of both magnesium and

Table 19. Uncoupling by calcium in sucrose suspended granules.

Experimental conditions were the same as in Table 11.

Metal ions added as shown (0.01M).

Metal ion added	Phosphate Uptake /umoles	Oxygen Uptake /natoms	P/O
None	1.6	5.6	0.29
$MgCl_2$	2.8	5.3	0.53
$CaCl_2$	0.4	5.0	0.08
$MgCl_2$, $CaCl_2$	1.2	5.8	0.21

calcium increases the inorganic phosphate uptake, but not to the level of the flask in which no metal ion was added at all, and to only 43% of the uptake recorded in the flask containing only magnesium.

Unlike 2.4-DNP, the uncoupling observed with calcium in granules suspended in sucrose-EDTA is not reversible, i.e. when the granules are held at $0^{\circ}C$ for 10 minutes in the presence of 0.01M calcium, then centrifuged to remove the calcium and later resuspended in sucrose-EDTA solution, uncoupling can still be observed.

Chapter 5

BALANCE STUDIES

Introduction.

Up to this point, the only evidence that the system under investigation was oxidative phosphorylation has been that during succinate oxidation, a disappearance of inorganic phosphate from the reaction mixture could be observed; the two processes being uncoupled by 2.4-DNP. No attempt had been made to determine:

(a) whether or not the succinate oxidation was, in fact, a one-step reaction, viz.,

succinate + O ⟶ fumarate + H_2O ⟶ malate ..... (1)

(b) the fate of the inorganic phosphate disappearing from the reaction medium.

An investigation was undertaken to measure the products of succinate oxidation, to demonstrate that glucose-6-phosphate was the main phosphorylated product and also to determine how valid the P/O ratio was in expressing results by the phosphorylation efficiency of succinate oxidation.

Results.

Respiration in the absence of added substrate could only occasionally be observed. Even then the oxygen uptake seldom exceeded 0.5 microatoms during the 30 minutes incubation period. When the blank respiration was observed there was a coincident inorganic phosphate uptake.

If the stoichiometric relationship shown in reaction (1) existed, the number of micromoles of succinate disappearing or fumarate plus malate formed should equal the number of microatoms of oxygen taken up. To check this, it was necessary to isolate the organic acids before and after the incubation period during which oxygen uptake was measured.

For the organic acid balance experiment, only 18 micromoles of succinate were used per Warburg vessel. The reaction was stopped with 0.1 ml. 10N H_2SO_4 in the usual manner. The contents of three zero-time and three experimental vessels were pooled after 1.0 ml. aliquots were removed for phosphate estimations. 4.0 ml. were made 2N with respect to H_2SO_4, 3 ml. titrated with celite, and the organic acids Soxhlet extracted with ether. The ether extract was evaporated to dryness and the acids separated on a silica gel column and estimated by titration against CO_2-free NaOH.

Only succinate, fumarate and malate could be recovered from the reaction mixture, suggesting that as far as the Krebs cycle acids were concerned, the reaction was in fact a one-step oxidation reaction and that malate was not further metabolised. This is in agreement with results obtained with malate as substrate and also the findings of Nossal *et al.* (1956). Under slightly different conditions, mainly with regard to the disintegration medium and the pH of the reaction mixture, Nossal found that the most notable defect of the yeast granules was the low rate of malate oxidation, in spite of the fact that appreciable dehydrogenation of malate occurred in the whole cell-free

extracts and particles (Nossal, 1954).

From Table 20, it can be seen that the average of 5.8 micro-atoms oxygen taken up during the experimental period exceeded the amount theoretically necessary to account for the accumulation of 3.3 micromoles fumarate plus malate. Although not shown in Table 20, it should be pointed out that the amount of fumarate plus malate formed during the experiment, was the same as the amount of succinate which disappeared from the reaction mixture. A possible explanation for the difference between the oxygen taken up and the succinate utilised is discussed below.

The discrepancy between the manometric results and the chromatographic results reduces the value of using a P/O ratio to assess the effect of varying the assay constituents and conditions. Since no other more suitable method was available for expressing the results, the use of the ratio was continued although it was realised that the ratio was not a true reflection of phosphorylation due only to succinate oxidation.

Apart from determining the validity of the P/O ratio, it was desirable to demonstrate that the inorganic phosphate removed from the reaction medium during succinate oxidation was in fact converted to an organic phosphate compound. Since hexokinase and glucose were in excess in the reaction mixture, as soon as inorganic phosphate was esterified to ATP, it should immediately be converted to glucose-6-phosphate. The amount of glucose-6-phosphate formed during the incubation period should equal the amount of inorganic phosphate removed from the incubation medium.

Table 20. Products of Succinate Oxidation.

Each Warburg vessel contained 18 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.8, 2 micromoles ATP, 20 micromoles sodium fluoride, 25 micromoles glucose, 10 magnesium chloride, yeast hexokinase, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.

Vessel	Phosphate Uptake μmoles	Oxygen Uptake μatoms	P/O	Fumurate + malate produced. moles
1	4.3	6.1	0.70	
2	3.9	5.5	0.71	
3	4.2	5.8	0.72	
Average	4.1	5.8	0.71	3.3

To ensure that all the hexose esters formed would be derived from the inorganic phosphate esterified, the two micromoles ATP normally used in the assay system were replaced with one micromole ADP. Two ml. aliquots were removed from the trichloroacetic acid extract described under "Methods", neutralised with NaOH and made up to volume. Phosphate estimations were carried out on this solution as described under "Methods".

The results in Table 21 show that the amount of glucose-6-phosphate formed at various time intervals during oxidative phosphorylation, closely paralleled the amount of inorganic phosphate taken up from the reaction mixture. However, in the absence of succinate, a small amount of phosphate ester (0.65 micromoles) was formed in 30 minutes suggesting that the yeast granules contain some myokinase activity. Therefore, if the rate of myokinase activity in the experimental systems was comparable with the blank system, then one would expect the glucose-6-phosphate formation to exceed the inorganic phosphate uptake. This was not the case, in fact, ester formation was slightly less than phosphate uptake.

Evidence has been obtained suggesting that the granules contain some Zwischenferment activity. The reactions explaining discrepancies in Tables 20 and 21 are as follows:

(1) Trapping system:

$$\text{glucose} + \text{ATP} \longrightarrow \text{glucose-6-phosphate} + \text{ADP}.$$

(2) Myokinase:

$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}.$$

This excess ATP formation then causes glucose-6-phosphate formation

Table 21. Formation of Glucose-6-phosphate.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 1 micromole ADP, 20 micromoles sodium fluoride, 25 micromoles glucose, yeast hexokinase, 10 micromoles magnesium chloride, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.

Time Interval mins.	Substrate	Glucose-6-phosphate formed. (Corrected for endogenous). µmoles	Phosphate Uptake µmoles	Oxygen Uptake µmoles	P/O
0	None	0	0	0	-
30	"	0.65	0	0	-
0	Succinate	0	0	0	-
10	"	1.86	1.55	1.25	1.24
20	"	2.10	2.25	4.10	0.55
30	"	2.42	2.65	5.8	0.46

in the blank.

(3) Glucose-6-phosphate + TPN^+ ⟶ 6-phosphate-gluconate + TPNH + H^+.

(4) $TPNH + H + \frac{1}{2}O_2 \xrightarrow[\text{transport system}]{\text{electron}} TPN^+ + H_2O$.

This gives a higher than expected oxygen uptake.

Further evidence for this hypothesis has been obtained by using 2-deoxy-glucose in place of glucose in the trapping system. Woodward and Hudson (1955) have shown that yeast hexokinase phosphorylates 2-deoxy-glucose and glucose at comparble rates. Since further metabolism of 2-deoxy-glucose-6-phosphate is much slower than glucose-6-phosphate, deoxy-glucose is the preferred phosphate acceptor. Utter, Keech and Nossal (in press) using a yeast granule system similar to the one described here and 2-deoxy-glucose were able to recover fumarate and malate to the extent of 85 per cent of the oxygen uptake as compared with 58 per cent in Table 20.

These observations, viz., that the oxidation of succinate is mainly a one-step reaction, that phosphorylation is dependent on the presence of succinate, and that glucose-6-phosphate is the major phosphorylated product provides evidence independent of the 2.4-DNP experiments that the phosphorylation under study is really connected with electron transport.

Chapter 6

THE PHOSPHATE TRAPPING SYSTEM

Introduction.

Virtually all experiments on oxidative phosphorylation employ a "high energy phosphate" trapping system. In the original experiments of Belitzer and Tsibakowa (1939) with minced muscle suspensions the ortho phosphate esterified was recovered as phosphocreatine. Although the realisation that electron transport phosphorylation was associated with mitochondria proved to be of great practical importance since the elimination of soluble enzymes by washing the mitochondria resulted in fewer complicating side reactions, an external trapping system was required. The availability of purified yeast hexokinase (Berger et al., 1946) made possible the introduction of hexokinase plus glucose as a tool for trapping phosphate from any ATP generated. Both of these systems, however, implicate ADP as the primary nucleotide phosphate acceptor. For optimum phosphorylating conditions catalytic amounts of ADP are invariably added to the mitochondria.

Using rat liver mitochondria, Lindberg and Ernster (1954) and Lehninger and Smith (1949) found that AMP could replace ADP on their systems. Herbert et al. (1954) obtained evidence that UDP was phosphorylated to UTP in liver mitochondria, provided the mitochondria were supplemented with a supernatant fraction.

Sanada et al. (1954) discovered that GDP was the primary phosphate acceptor in the substrate level phosphorylation which occurs during the oxidation of α-ketoglutarate:-

α-Ketoglutarate + CoA + DPN^+ ⟶ succinyl- CoA + DPNH + H^+

Succinyl- CoA + Pi + GDP ⟶ succinate + GTP

GTP + ADP ⟶ GDP + ATP.

Of a series of nucleotides tested by Sanadi, only IDP could replace GDP in the primary reaction.

Results.

The requirement for a nucleotide phosphate acceptor for optimum phosphorylation was well known from previous work with mammalian. The data shown in Table 1 confirmed this necessity for yeast granules also. In the absence of ATP, very little phosphorylation occurred. The fact that some phosphate uptake could be observed at all suggested that the yeast particles contain endogenous adenine nucleotides but at a level at which optimum phosphorylation was not attained. Direct analysis of the adenine nucleotide content of yeast granules was obtained by preparing yeast granules according to the normal procedure except that they were much more concentrated. An equal volume of 0.1M EDTA was added and the solution neutralised to phenolphthalein. After boiling for two minutes, the solution was centrifuged and aliquots of the clear supernatant were added to a Dowex 1 Cl^- column. The nucleotides were separated and assayed as described in Chapter 2. It was found by this method that 1 ml. of yeast granules as used in these experiments contained:-

10.86 mμ moles AMP

5.82 mμ moles ADP

2.76 mμ moles ATP.

The data in Table 1 also demonstrates the necessity for an efficient trapping system, since only when hexokinase was added to the reaction mixture could a net phosphorylation be shown. In fact, when ATP only was used and hexokinase omitted, there was a liberation of inorganic phosphate during the incubation period. This was attributed to the ATPase activity in the yeast granules.

When hexokinase was added to the reaction mixture (Table 1), a marked increase in phosphorylation and an appreciable decrease in respiration was found. This finding contrasted with the marked increase in oxygen consumption on addition of hexokinase found by other workers (Lardy et al., 1952; Rabinovitz, Stuberg and Boyer, 1951; Siekevitz and Potter, 1953). This stimulation has been attributed by them to the rapid conversion of ATP to ADP since Britton Chance (1954) has shown that the respiration of mitochondrial preparations is blocked by the lack of a phosphate acceptor. It has already been pointed out that there is no evidence for obligatory coupling in yeast granules and so the observation that hexokinase did produce a pronounced stimulation of respiration in yeast granules was not unexpected.

Omission of ATP from the reaction mixture caused a very slight increase in respiration. This observation differs from the results of similar experiments with mammalian preparations. Lehninger (1949) reported that the rate of β-hydroxy-butyrate oxidation was not diminished by omitting AMP, while Green (1949) found that α-ketoglutarate oxidation was retarded about 60 per cent in the absence of AMP. To confuse the situation still further, Lardy and Wellman (1952) described experiments in which curtailments, stimulations or no differences of respiration

were obtained when a comparison was made between the absence or presence of ATP in the reaction mixture. Additional experiments are needed to settle this question.

Of the nucleotides investigated in yeast granules, ADP was the only nucleotide which could act as a phosphate acceptor (Table 22). When used in conjunction with ADP, AMP gave a slight increase in the P/O ratio (Table 22, Experiment 3); since AMP alone was inactive, the effect shown by AMP was attributed to its shifting the myokinase equilibrium away from ADP decomposition:

$$2\ ADP \rightleftharpoons ATP + AMP.$$

Myokinase activity has previously been shown to be present in the yeast granules.

At this point, further evidence in favour of this interpretation was obtained. It was found that when an additional 2 micromoles ATP was added to the reaction mixture during the experiment (i.e. 10 minutes from zero-time, Table 23, experiment 2) an increase in the P/O ratio was obtained. This suggested that the inability to maintain a constant P/O ratio with increasing time (Table 23, experiment 1) was due to the progressive loss of the specific phosphate acceptor, viz., ADP. Although this point was later disproved, this, and similar experiments showed that the phosphorylating enzyme complex was stable for at least 40 minutes at 22°C.

The preceding experiments had shown that yeast granules, in the presence of ADP (or ATP) and supplemented by hexokinase and glucose could effect oxidative phosphorylation. It has been suggested (Barkulis and Lehninger, 1951; Keilley and Keilley, 1951) that ADP

Table 22. Effect of various nucleotide polyphosphates.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 20 micromoles sodium fluoride, 25 micromoles glucose, yeast hexokinase, 10 micromoles magnesium chloride, 1 ml. yeast granules and water to a final volume of 2.25 ml. Nucleotides, 2 μmoles, were added as indicated. Temperature 22°C. Time 30 minutes.

Expt. No.	Nucleotide added	Phosphate Uptake μmoles	Oxygen Uptake μmoles	P/O
1	ADP	3.4	7.1	0.48
	ITP	0	6.4	-
	GDP	0	6.4	-
	ITP + ADP	3.4	8.3	0.41
	GDP + ADP	3.9	8.5	0.46
2	ADP	2.9	5.5	0.53
	UDP	0	6.4	-
	CDP	0	6.9	-
	UDP + ADP	2.6	7.5	0.35
	CDP + ADP	3.2	7.6	0.42
3	ADP	4.1	6.0	0.68
	AMP	0	4.8	-
	AMP + ADP	4.5	5.5	0.82

Table 23. Effect of adding excess ATP on P/O ratio.

Experimental conditions were the same as in Table 22. In experiment 2, 2 μ moles ATP were added after the 10 minute experimental period.

Expt. No.	Time Interval mins.	Phosphate Uptake Total μmoles	Phosphate Uptake Δ μmoles	Oxygen Uptake Total μmoles	Oxygen Uptake Δ μmoles	P/O
1	10	1.3	1.3	1.7	1.7	0.77
	20	1.8	0.5	3.2	1.5	0.56
	30	2.1	0.3	4.8	1.6	0.44
2	10	1.0	1.0	1.7	1.7	0.59
	20	2.3	1.3	3.0	1.3	0.77
	30	3.4	1.1	4.0	1.0	0.85

was the primary phosphate acceptor and that the phosphorylation of AMP observed by various workers was a secondary process. Thus the use of AMP is first of all dependent on its prior conversion to ADP by means of the adenylate kinase reaction. Since it was also known that fluoride inhibited adenylate kinase activity (Barkulis et al., 1951), the negative results shown in Table 22 could be interpreted to indicate that AMP is not a phosphate acceptor, at least in yeast granules, and also the fluoride present inhibited the adenylate kinase reaction sufficiently to prevent the formation of sufficient ADP to produce a detectable phosphate uptake.

Table 24 shows the effect of decreasing the fluoride concentration with AMP as the phosphate acceptor. The vessels containing ATP were included as controls. It can be seen that the presence or absence of sodium fluoride made very little difference in the amount of phosphorylation in the vessels containing ATP, whereas, in the vessels containing AMP, no phosphate esterification occurred in the presence of sodium fluoride. In the vessel containing AMP but no sodium fluoride, a phosphate uptake could be demonstrated. In the absence of sodium fluoride, myokinase apparently competes with hexokinase for the available ATP to regenerate more ADP. It was not surprising that the amount of phosphorylation occurring with AMP as the phosphate acceptor was only 25 per cent of the ATP control. Since phosphorylation would be dependent on the concentration of ADP, which in turn would be dependent not only on the AMP concentration but also the ATP, it was not surprising that phosphorylation was so low. Furthermore, although myokinase activity was demonstrated in the previous chapter with ADP as substrate

Table 24. Effect of varying fluoride concentration with AMP as phosphate acceptor.

Experimental conditions were the same as in Table 22 except that the adenine nucleotides and fluoride were varied as indicated.

Nucleotide 0.9×10^{-3}M	Sodium fluoride concentration	Phosphate Uptake /umoles	Oxygen Uptake /uatoms	P/O
ATP	9×10^{-3}M	2.9	4.6	0.63
ATP	4.5×10^{-3}M	2.9	4.8	0.61
ATP	--	2.7	4.8	0.56
AMP	9×10^{-3}M	0	4.7	--
AMP	4.5×10^{-3}M	0	5.8	--
AMP	--	0.7	6.5	0.11

and in the presence of sodium fluoride, it should be pointed out that the equilibrium for this enzyme is reached when approximately two-thirds of the added ADP has been converted to AMP and ATP (Kalckar, 1943).

Chapter 7

EFFECT OF VARIOUS REAGENTS

Introduction.

Although detailed studies have been made of the metal ion requirement for various oxidising systems, the recognition of magnesium as a necessary cofactor for oxidative phosphorylation has been so unquestionably accepted that no detailed studies have been reported on its mode of action.

Data by Lipmann (1939) indicated that magnesium was essential for pyruvic dehydrogenase, and Ochoa (1944) reached the same conclusion for α-keto-glutaric dehydrogenase. However, Stumpf, Larunduaya, and Green (1947) observed that such dehydrogenases could be prepared, perhaps by more drastic procedures, in a form not requiring magnesium. Since these preparations did not require phosphate, there seemed to be a possibility that the metal ion is essential only when the phosphorylating mechanism is intact.

The observation of Lehninger (1949), that magnesium was not necessary for β-hydroxy-butyrate oxidation in liver suggests that the electron transfer mechanisms at redox potentials higher than DPN^+ do not require this metal. However, since magnesium was essential for phosphate esterification in such a system, it was concluded that magnesium is either necessary for such phosphate esterification or for trans-phosphorylation to the adenylic system.

From these and other observations, including the fact that

optimum P/O values are obtained in its presence, the assumption has grown that magnesium is a cofactor in oxidative phosphorylation. More recently Lindberg and Ernster (1954) showed that the inactivation of glutamate oxidation by liver mitochondria caused by incubation of the latter with calcium can be prevented by the addition of manganese, ATP and DPN^+. These authors were able to restore both respiration and phosphorylation to a system thus inactivated and concluded that manganese is a cofactor of oxidative phosphorylation.

Slater (1955) tested the hypothesis of Lindberg and Ernster and the results obtained were not, as Slater stated, what would be expected if manganese were a cofactor of oxidative phosphorylation. Under the conditions used by Slater, manganese primarily increased oxygen uptake. Although Lindberg and Ernster clearly showed that manganese (in the presence of ATP, DPN^+ and glutamate) can activate mitochondria which had previously been inactivated by incubation in the presence of calcium, Slater is of the opinion that manganese acts only indirectly on phosphorylation since it was not essential for reactivation when succinate was the substrate.

It has frequently been observed that the addition of calcium depresses the respiration of tissue minces and dispersions. An exception is the oxidation by homogenates of succinate which was found by Axelrod, Swingle and Elvehjem (1941) to be stimulated by calcium. This was shown by Swingle *et al.* (1942) to be due to the activating effect of calcium on the enzyme hydrolysing DPN^+. In the presence of DPN^+, malate, formed from succinate oxidation and hydration of the resulting fumarate, is oxidised to oxaloacetate, which is a strong

inhibitor of succinic dehydrogenase (Das, 1937; Keilin and Hartree, 1940; Paudee and Potter, 1948). The destruction of DPN^+, accelerated by calcium, prevents the formation of oxaloacetate and therefore allows an increased oxidation of succinate.

Keilin and Hartree (1949) have described another type of calcium activation with heart muscle succinic oxidase, which did not oxidise fumarate. In this case, it is believed that the activation observed in phosphate buffer was caused by the formation of calcium phosphate gel which, like similar precipitates, activated the dilute heart muscle preparations by some physical action.

Results.

Yeast granules have been shown to oxidise succinate with concurrent esterification of inorganic phosphate in the absence of added divalent metal ions (Table 1). It was also found that by ashing, dissolving the residue in HCl and titrating with standard EDTA, the granules contained less than one micromole of divalent metal ion per ml. of suspension. The metal ions found to be present in the ashed material using BDH spot-test reagents were Ca, Mg, Fe, Cu and Zn. Hoch and Vallee (1955) using emission spectrography also identified these cations in cell-free yeast extracts. The presence of these metals could account for the observed activity in the absence of added divalent ions.

The addition of 10 micromoles $MgCl_2$ to this system (Table 25), although not greatly affecting the respiration, markedly increased phosphorylation. Optimum concentration for phosphorylation was reached

Table 25. Effect of divalent metal ions on oxidative phosphorylation.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 2 micromoles ATP, 20 micromoles sodium fluoride, 25 micromoles glucose, yeast hexokinase, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes. Metal ions were added as indicated.

Metal Ion Conc.	Magnesium			Manganese			Calcium		
	Phosphate Uptake	Oxygen Uptake	P/O	Phosphate Uptake	Oxygen Uptake	P/O	Phosphate Uptake	Oxygen Uptake	P/O
x 10^{-3}M	/umoles	/uatoms		/umoles	/uatoms		/umoles	/uatoms	
	1.4	5.3	0.26	1.1	4.4	0.25	0.8	5.9	0.14
.44	1.8	5.2	0.35	2.3	6.8	0.34	0.9	5.9	0.15
1.1	1.6	5.7	0.28	2.2	5.9	0.37	0.8	5.7	0.14
2.2	2.7	5.5	0.49	1.8	5.2	0.35	1.2	4.9	0.25
3.3	2.6	5.4	0.48	2.2	4.5	0.49	1.6	4.6	0.36
4.4	2.7	5.1	0.53	2.4	3.6	0.66	1.6	4.6	0.36

at 2.2×10^{-3}M. The oxidative capacity did not appear to be affected over the whole range of concentrations used.

With manganese, optimum phosphorylating activity was obtained at a lower concentration (4.4×10^{-4}M) at which oxidation also proceeds at a higher rate than in the absence of added metal ion. Further increases in manganese concentration reduced oxygen uptake while the inorganic phosphate uptake remained constant (Table 25). 4.4×10^{-3}M $MnCl_2$ consistently gave higher P/O values than $MgCl_2$ at the same concentration, not by stimulating phosphorylation but rather by reducing respiration. It is not uncommon for manganese to be effective at a lower concentration than magnesium and inhibition of various enzyme systems by higher concentrations of manganese has been reported.

The addition of calcium to yeast granules suspended in NaCl-EDTA solution did not uncouple phosphorylation but in fact gave a slightly higher inorganic phosphate uptake than in the system containing no added metal ion (Table 25). Optimum conditions for supporting phosphorylation with calcium were reached at a final concentration of 3.3×10^{-3}M. The reduction in the rate of respiration with increasing calcium ion concentration is not unexpected since the activation observed by Swingle et al. (1942) would not apply to the one-step oxidation found with these granules. Similarly, the activation described by Keilin and Hartree (1949) would not apply, since the phosphate concentration is too low to form a gel.

However, with granules suspended in 10% sucrose-EDTA, calcium failed to increase phosphorylation over the metal-free control

and partially uncoupled the phosphorylation obtained in the presence of magnesium (Table 26). Very little inorganic phosphate esterification occurred when calcium was the sole divalent metal ion added. Unlike 2.4-DNP, this uncoupling by calcium was not reversed by washing, i.e., when the granules were held at $0^{o}C$ for 10 minutes in the presence of 10 micromoles calcium per ml., centrifuged and resuspended in sucrose, little phosphorylation was obtained (Table 27).

The findings with NaCl-EDTA suspended granules differ from the known uncoupling effect of calcium on heart muscle sarcosomes (Slater and Cleland, 1953), but resemble those of Lehninger and Cooper (1955) who observed that calcium did not uncouple "digitonin" sub-particles of rat liver mitochondria.

Of the other divalent metal ions tested, 4.4×10^{-3}M, copper and iron had no effect on the P/O value although they both reduced oxidation and phosphorylation markedly, and nickel and cobalt strongly inhibited both processes.

The finding that oxidative phosphorylation would proceed in the absence of added metal ions raised the question as to what function magnesium has in the system. Since hexokinase requires magnesium for optimum activity, it was realised that the cation may only have been providing optimum conditions for the trapping system. Therefore, an experiment was conducted in which the usual trapping system (i.e., glucose, hexokinase and ATP) was replaced with ADP. Various combinations of sodium fluoride and magnesium chloride were used and AMP was added to reduce any myokinase activity that may have been present. The results (Table 28) show that in the presence of hexokinase and

Table 26. Effect of calcium on sucrose-EDTA suspended granules.

Experimental conditions were the same as in Table 25. After disintegration and washing, the granules were suspended in a medium consisting of 10% sucrose containing 0.001M EDTA and 0.015M phosphate. Metal ions were added as indicated, each at 4.4×10^{-3}M.

Metal Added	Phosphate Uptake µmoles	Oxygen Uptake µatoms	P/O
None	1.6	5.6	0.29
Magnesium	2.8	5.3	0.53
Calcium	0.4	5.0	0.10
Magnesium and Calcium	1.2	5.8	0.21

Table 27. Irreversibility of calcium uncoupling.

Experimental conditions were the same as in Table 25.

Pretreatment of granules			Ca^{++} present during expt.	Phosphate Uptake µmoles	Oxygen Uptake µatoms	P/O
0.01M Ca^{++} added	Time Minutes	Temp. °C.				
-	10	0	-	3.0	5.0	0.60
-	10	22	-	3.2	5.1	0.63
+	10	0	+	0	4.8	---
+	10	0	-	0.2	3.9	0.05
+	10	22	-	0.11	4.7	0.02

The granules were prepared in 10% sucrose containing 0.001M EDTA and 0.015M phosphate (pH 6.7) and treated as in Table 26.
Where calcium was not present during the experiment, 10 micromoles of magnesium were added.

Table 28. Oxidative Phosphorylation in the absence of a trapping system.

Each Warburg vessel contained 50 micromoles succinate, 50 micromoles glycyl-glycine, pH 6.7, 4 micromoles ADP, 1 micromole AMP, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes. Additions as shown.

Additions	Phosphate Uptake μmoles	Oxygen Uptake μatoms	P/O
H'hane, glucose, NaF, $MgCl_2$	2.6	6.3	0.41
NaF	0.4	4.2	0.10
NaF, $MgCl_2$	-0.2*	3.7	-
$MgCl_2$	-2.7	4.05	-
None	-1.0	4.55	-

* A negative sign implies liberation of inorganic phosphate.

glucose, good phosphorylation occurred with ADP as phosphate acceptor. When hexokinase and glucose were omitted, there was an appreciable liberation of inorganic phosphate. When magnesium was also omitted, phosphate liberation was reduced although a still greater reduction in phosphate liberation was observed in the system to which fluoride was added. In fact, a phosphate uptake could be observed when the cation was omitted and fluoride added, indicating that added metal ions were not required for the demonstration of a phosphate uptake. Later experiments show that the yeast granules possess a phosphatase activity even in the absence of added metals suggesting that the phosphate esterification may have been even better if the phosphatase had been successfully inhibited. In any case, the results indicate that the addition of magnesium to the reaction mixture stimulates phosphate liberation more than phosphate esterification.

Slater and Cleland (1953) introduced EDTA into the medium used for isolating heart muscle sarcosomes, because this compound was able to form a stable chelate complex with calcium. By removing calcium from the reaction mixture, Slater demonstrated a stability in the sarcosomes towards oxidative phosphorylation not previously described by other workers. Furthermore, Hunter and Ford (1955) reported that in a DPN-dependent system, viz., α-hydroxy-butyrate oxidation in rat liver mitochondria, inorganic phosphate had an inhibitory effect on both oxidation and phosphorylation. The addition of EDTA protected against this inhibition.

The protective action of EDTA on phosphorylation in yeast granules has been discussed. Furthermore, it has been shown that

for granules suspended in a NaCl-EDTA solution calcium did not uncouple the oxidative and phosphorylative mechanisms. Therefore, the action of EDTA in yeast granules required further investigation. Irrespective of how the granules were prepared, either with or without EDTA in the disintegrating or washing media, the presence of EDTA in the reaction mixture invariably stimulated the rate of both oxidation and phosphorylation. In addition, it prevented the loss of activity when the granules were preincubated at 22°C for 15 minutes (Table 11).

The stimulating effect of EDTA was compared with two other compounds, viz., histidine and ethylene-diamine. EDTA chelates with divalent cations through four carboxyl groups and the two amino nitrogens; histidine chelates through a carboxyl and an amino group; and ethylene-diamine through two amino groups. The yeast granules were disintegrated and washed in the normal manner, and finally suspended in suspending solutions containing equal amounts (0.001M) of the different chelating agents. The ability of histidine to retain both the oxidative and phosphorylative mechanisms of the granules compared favorably with EDTA (Table 29). Ethylene-diamine on the other hand, did not stimulate either mechanism over the basic system, i.e. the system containing no chelating agent.

The results shown in Table 30 throw some light on the action of EDTA. In experiment 1, the granules were equilibrated in the presence of ATP and various combinations of EDTA and ortho phosphate. The control, vessel 1, was treated in the same manner as the usual oxidative phosphorylative experiments, i.e. all reagents of the

Table 29. Effect of other chelating compounds.

Experimental conditions were the same as in Table 25 except that 10 micromoles magnesium were present. The granules were disintegrated and washed in the normal manner, and finally suspended in suspending solutions containing equal amounts (0.001M) of the different chelating agents.

Chelating agent 1×10^{-3}M	Phosphate Uptake /µmoles	Oxygen Uptake /µatoms	P/O
None	1.9	3.8	0.50
EDTA	3.6	4.8	0.75
Histidine	3.2	5.1	0.65
Ethylene-diamine	1.6	3.5	0.46

Table 30. Effect of EDTA, phosphate and ATP on oxidative phosphorylation.

Experimental conditions were the same as in Table 29.

Expt.	Vessel No.	Reagents present during equilibration	Reagents tipped after equilibration	Phosphate Uptake /umoles	Oxygen Uptake /uatoms	P/O
1	1	ATP, Pi, EDTA	-	3.0	4.7	0.64
	2	ATP	Pi, EDTA	3.0	5.1	0.59
	3	ATP	Pi	1.6	3.4	0.47
	4	ATP, Pi	EDTA	1.0	4.1	0.25
	5	ATP, EDTA	Pi	2.5	4.8	0.52
	6	ATP, Pi	-	0.6	4.8	0.12
2	1	ATP, Pi, EDTA	-	2.6	5.7	0.46
	2	Pi, EDTA	ATP	3.0	5.1	0.59
	3	Pi	ATP, EDTA	2.2	5.3	0.42
	4	Pi, ATP	EDTA	0.6	3.7	0.17

reaction mixture were equilibrated in the presence of the yeast granules. In vessel 2, EDTA and phosphate were omitted from the reaction mixture during the equilibration period and added from the side bulb at zero time. This treatment caused little change in activity. When EDTA was left out of the reaction mixture entirely and ortho phosphate was placed in the side bulb during the equilibration period (vessel 3), there was a considerable reduction in activity and a lowering of the P/O value. This was similar to the data shown in Table 11. Vessel 6 shows that when ATP and ortho phosphate were equilibrated with the yeast granules in the reaction mixture, there was a marked decrease in phosphorylation, which was only partly reversed by the subsequent addition of EDTA (Vessel 4). Vessel 5 demonstrates that the P/O value is not seriously affected by equilibrating the granules in the reaction mixture in the absence of phosphate.

In the second experiment, Table 30, the time of ATP addition was also varied. Again the biggest loss of activity was noted when the yeast granules were equilibrated with ATP and inorganic phosphate but without EDTA (Vessel 4). Vessels 2 and 3 show that equilibrating the granules with inorganic phosphate in the absence of ATP does not greatly affect phosphorylation. Thus, the loss of activity during equilibration under certain conditions requires contact between the granules, inorganic phosphate and ATP in the absence of EDTA. The inactivation therefore seemed to concern ATP rather than the actual phosphorylating mechanism. The results discussed below further support such a view.

P/O values steadily decrease with increasing incubation

periods (Table 31). This is not uncommon in oxidative phosphorylation systems, and is usually ascribed to deterioration of labile enzyme complexes. In this case, however, loss of activity was immediately reversed by a second addition of ATP (Table 31). This, together with the above experiments, strongly suggests that decomposition of ATP or ADP accounts for the time-loss of phosphorylating activity in yeast particles.

From the above results, it was apparent that at least one reason for the relatively low P/O values observed in yeast granules could be the progressive loss of the specific nucleotide phosphate acceptor. Since it has also been shown that AMP, in the presence of sodium fluoride, would not act as a phosphate acceptor, conversion of ADP to AMP would cause a cessation of phosphate esterification.

Further evidence for this hypothesis was obtained from another direction. When the yeast granules were incubated in the presence of ATP, sodium fluoride and buffer, a liberation of inorganic phosphate was observed (Table 32). The incubation period chosen (10 minutes at 22°C) equalled the equilibration period of oxidative phosphorylation experiments. Phosphatase activity was greatly stimulated by the addition of magnesium chloride to the reaction mixture. The stimulation was only partly reduced by the addition of sodium fluoride. The inhibition of phosphatase activity by sodium fluoride was not unexpected since fluoride is known to inhibit phosphatase activity in other systems. Yet when fluoride was left out of the reaction mixture used for oxidative phosphorylation studies, it did not have any appreciable effect on phosphorylation (Table 1).

Table 31. Effect of time on the P/O ratio.

Experimental conditions were the same as in Table 29. Vessels 1, 2, 3 and 4 were treated according to the procedure outlined under "Methods" (Chapter 2). Vessels 5 and 6 contained in the side-bulb, an additional 2 micromoles ATP which were tipped 10 minutes after the reaction was initiated, i.e. 20 minutes from the time the vessels were put into the 22°C water bath.

Vessel No.	Time at 22°C Mins.	Phosphate Uptake µmoles	Oxygen Uptake µatoms	P/O
1	10 - 20	1.3	1.7	0.77
2	20 - 30	0.5	1.5	0.33
3	30 - 40	0.3	2.1	0.14
4	10 - 20	1.0	1.7	0.59
5	20 - 30	1.3	1.3	1.00
6	30 - 40	1.1	1.0	1.10

Table 32. Phosphatase activity in yeast granules.

Each tube contained 4 micromoles ADP, 50 micromoles glycyl-glycine, pH 6.7, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 10 minutes. Additions as shown. 20 micromoles NaF, 10 micromoles $MgCl_2$.

Additions	Phosphate Liberation µmoles
None	0.4
NaF	0.4
$MgCl_2$	3.2
NaF, $MgCl_2$	2.1

It will be recalled that calcium did not uncouple oxidative phosphorylation in yeast granules prepared in a NaCl-EDTA medium. Therefore, the effect of this metal ion on the phosphatase activity was examined. The experiments showed (Table 33) firstly that granules suspended in sucrose showed less phosphatase activity than those suspended in NaCl. Secondly, that calcium stimulation of phosphatase in both preparations is about 100 per cent. Since magnesium, which increases oxidative phosphorylation, stimulated phosphatase activity more than calcium, it seems unlikely that the uncoupling of oxidative phosphorylation by calcium in sucrose suspended particles can be explained on the basis of phosphatase stimulation.

The rate of liberation of inorganic phosphate is dependent on the pH of the reaction mixture. Under alkaline conditions, the reaction is faster than at neutrality (Table 34). This could be the reason why optimum P/O values were obtained at pH 6.8 (Table 2). It would then follow that the pH used for normal oxidative phosphorylation experiments is not necessarily optimum for the phosphorylating system but rather that the balance between phosphate esterification and liberation is in favour of the former at this pH.

Phosphatase activity in yeast granules was markedly affected by the presence or absence of both ortho phosphate and EDTA. Inorganic phosphate greatly stimulated, while EDTA reduced the extent of stimulation (Table 35). Further, there was a slightly higher phosphatase activity with ADP as substrate than with ATP (which normally would not be present in the reaction mixture during oxidative phosphorylation experiments due to the excess of yeast hexokinase

Table 33. Phosphatase activity in NaCl and sucrose media.

Each tube contained 4 micromoles ADP, 50 micromoles glycyl-glycine, pH 6.7, 10 micromoles magnesium chloride, 1 ml. yeast granules, and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes. Granules were suspended in either 1% NaCl containing 0.001M EDTA and 0.015M phosphate, or 10% sucrose containing 0.001M EDTA and 0.015M phosphate.

Suspending medium	Metal ion 4.4×10^{-4}M	Phosphate liberated µmoles
NaCl	None	1.1
NaCl	Mg	2.8
NaCl	Ca	2.0
Sucrose	None	0.5
Sucrose	Mg	1.8
Sucrose	Ca	1.2

Table 34. Variation of phosphatase activity with pH.

Each tube contained 4 micromoles ADP, 50 micromoles glycyl-glycine, pH varied as shown, 20 micromoles sodium fluoride, 10 micromoles magnesium chloride, 1 ml. yeast granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.

pH	Phosphate Liberation µmoles
6.8	2.4
7.1	2.8
7.4	3.4
7.7	3.8

Table 35. Effect of inorganic phosphate and EDTA on phosphatase activity.

Each tube contained 50 micromoles glycyl-glycine, pH 6.7, 20 micromoles sodium fluoride, 10 micromoles magnesium chloride, 1 ml. yeast granules suspended in 1% NaCl. EDTA and inorganic phosphate were added as shown. Water added to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes. In experiment 1, 15 micromoles inorganic phosphate were present in all tubes. In experiment 2, 1 micromole EDTA was present in all tubes.

Experiment No.	Additions		Phosphate liberation
	Reagent	µmoles	µmoles
1	EDTA	None	2.8
		1	1.8
		2	1.2
		3	0.7
		4	0.5
		5	0.5
2	Phosphate	None	0.7
		5	2.0
		10	2.1
		15	2.1

and glucose). The possibility of the phosphate solution introducing a trace-metal was eliminated by passing the solution through Zeo-Karb 225 (Na form). Phosphate solutions treated in this manner had the same effect on phosphatase activity and P/O values as the original solutions. Although 15 micromoles of phosphate were normally used in the assay mixture, no difference in activity could be observed with as little as 5 micromoles - the lowest amount which could be used in phosphorylation experiments.

The uncoupling activity of 2.4-DNP has been discussed. In isolated mitochondria the action of 2.4-DNP is complex. It has been shown (Hunter, 1951; Lardy, 1945) that freshly prepared mitochondria has a low ATP-ase activity towards added substrate, but on addition of low concentrations of 2.4-DNP, ATP is hydrolysed at a rapid rate. In isolated mitochondria, the action of this compound is apparently complex and may involve, amongst other things, effects on the permeability of the membrane (Tapley, 1956). However, from the work of Lardy and Wellman (1953) on the activity of aqueous extracts of mitochondrial acetone dried powders it appears that 2.4-DNP has a specific stimulatory effect on the ATP-ase enzyme itself. In yeast granules, a stimulation of activity could not be observed with this nitro-phenolic compound either in the presence or absence of sodium fluoride under the conditions described in Experiment 1, Table 36. In this case, the lack of stimulation could have been due to the fact that the magnesium stimulated ATP-ase activity was so high that a further stimulation by 2.4-DNP would not be apparent. However, the addition of 2.4-DNP to a system not previously activated by magnesium did not result in a

Table 36. Effect of 2.4-DNP on phosphate activity.

Each tube contained 4 micromoles ADP, 50 micromoles glycyl-glycine, pH 6.7, 10 micromoles magnesium chloride, 1 ml. yeast granules, and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.
20 micromoles sodium fluoride and 1.2 micromoles 2.4-DNP added as shown.

Expt. No.	Additions	Magnesium 4.4×10^{-3}M	Phosphate Liberation /umoles
1	None	+	2.8
	2.4-DNP	+	2.9
	NaF	+	1.7
	2.4-DNP, NaF	+	1.8
2	NaF	+	1.8
	2.4-DNP, NaF	+	1.6
	2.4-DNP, NaF	-	0
	NaF	-	0.2

stimulation of ATP hydrolysis.

Harman and Kitiyakra (1955) studying ATP-ase activity in rat liver mitochondria reported that mechanical dispersion resulted in a minimal release of the enzyme. This was found to be the case with yeast granules also. The specific ATP-ase activity did not vary with increasing disintegration periods.

Certain sulphydryl blocking agents, such as iodo-acetamide and p-chloro-mercuribenzoate are known to uncouple phosphorylation, suggesting the participation of a sulphydryl substance in the phosphorylation process (Lehninger, 1951). Therefore, the finding that low concentrations of p-cmba (4×10^{-5}M) increased the amount of phosphate esterified during oxidative phosphorylation (Table 37) appeared to be contrary to the findings obtained with animal mitochondria. The fact that cysteine ethyl ester partially abolished phosphorylation with succinate as substrate confirmed the finding obtained with the sulphydryl blocking agent. However, when p-cmba (4×10^{-5}M) was tested on the phosphatase activity of the yeast granules, a 50 per cent reduction in phosphate liberation was observed.

Thus, it appeared most likely that the increased phosphorylation observed with p-cmba present in the assay system, was due to the inhibition of phosphatase rather than a stimulation of the phosphorylation mechanism although it is interesting to note that at 2×10^{-4}M, p-cmba exerted a marked inhibition of both oxidation and phosphorylation.

The demonstration of both a phosphatase activity and a myokinase activity contained within the yeast granules seemed to

Table 37. Effect of -SH groups on oxidative phosphorylation.

Experimental conditions were the same as in Table 29. Granules were prepared by the standard procedure and additions were made as indicated.

Expt. No.	Compound added		Phosphate Uptake µmoles	Oxygen Uptake µatoms	P/O
1	None		1.7	3.6	0.48
	p-cmba	4×10^{-5}M	3.6	4.7	0.76
	"	2×10^{-4}M	0.8	1.3	0.62
	"	1×10^{-3}M	0	0	—
2	None		1.7	3.8	0.45
	CEE	1×10^{-4}M	1.4	3.3	0.42
	"	1×10^{-3}M	1.3	3.6	0.36

explain partially the low P/O ratios obtained in earlier experiments. Also, the time-loss in phosphorylating activity apparently was due to the loss of the phosphate acceptor since these two enzymes could account for a loss of ADP from the reaction mixture. Optimal conditions for the observation of phosphate esterification require that a high ADP/ATP ratio be maintained throughout the experimental period. Normally this is achieved by adding glucose and hexokinase to ensure a low level of ATP, in the reaction mixture, but if the level of ADP is also reduced by phosphatase activity, then optimum conditions would no longer be preserved. Therefore, to test further the hypothesis that the phosphate acceptor became limiting during the 30 minute experimental period, recoveries of adenine nucleotides from the reaction mixture were made. Samples were withdrawn from the reaction vessels at various time intervals and added to an equal volume of 0.1M EDTA to inhibit myokinase activity; the solution was neutralised to phenolphthalein and boiled for 2 minutes. The solution was made up to volume, the protein removed by centrifugation and samples placed on a Dowex 1, Cl^- column. The nucleotides were separated and assayed as described in Chapter 2.

Strangely enough, during the 30 minute oxidative phosphorylation experiment, not only did the total nucleotide concentration remain constant, but the concentration of the individual nucleotides did not vary, i.e., the ADP/ATP ratio remained high without any marked change in either constituent (Table 38).

Table 38. Recovery of added nucleotides from the oxidative phosphorylation reaction mixture.

Experimental conditions were the same as in Table 29.

Time Interval mins.	Phosphate Uptake µmoles	P/O	AMP µmoles	ADP µmoles	ATP µmoles	Total Nucleotides µmoles
			Nucleotides Recovered			
0	0	-	0.42	1.69	0.11	2.22
0 - 10	1.65	0.72	0.48	1.48	0.14	2.10
10 - 20	0.65	0.63	0.38	1.53	0.05	1.96
20 - 30	0.30	0.48	0.49	1.56	0.12	2.17

Chapter 8

FACTORS AFFECTING MORPHOLOGICAL CHANGES

Introduction.

Previously Price, Fonnesu and Davies (1956) had shown that the typical osmotic behaviour (i.e. hypotonic swelling) of respiring liver mitochondria was observed only under detrimental conditions, e.g. deficiency of magnesium or phosphate acceptors, or the presence of inorganic phosphate. Harman and Feigelson (1952, a,b) concluded from visual comparisons that the morphological state of swelling not only influenced, but was also in turn influenced by the rates of oxidation and oxidative phosphorylation. Fonnesu and Davies (1956) showed that neither an active state of metabolism nor the presence of "high energy" phosphate esters was required to maintain a low mitochondrial water content since EDTA, citrate oxalate, AMP and magnesium were effective in maintaining a contracted mitochondrial structure while inorganic phosphate proved deleterious, i.e., it stimulated the spontaneous swelling process.

The effect of these compounds on the osmotic behaviour of mitochondria was also reflected in their effects on oxidative phosphorylation. EDTA and magnesium have been shown to provide improved P/O values, while inorganic phosphate has been shown by Hunter and Ford (1955) to reversibly inhibit oxidative phosphorylation.

Apparently, a precise mitochondrial structure is necessary before phosphate esterification can be demonstrated and agents, such as calcium, thyroxine and inorganic phosphate, which are capable of

causing the mitochondrion to swell (Tapley, 1956), will also cause a decrease in the rate of phosphorylation. Since the mode of action of EDTA, inorganic phosphate and magnesium chloride on the oxidative and phosphorylative processes in yeast granules remained unexplained, their effects on the structure or size of the granules was investigated.

Results.

Various methods have been utilised to measure the swelling or uptake of water by animal mitochondria. Visual observation under a phase-contrast microscope is specific but only qualitative (Harman et al., 1952). The most convenient method is the optical density method (Cleland, 1952) which merely involves following changes in the optical density of the mitochondrial suspension. The uptake of water by the mitochondria produces a less dense body which can be observed by a decrease in optical density while contraction, i.e., a loss of mitochondrial water is reflected in the increase in optical density. A good correlation with this technique and the more direct method of high-speed centrifugation followed by wet- and dry- weight determinations has been observed by Price et al. (1956). In view of these findings, the optical density method was used in these studies.

Initially, yeast granules were prepared throughout in 1 per cent NaCl solution. The yeast cells were disintegrated using a 10-sec. shaking period as described under "Methods" and the granules were isolated and washed according to the standard procedure. The granules (1 to 1.5 mg. dry-weight) were pipetted into cuvettes and the changes in optical density followed at 520 mμ in a spectrophotometer.

Experiments were conducted to determine the conditions necessary to demonstrate a slow rate of spontaneous swelling. However, neither variations in the tonicity of the suspending medium nor changes from pH 6.5 to 7.5 were capable of causing any change in the optical density of the yeast particle suspension. Even the addition of inorganic phosphate, which is known to stimulate the spontaneous swelling of isolated rat liver mitochondria, failed to promote any reduction in optical density.

The inability to observe swelling under these conditions suggested that the yeast granules were fully distended before their optical density was measured. Since sucrose has been used as the medium of preference for the isolation of animal mitochondria, hypertonic sucrose (0.5M) was used to isolate the yeast granules instead of NaCl. As was the case with NaCl prepared granules, very little spontaneous swelling was observed in this medium even after the granules were added to very dilute sucrose solutions at room temperature. Similarly, addition of inorganic phosphate or variations in pH failed to produce any change in optical density.

The most striking observation, however, was the large, almost instantaneous increase in optical density produced by the addition of magnesium chloride to the granule suspension (Fig. 1). Price et al. (1956) showed that AMP largely prevented the swelling of mitochondria while the further addition of magnesium caused an almost complete reversal of this process, while Tapley (1956) was able to demonstrate that magnesium prevented mitochondrial swelling without the presence of AMP. Therefore, it can be reasonably assumed that the increase in

optical density shown in Figure 1 represented a "contraction" in the size of the yeast granules.

In the contracted state, i.e. after the granules had been treated with magnesium, it was possible to demonstrate a decrease in optical density by the addition of inorganic phosphate (Fig. 1). The magnesium induced contraction was antagonised by inorganic phosphate, i.e. when the granules were treated with inorganic phosphate before magnesium, the change in optical density was only 13 per cent of that recorded without phosphate treatment. Furthermore, the order in which inorganic phosphate and magnesium was added apparently determined the ultimate size of the particles, although both treatments yielded particles smaller in size than the untreated controls (Fig. 1).

All the evidence obtained at this stage suggested that the granules were already swollen, irrespective of the tonicity of the solution in which the granules were suspended. In all these experiments, the yeast cells used had been commercially processed and were obtained in a pressed yeast cake, hence it was assumed that before disintegration, the cells were in a resting state. Therefore, it was still a possibility that the granules were already swollen inside the cell prior to disintegration. This was quite different to the way in which animal mitochondria were processed. The mitochondria used in oxidative phosphorylation experiments and experiments involving water exchange were isolated from actively metabolising cells and used immediately. Therefore, to simulate more closely the conditions under which mitochondria were isolated, yeast cells were incubated in 0.5M sucrose for 60 minutes at 22°C and then disintegrated.

The most striking difference between granules prepared after incubation of the cells in sucrose and granules prepared from the yeast cake was the fact that, in the former case, the increase in optical density on the addition of magnesium was markedly reduced (Fig. 2). Furthermore, swelling could be observed after the addition of inorganic phosphate without prior addition of magnesium. Although these granules did not exhibit the spontaneous swelling observed with isolated animal mitochondria, even in extremely hypotonic sucrose solution (0.065M), the addition of magnesium chloride followed by inorganic phosphate resulted in granules having an optical density less than the untreated control. This was in direct contrast to granules isolated from cells not previously incubated in sucrose where the optical density of the control remained lower than the treated granules. These results suggested that allowing the cells to metabolise prior to disintegration yielded granules in a much more contracted state and thus more closely resembling animal mitochondria.

No difference could be observed in either the phosphate swelling or the magnesium induced contraction in the presence or absence of AMP, ADP, ATP, EDTA or 2.4-DNP. Although the granules isolated from actively metabolising cells were in a more contracted state than granules isolated from resting cells, they did not appreciably alter the P/O value.

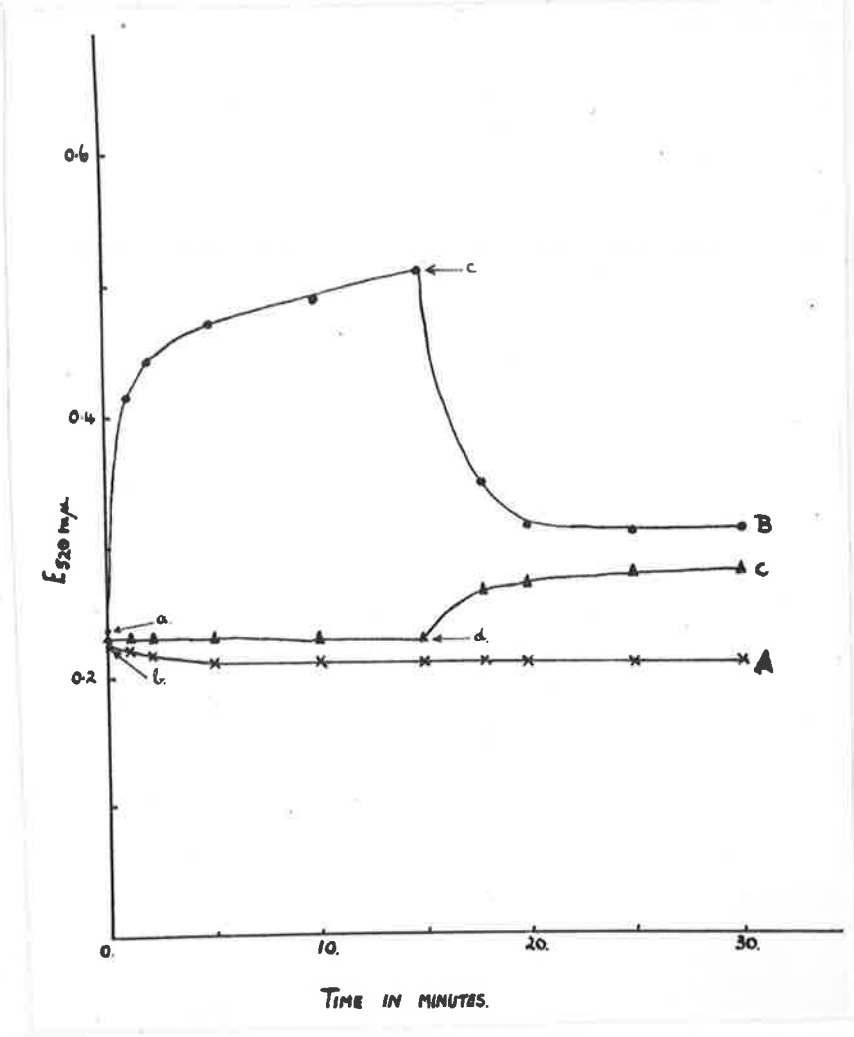


Fig. 1. Effect of magnesium and ortho phosphate on optical density of yeast granule suspensions.

A. control; no additions.

B. (a) 10 micromoles Mg^{++} added; (c) 15 micromoles phosphate added.

C. (b) 15 micromoles phosphate added; (d) 10 micromoles Mg^{++} added.

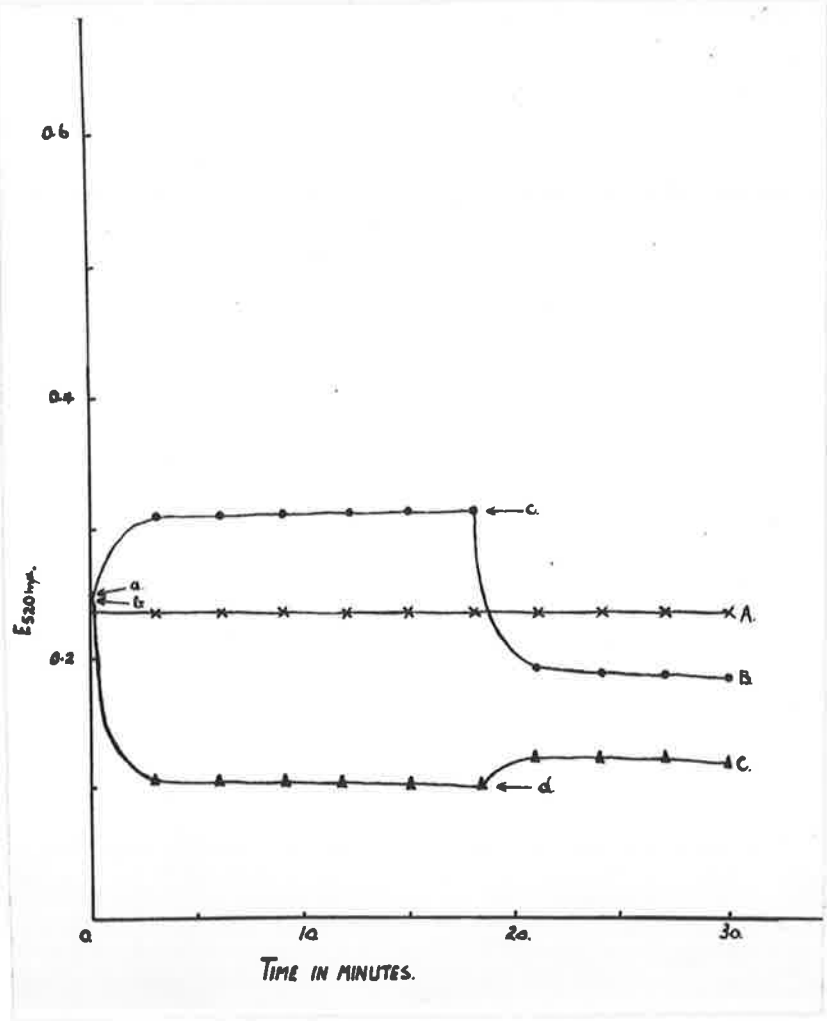


Fig. 2. Effect of magnesium and ortho phosphate on the optical density of yeast granules isolated from metabolising cells.

A. control; no additions.

B. (a) 10 micromoles Mg^{++} added; (c) 15 micromoles phosphate added.

C. (b) 15 micromoles phosphate added; (d) 10 micromoles Mg^{++} added.

Chapter 9

OTHER SUBSTRATES

Introduction.

Evidence for all the enzymes of the tricarboxylic acid cycle in yeast is now beyond question. Some of this evidence relates to the incorporation of isotopic acetate into amino acids by growing yeast cells, indicating that the Krebs' cycle is indeed a mechanism for synthesis. Other evidence for the individual reactions has been summarised by Krebs, Gurin and Eggleston (1952) and by Nossal, Hanson and Ladd (1957). However, not a great deal is known about the distribution of the enzymes involved in these reactions within the yeast cell.

Slonimsky and Ephrussi (1949) and Slonimsky and Hirsch (1952 a, 1952 b) have prepared a particulate fraction from cell-free yeast extracts which contains virtually all the cytochrome oxidase, succinic and α-glycerophosphate dehydrogenase and cytochrome c reductase activities of the whole extract. Although Nossal (1954), using the same mechanical disintegrator as used in this work, failed to prepare actively respiring yeast "mitochondria", he reported that several dehydrogenases, aconitase and fumarase were largely associated with the granular fraction of cell-free extracts. Since all the previous experiments had been concerned with the oxidation of succinate, attempts were made to substitute other substrates including lactate, α-keto-glutarate, ethanol, iso-citrate and malate.

Results.

Malate.

Although Nossal (1954) was able to observe a conversion of methylene blue to the leuco form with malate as substrate, it has not been possible to observe any oxidation of this substrate in a respirometer (Nossal et al., 1956). Furthermore, in the balance experiments (Chapter 5) using ^{14}C carbory labelled succinate it was found that complete recovery of radio-activity could be found in the unused succinate and in the fumarate and malate accumulated during the experiment. This evidence suggested that fumarasewas present in the granules but malic acid dehydrogenase was either not present or was non-functional.

Lactate.

The phosphorylation associated with the oxidation of lactate has a sensitivity of 2.4-DNP which corresponds very closely to that of the oxidation of succinate (Table 39). Uncoupling is about 75 per cent for both substrates at a concentration of 3.6×10^{-4}M 2.4-DNP and is complete at 4×10^{-4}M.

The results obtained by adding magnesium to the reaction mixture when lactate is the substrate varied from the results obtained with succinate. The addition of 10 micromoles magnesium to the reaction mixture containing succinate affected only the phosphorylation while oxidation was very little affected. With lactate as substrate, the rate of both oxidation and phosphorylation was increased, the stimulation being most noticeable in the rate of oxidation, hence a

Table 39. Uncoupling by 2.4-DNP during lactate and succinate oxidation.

Each Warburg vessel contained 50 micromoles substrate, 50 micromoles glycyl-glycine, pH 6.7, 2 micromoles ATP, 20 micromoles sodium fluoride, 25 micromoles glucose, 10 micromoles magnesium chloride, yeast hexokinase, 1 ml. granules and water to a final volume of 2.25 ml. Temperature 22°C. Time 30 minutes.

Substrate	2.4-DNP molarity	Phosphate Uptake /umoles	Oxygen Uptake /umoles	P/O	Inhibition by 2.4-DNP %
Lactate	none	2.1	4.7	0.45	-
Lactate	1.8×10^{-4}	1.3	4.5	0.29	35
Lactate	3.6×10^{-4}	0.4	3.2	0.12	73
Succinate	none	2.8	5.6	0.50	-
Succinate	1.8×10^{-4}	1.3	5.4	0.24	52
Succinate	3.6×10^{-4}	0.5	4.4	0.11	78

lower P/O value was observed in the presence of magnesium than when the divalent cation was omitted (Table 40).

Crystalline yeast lactic dehydrogenase has been shown by Appleby and Morton (1954) to be a flavo-protein with many properties analogous to the succinic dehydrogenase of heart muscle. When these facts are considered together with the present observations, the presence of a lactoxidase as well as a succinoxidase in yeast can be postulated and the present evidence suggests that the two systems are located in the particulate fraction of cell-free extracts. In contrast to the classical succinoxidase system from heart tissue (Keilin and Hartree, 1947) the yeast complexes retain at least part of their phosphorylative activity. It is interesting to note that the present demonstration of phosphorylation linked to lactate oxidation in yeast is to the author's knowledge, the only flavo-dehydrogenase linked phosphorylation other than succinate to be reported.

Ethanol.

The oxidation of ethanol cell-free extracts of bakers' yeast has been reported (Nossal, 1954). Later work has shown that most of this oxidation was associated with the yeast granules (Nossal et al., 1956), although disintegrating the yeast for periods longer than 10-seconds caused a decrease in the rate of oxidation in the particles and a corresponding increase in ethanol oxidation in the soluble fraction after the granules had been removed.

Table 41 presents data showing that ethanol oxidation by yeast granules washed and suspended in 10% sucrose could be coupled to

Table 40. Effect of magnesium on phosphorylation associated with lactate oxidation.

Experimental conditions were the same as in Table 39 except that magnesium was added as indicated.

Expt. No.	Magnesium 4.4×10^{-4} M	Phosphate Uptake /umoles	Oxygen Uptake /uatoms	P/O
1		1.8	4.6	0.39
	-	1.5	3.3	0.46
2		1.4	3.3	0.43
	-	1.0	1.5	0.67
3		2.2	4.9	0.45
	-	1.3	2.3	0.57

Table 41. Ethanol oxidation and phosphorylation.

Experimental conditions were the same as in Table 39 except that the yeast granules were washed and suspended in 10% sucrose.

The final concentrations of the additions were: DPN, 1×10^{-4}M; CEE, 4×10^{-5}M.

Additions	Phosphate Uptake /umoles	Oxygen Uptake /umoles	P/O
None	1.3	2.7	0.47
DPN	2.2	3.8	0.58
CEE	2.3	3.9	0.59
DPN, CEE	2.9	4.9	0.59
2.4-DNP	0	2.6	—

phosphorylation. The addition of 0.2 µmole DPN to the reaction mixture caused an increase in activity similar to that obtained by the addition of CEE. The existence of some 22 free-SH groups in yeast alcohol dehydrogenase has been reported (Barron and Levine, 1952) and it has been postulated that sulphydryl groups bind DPN to liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951). Whether this is so in the yeast enzyme also is not known though it was noted that the stimulations by DPN and CEE were additive.

Vallee and Hoch (1955) demonstrated that yeast alcohol dehydrogenase was a zinc metalloenzyme containing four moles of zinc firmly bound to one mole of protein. Zinc ions themselves, however, inhibited the enzymic reaction. This was found to be the case in this system also.

2.4-DNP completely uncoupled the phosphorylation associated with ethanol oxidation. It may be pointed out at this point that, like lactate, oxidative phosphorylation has not been described with this substrate.

Iso-citrate and α-ketoglutarate.

Both iso-citrate dehydrogenase and α-ketoglutarate dehydrogenase have been shown to be associated with the particulate fraction of cell-free yeast extracts. These oxidations can be coupled to the phosphorylating mechanism where suitable conditions are provided. Iso-citrate oxidation, without added cofactors, was very slow and like ethanol oxidation, was markedly increased by the addition of either CEE or DPN or both. Similarly, the rate of phosphate esterification

increased also.

α-Ketoglutarate oxidation proceeded satisfactorily in the absence of added coenzymes and although various cofactors were used, their effects were both variable and inconsistent. No evidence could be obtained to suggest that GDP may function as a primary phosphate acceptor during α-ketoglutarate oxidation (Sanadi et al., 1954) in yeast granules. The addition of malonate to inhibit succinate oxidation failed to increase the P/O value with α-ketoglutarate as substrate.

Oxidation of substrates other than succinate and lactate was complicated by the fact that particles prepared from yeast by different procedures possess different abilities to oxidise various substrates. Nossal et al. (1956) have found that particles obtained by the disruption of yeast in phosphate buffer (pH 7.6) can oxidise ethanol, iso-citrate, α-ketoglutarate and lactate but not malate. For reasons discussed earlier, particles prepared in phosphate are not suitable for studies of oxidative phosphorylation. Nossal, Keech and Utter (1956) found that particles prepared by disruption in NaCl and washed in sucrose were also able to oxidise ethanol, iso-citrate, α-ketoglutarate and lactate when appropriate cofactors were provided. Although phosphorylation accompanied these oxidation reactions, the results of these experiments were highly variable, and generally suggest that factors necessary for oxidation were retained to varying degrees in different batches of particles. Particles prepared by washing and suspending in NaCl oxidised only succinate and lactate with any consistency.

Chapter 10

DISCUSSION

Introduction.

The present experiments establish that yeast cells contain a sedimentable sub-cellular unit able to couple phosphorylation with respiration. This conclusion is based on several independent avenues of investigation:

(a) Inorganic phosphate disappearance from the reaction mixture could be observed when the particulate fraction from whole cell-free extracts was incubated in the presence of an oxidisable substrate, ATP, hexokinase, glucose and inorganic phosphate. Omitting any one of these reagents from the reaction mixture either drastically reduced or completely abolished the phosphate uptake.

(b) The addition of an uncoupling compound to the reaction mixture allowed oxidation to proceed without phosphate esterification.

(c) Glucose-6-phosphate has been shown to be the main phosphorylated compound formed during phosphate esterification.

Thus far, the nature of the particulate elements responsible for oxidative phosphorylation has not been fully established. Considerable evidence has been presented to suggest that the particles isolated from yeast cells in this work are mitochondria. The electron micrographs reveal mitochondrion-like structures with some suggestion of internal structure. Technically, the photographs presented in Chapter 3 were not ideal; they were taken of sections prepared from granules isolated in 1% NaCl since most of the oxidative phosphorylation

experiments reported in this work have been conducted in this medium. Hypertonic sucrose would have been a better medium but very few experiments were conducted in this medium. Other evidence includes the variations in size of the granules by appropriate treatments indicating the existence of a semipermeable membrane. Finally, although other enzymes and enzyme systems have been found, the most fundamental property of animal mitochondria is the existence of an electron transport mechanism with its coupled phosphorylations.

A major portion of the active material sediments at surprisingly low centrifugal forces considering the size of the particles, suggesting that a considerable degree of organisation may exist in the particulate fraction. This hypothesis is supported to some extent by microscopic studies. Under phase contrast, the majority of the granules appeared to be attached to a membrane, probably the vacuole membrane. Sections viewed through the electron microscope supported such a view. This then could account for the fact that most of the enzymic activity was sedimented relatively easily. The fact that at higher centrifugal forces, additional enzymic activity could be recovered suggested that the size of the "aggregates" was variable.

The failure in the present experiments to demonstrate an obligatory coupling of oxidation and phosphorylation may be explained by assuming that the yeast particles were not isolated in their native state, but the possibility must also be considered that in yeast granules no obligatory coupling exists. An explanation of the low P/O values could be that the yeast granules represent a mixture of active and inactive particles, but it is difficult to see why such a

mixture would not show some degree of obligatory coupling between oxidation and phosphorylation.

The balance study experiments elucidated several points. Under conditions in which yeast granules exhibited phosphorylation, a one-step succinate oxidation was demonstrated. Glucose-6-phosphate was shown to be the main phosphorylated compound formed during phosphorylation. The inability to balance the various reactions was shown to be due to the presence of at least two interfering enzymes. These enzymes caused conflicting side reactions to occur, considerably reducing the worth of using a P/O ratio for assay purposes and provide difficulties in arriving at the actual ratio for any substrate.

The rather low P/O values obtained for succinate oxidation by the yeast granules could be due to several factors. The most obvious explanation is that the particles have been damaged during the isolation procedure and are therefore not exhibiting optimum phosphorylation. In support of this argument, it should be mentioned that Kielley and Bronk (1957), using particles prepared by sonic disintegration of rat liver mitochondria, were unable to demonstrate ratios greater than unity with either DPNH or succinate as substrate. These authors believed that, in their preparations, only one stage of phosphorylation was operating but attempts to identify which stage was functional were unsuccessful. Further evidence that the isolation procedure used in this work was probably not the ideal came from the results which showed that increasing disintegration periods progressively reduce the P/O ratio in yeast granules.

However, other factors are also implicated. Some of the

observed oxidation may not have been linked to the phosphorylation mechanism. During the balance experiments, it was shown that the oxygen uptake was greater than expected, calculated from the amount of substrate disappearing. Also, although glucose-6-phosphate accumulated during the experiment, it was less than the amount expected from the inorganic phosphate disappearance. The interpretation placed on these findings was that some glucose-6-phosphate was being further metabolised by the Zwischenferment reaction. To the author's knowledge, there have been no reports in the literature of the TPNH formed in this reaction being coupled to phosphorylation during its reoxidation. This interpretation is further supported by the finding that 2.4-DNP reduces the rate of respiration in yeast granules, i.e., in the presence of 2.4-DNP, no glucose-6-phosphate is formed during the experimental period and therefore the oxygen uptake is reduced.

Finally, the possibility must also be considered that the P/O value for succinate oxidation in yeast particles is one rather than two. In a wide variety of preparative and assay procedures, each designed to provide optimum conditions, very few experiments have yielded values exceeding unity.

Uncoupling.

Of great interest in this work was the action of agents known to uncouple oxidative phosphorylation. Although it has often been suspected that the many different uncoupling agents known may have different modes of action, little direct evidence on this point is available. Tapley (1956) grouped a number of typical uncoupling

agents into three categories on the basis of their effects on mitochondrial swelling:

(a) Those which retard mitochondrial swelling when suspended in hypotonic solution.

(b) Those which accelerate mitochondrial swelling.

(c) Those which do not have a measurable effect on swelling.

In the experiments described here, examples from each of these groups were utilised.

2.4-DNP was used as an uncoupler. This nitrophenol was used as an example from the first group of compounds. The concentration required for complete uncoupling in yeast granules (3 to 5 x 10^{-4}M) was five to tenfold higher than that reported for animal mitochondria by Cross et al. (1949). The only reason which can be advanced for the requirement of a higher concentration is that a large percentage of the 2.4-DNP is adsorbed onto inactive material present in the yeast granule preparation. Cross et al. established the need for a phenolic grouping in uncouplers of the 2.4-DNP type. This was found to apply to yeast particles also; dinitro-aniline was inactive. However, mono-nitrophenols were active uncouplers for yeast oxidative phosphorylation, but inactive with animal mitochondria.

The mechanism of 2.4-DNP uncoupling is not fully understood. In aqueous extracts of acetone dried mitochondrial powder and in digitonin extracted sub-particles isolated from mitochondria, 2.4-DNP is known to stimulate ATPase activity but yeast granules, prepared in the manner described in Chapter 2, do not show a stimulation of ATP hydrolysis. However, the uncoupling experiments with this compound indicate that the phosphorylation occurring in yeast particles

closely resembles that of animal mitochondria.

Compounds which fall into the second group of uncouplers, i.e., those which accelerate the swelling of mitochondria in hypotonic solution are thyroxine and its derivatives. The fact that such compounds as thyroxine and triiodothyronine uncouple in yeast granules is of particular interest. The swelling of the mitochondria apparently disrupts the complex multi-enzyme structure involved in phosphorylation. All indications point to this structure being essential for oxidative phosphorylation. Therefore, the mechanism of thyroxine uncoupling can be visualised as being of an indirect type, acting via structure. This is borne out by the finding that digitonin sub-particles (Tapley and Cooper, 1956) are not uncoupled by thyroxine. Neither is the ATPase activity of this isolated complex affected by the hormone. Hence, the observed uncoupling by thyroxine in yeast granules provides strong biochemical but circumstantial evidence that the yeast granules contain some organised structure. On the other hand, most workers have encountered some difficulty in demonstrating an uncoupling effect of thyroxine in rat liver mitochondria. Martius and Hess (1955) were able to obtain reproducible results with rat liver mitochondria only when they preincubated the mitochondria with the hormone. Even with such preincubation Hoch and Lipmann (1954) failed to obtain consistent results. Tapley *et al.* (1956) found that a brief exposure to a hypotonic medium prior to the phosphorylation test renders the phosphorylation susceptible to uncoupling by thyroxine. Since the uncoupling could be observed in yeast granules without any such treatment, it is assumed that the

yeast particles are more permeable than rat liver mitochondria. Alternatively, it has been suggested that thyroxine is first deaminated and decarboxylated to produce a hypothetical uncoupling compound, hence the necessary incubation. In this respect, Dickens and Salmony (1956) obtained an immediate effect in inhibiting phosphorylation with triiodothyroacetic and tetraiodoacetic acid. It could be argued that the yeast granules rapidly deaminate thyroxine thus giving rise to the uncoupling agent.

The magnesium antagonism toward thyroxine uncoupling is similar to results obtained with mitochondria.

An example of the compounds which fall into the third group, i.e., those which do not appear to have any effect upon mitochondrial structure, is gramicidin. The uncoupling observed by this compound adds further to the evidence that the animal and yeast phosphorylating systems are similar in many respects.

It is interesting to note that no oxidative phosphorylation can be observed in unfractionated crude cell-free yeast extracts. This was at first thought to be due to the fact that the granules were too dilute to provide measurable phosphorylation figures and it was only after the particles were removed from the soluble fraction by centrifugation and concentrated several times that phosphorylation could be demonstrated. Later, it was shown that increasing disintegration periods caused a progressive loss of phosphorylation. It was then considered possible that perhaps all or part of the phosphorylating mechanism was being solubilised, i.e., reconstruction of the phosphorylating mechanism became a distinct possibility. To test this hypothesis,

batches of yeast granules were prepared by disintegrating the yeast cells for 15 seconds. These granules were known to have a reduced phosphorylating activity, and hence, on adding back supernatant fractions, changes in either direction, i.e., stimulatory or inhibitory, would be observed. Another sample of cells were disintegrated for 30 seconds. The particles prepared by this procedure were known to possess virtually no phosphorylating activity and the supernatant from this procedure should have been rich in phosphorylating enzymes if they were being solubilised. Therefore, the soluble fraction from the cells disintegrated for 30 seconds was added to the granules prepared from cells disintegrated for 15 seconds. Complete uncoupling resulted (Nossal, Keech, and Utter, 1956).

Uncoupling could be prevented by heating the soluble fraction before adding it to the yeast granules. It could not be removed by dialysis or treatment with Dowex resins. This phase of the project has since been extended by Dr. Nossal, who has prepared ammonium sulphate fractions which have extremely potent uncoupling capacities. Dr. Utter (private communication) has shown that one of these fractions contained a very high myokinase activity. Before any further reconstitution experiments can be attempted, either the uncoupling agent or agents will have to be removed. This aspect is under further investigation.

Other factors effecting oxidative phosphorylating.

The major factors described in Chapter 7 were magnesium, calcium, inorganic phosphate and EDTA. The effects of each have been investigated in relation to two enzymic processes; the whole complex

catalysing oxidative phosphorylation and the presumably simpler phosphatases responsible for the liberation of inorganic phosphate from ATP.

As is the case with animal mitochondria (Tapler et al., 1956) both oxidation and phosphorylation will proceed in yeast granules in the absence of any added divalent metal ion. Since the yeast cells were disintegrated in a solution containing 0.01M EDTA, and the granules washed and suspended in a solution containing 0.001M EDTA, free metal ions have presumably been removed. Analysis after ashing shows that there is less than one micromole of divalent metal present in 1.0 ml. of granule suspension. It is conceivable that these metals could be chelated by EDTA, and rendered ineffective for oxidative phosphorylation. Alternatively, the granular metal could already be bound to sites in such a way that it would not be affected by EDTA, and this could explain the observed oxidative phosphorylation in the absence of added metal.

From the results described, it is tempting to suggest that in yeast granules, added metal ions are not required for the actual process of oxidative phosphorylation. The observed effect of added magnesium would then be explicable either by activation of hexokinase or by maintenance of granular structure. The first alternative can normally be tested by substituting ADP for the hexokinase, ATP trapping system. However, in the case of yeast granules, the phosphatase activity described is so high that, even in the presence of sodium fluoride, very little phosphorylation could be observed. Nor could the phosphatase activity be inhibited completely. Furthermore, magnesium activates phosphatase activity very strongly and yet increased the rate of phosphorylation. It should be pointed out that

the phosphatase experiments were conducted under conditions where no oxidative phosphorylation was occurring.

As far as is known, the only case reported where added magnesium was an obligate component for coupled phosphorylation was in the sub-particles isolated from sonic extracts of rat liver mitochondria (Kielley and Bronk, 1957).

The second alternative, i.e., the maintenance of granular structure, receives considerable support both from the findings described here and from the findings by workers using rat liver mitochondria. For example, Baltscheffsky (1956) found that respiratory control and a high phosphorylation efficiency are retained for several minutes without the need for added magnesium, but after that period, structural changes and a loss of respiratory control occur. Thus, magnesium may be needed only to preserve the mitochondrial structure so necessary for respiration and a high phosphorylation efficiency. In this sense, the magnesium-thyroxine antagonism finds a logical explanation (Tapley et al, 1956). This role of magnesium is further supported by the studies of Lehninger and Cooper (1956) using small particle "assemblies" in which calcium had no effect on phosphorylation and magnesium was inhibitory.

It is, however, impossible to discuss the maintenance of mitochondrial structure by magnesium without also considering the effects of EDTA and inorganic phosphate. The loss of phosphorylating activity caused by ortho-phosphate and the protection by EDTA again appear to be related to the structure of the granules. Fonnesu and Davies (1956) and Price, Fonnesu and Davies (1956) while studying

the movement of ions and the prevention of swelling of liver mitochondria showed that adenine nucleotides largely prevented swelling. Both magnesium and manganese ions enhanced the action of the nucleotides although both of these ions were antagonised by inorganic phosphate. The addition of EDTA also helped protect the mitochondria from swelling. The deleterious effect of inorganic phosphate on the water content (or swelling) of the mitochondria, confirmed the results of Raaflaub (1953) and agreed with the results of Hunter and Ford (1955) who found that inorganic phosphate can reversibly inhibit phosphorylation. The results described with yeast granules can be interpreted in the light of these facts.

Although it was not possible to demonstrate any effect with EDTA, it was shown that this compound protected the granules against aging, stimulated both the oxidative and phosphorylative capacity and considerably reduced phosphatase activity. Slater et al. (1953) showed that the addition of EDTA to the washing medium simply removed calcium from the system and thus protected the sarcosomes from inactivation. From the results obtained with yeast particles, it is doubtful whether this is the only function of EDTA since added calcium did not inactivate NaCl prepared granules yet EDTA was shown to be necessary in the reaction mixture during the experimental period. The observed stimulation of succinate respiration by EDTA has been described previously in other systems. Altmann and Crook (1953) have described a similar stimulation of succinate oxidation and concluded that EDTA removed trace-metals. However, succinate oxidation in Bonner's preparation (1954) was also stimulated by EDTA, although it could be reversed by subsequently washing with trace-metal free reagents,

or by dialysis against trace-metal free buffers; the author therefore showed that EDTA did not exert its effect merely by removing metal impurities.

The results obtained by Hunter and Ford (1955) and Raaflaub (1953) using mitochondria were confirmed in these studies. Inorganic phosphate reversibly inhibited phosphorylation, the reversal being accomplished by the addition of EDTA and ATP. Two other closely connected effects noted were that inorganic phosphate caused the yeast particles to swell provided they had previously been contracted by magnesium, and also, phosphate induced an increased nucleotide phosphatase activity. Harman and Feigelson (1952) have shown that changes in morphology and an increase in the size of the mitochondria are associated with ATPase activity.

All this emphasises the complexity of the situation, for it is apparent that the activity of the granules is regulated by a delicate balance of mutually interrelated factors such as structural integrity, phosphate acceptors, pH, phosphate and metal ion concentrations.

Conclusion.

In view of the known facts about the various systems which exhibit oxidative phosphorylation, it appears that biochemically the yeast particles occupy a position intermediate between rat liver mitochondria on the one hand and bacterial systems on the other hand. For the demonstration of oxidative phosphorylation in yeast granules, it is necessary to add only ADP, inorganic phosphate, an oxidisable substrate and sufficient NaCl or sucrose to maintain a minimum level of osmolarity. The addition of hexokinase and glucose ensures a high

ADP/ATP ratio while added magnesium and sodium fluoride provide optimal conditions. The yeast particles resemble rat liver mitochondria in many respects. The phosphorylating mechanism is very labile at room temperature although increasing stability is observed by the addition of EDTA to the reaction mixture. Electron transport phosphorylation is completely uncoupled by 2.4-DNP. Both systems require ADP as the specific phosphate acceptor during phosphate esterification since it has been shown with both materials (animal and yeast) that AMP will only act as an acceptor under conditions where adenylate kinase is not inhibited.

The two systems differ in that there is no evidence to suggest that during the oxidation of either succinate or lactate in yeast particles, oxidation is obligatorily coupled to phosphorylation. Furthermore, from the results described, the P/O values for yeast particles are much lower than those reported for mitochondria although it is interesting to note that Slater et al. (1953) using heart muscle sarcosomes have suggested that the ratio for succinate oxidation is only one. Also Sacktor (1953), using mitochondria isolated from the house fly, could only demonstrate ratios up to 1.8 with α-ketoglutarate as substrate. No evidence for obligatory coupling was observed with the insect mitochondria and unfortunately no uncoupling experiments were reported.

On the other hand, phosphorylation has been demonstrated in fractions isolated from cell-free bacterial extracts. With one exception, the P/O values observed are invariably low. These systems differ from the present study and animal mitochondrial systems also, in that recombination of two or more fractions is invariably required before both oxidation and subsequent phosphorylation can be observed.

Even more striking is the fact that in the soluble bacterial systems, the phosphorylating mechanism is insensitive to 2.4-DNP treatment. The significance of this point is not properly understood.

In considering these various points, it is tempting to place yeast particles in the lower portion of a crude phylogenetic scale. At the bottom of the scale would be the simple, soluble and relatively inefficient bacterial system. The yeast particle coming next showing a certain amount of structural organisation, exhibiting considerably more efficiency and responding to uncoupling agents although at a comparatively high concentration. Next would come insect mitochondria. Not a great deal is known about this material. Eventually the ultimate is reached in the mitochondria isolated from a most metabolically active mammalian tissue, the liver.

SUMMARY

1. The present experiments establish that yeast cells contain sedimentable sub-cellular particles which are able to couple phosphorylation with respiration.

Cell-free yeast extracts were prepared by high-speed reciprocal shaking of the yeast cells with glass beads. Various preparative and assay procedures have been described.

2. The nature of the granules, isolated by differential centrifugation, has been investigated. The isolated yeast granules resembled animal mitochondria in many respects. They were capable of oxidising several Krebs' cycle intermediates such as iso-citrate, α-ketoglutarate and succinate with oxygen as the terminal electron acceptor. In addition, lactate and ethanol were oxidised. Fumarase and aconitase were known to be present also.

3. The effect of inhibitors of electron transport on succinate oxidation has been reported. Oxidation was inhibited by antimycin A, cyanide and azide.

4. Concurrent phosphorylation occurred during the oxidation of various substrates under suitable conditions. Optimum phosphorylating conditions were obtained only after the addition of ATP, hexokinase, glucose and magnesium.

5. During the oxidative phosphorylation experiments, ADP was shown to be the specific high energy phosphate acceptor; and in the presence of glucose and hexokinase, glucose-6-phosphate has been shown to accumulate.

The P/O values observed during succinate oxidation, generally ranged from 0.5 to 0.9. No evidence for obligatory coupling between oxidation and phosphorylation could be observed.

6. Various compounds, e.g., 2.4-DNP, which are known to uncouple in animal mitochondria, have been investigated in yeast granules and the significance of the findings discussed.

7. The effect of various reagents on isolated yeast granules has been investigated with regard to both oxidative phosphorylation and response to swelling. These findings, in conjunction with electron micrographs which revealed some evidence of a particle with internal structure suggested that the yeast particle may be similar to the animal mitochondrion.

8. The existence of a mitochondrion-like particle in yeast capable of localising such fundamental metabolic processes as electron transport and coupled phosphorylation has been considered in relation to other biological materials known to possess similar properties.

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