

A STUDY OF SPORULATING CELLS  
OF  
SACCHAROMYCES CEREVISIAE

A STUDY OF PHYSIOLOGICAL AND MORPHOLOGICAL  
CHANGES IN SPORULATING CELLS OF  
SACCHAROMYCES CEREVISIAE

By

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SCOPE AND CONTENTS:

An aeration technique is described which gave high and consistent yields of yeast spores.

Nuclear structure and division were studied in both vegetative and sporulating cells. The variation in glycogen and fat content of such cells was followed. These features of internal morphology are described and illustrated by five plates of figures.

A comparison was made of the respiration of vegetative and sporulating cells in the presence and absence of substrate.

Attempts were made to detect dipicolinic acid (pyridine-2,6-dicarboxylic acid) in yeast spores but no positive results were obtained. The comparative physiological significance of this is discussed.

Correlations are made of certain physiological and cytological changes observed during the sporulation process.

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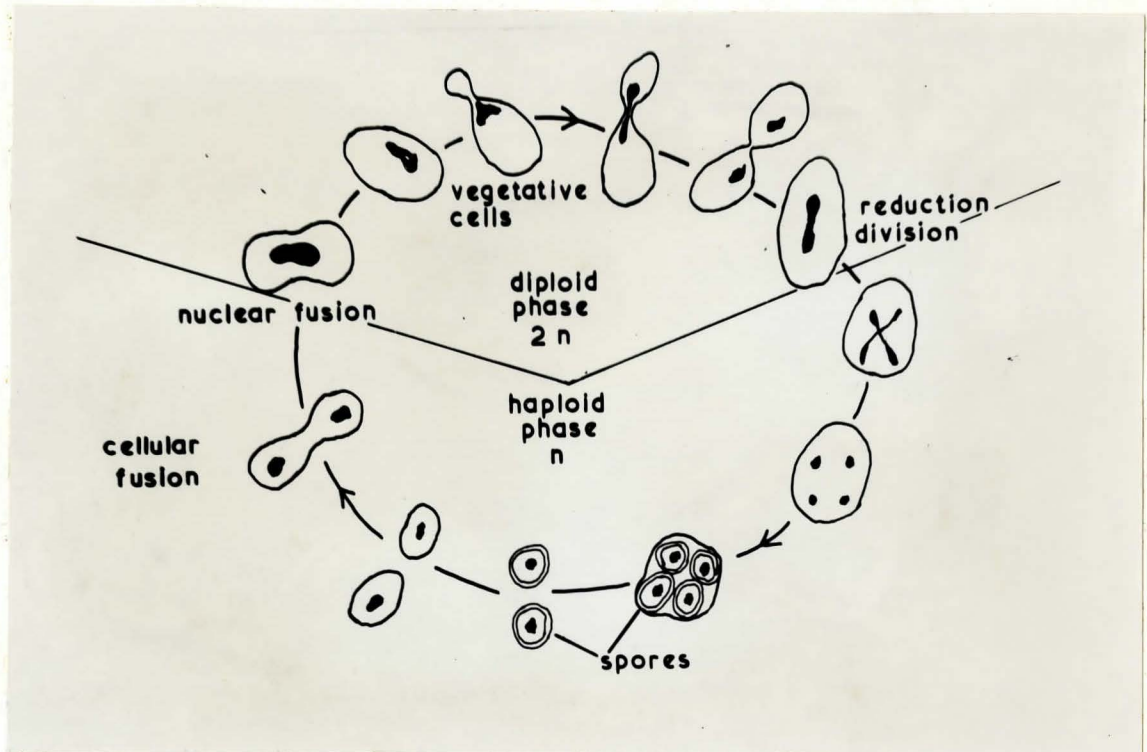
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## INTRODUCTION

The life cycle of Saccharomyces cerevisiae Hansen normally consists of an alternation between haploid and diploid generations, both of which can multiply vegetatively by budding. The diploid phase is the most important stage in the life cycle and it is in this stage that most of the vegetative growth takes place. The yeast cells can be maintained easily in this diploid vegetative phase by continually providing them with fresh nutrient. Under certain circumstances, however, these active diploid cells will cease growing, reduction division will occur, and the diploid cells will become converted into asci, each of which may contain one, two, three or four ascospores. These haploid cells may fuse in the ascus, immediately upon release from the ascus, or even after they have budded a number of times. The production of spores within the diploid cell occurs directly, without the complications of a mycelium or special fruiting structures. The foregoing features, plus the fact that sporulation can be initiated in yeast cells under strict laboratory control, make Saccharomyces cerevisiae an admirable experimental subject for sporogenesis studies. The complete life cycle is shown below.



The endospores of yeast cells were first noted almost one hundred years ago when De Seynes in 1868 observed that cells of a yeast which he identified as Mycoderma vini had tiny bodies (spores) inside them. Following De Seynes, Reess in 1869 and 1870 also observed that certain yeast cells undergo changes to produce spores, which he called ascospores because they resembled the endospores produced by Ascomycetes. Reess called the sac that held the spores and that had formerly been the yeast cell wall the ascus. A great deal of work was done on the growth and sporulation of yeast by Emil Christian Hansen, noted pioneer in yeast research, at the Carlsberg Laboratorium in Copenhagen. He developed methods

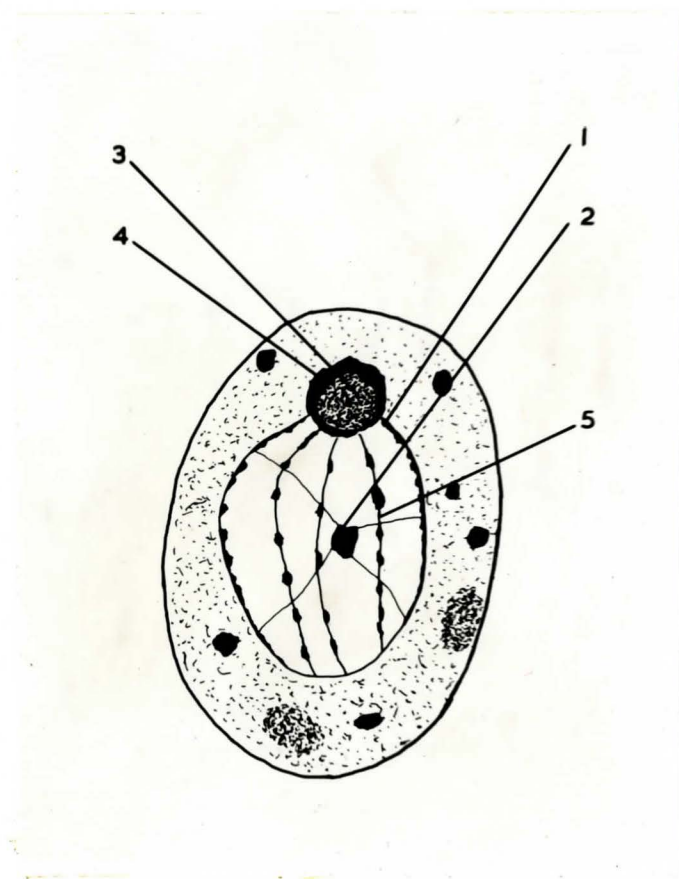
of inducing spore formation and germination. Guilliermond followed Hansen and his work dealt chiefly with the cytological aspects of spore formation in yeasts. Although Guilliermond realized that the yeast nucleus divided into four nuclei before the spores formed inside the ascus, he did not conclude that this division was a reduction division. In 1935, Winge, in a series of genetical experiments on Saccharomyces, demonstrated that haploid and diploid generations exist in this yeast and that reduction division occurs before the formation of ascospores.

The present study involves two independent fields of yeast research, physiology and cytology. Pertinent work done in these two fields will be considered in separate sections of this Introduction.

#### Cytological Studies

Even though yeasts have been the object of extensive cytological research, the cytological picture of the structure and mode of division of the yeast nucleus is still not clear. There are varying opinions concerning (1) the number of chromosomes present, if any, (2) whether the nuclear divisions are amitotic or show all stages of mitosis, and (3) the structure of the vegetative nucleus. There is general agreement that the nucleus is small and difficult to stain clearly. Details of the nuclear structure are not easily seen. The actual structure of the vegetative nucleus and its manner of division are the major areas of disagreement among yeast

cytologists. The number of workers who have dealt with this subject is much too large to go into details of all their work, but a few examples can be given to illustrate the wide divergence of opinions that were, and are, being held regarding the yeast nuclear structure. In the labelled diagram below a composite picture is presented to illustrate the concepts of the yeast nucleus held by a number of different workers. It will now be referred to in order to explain the various points of disagreement. The diagram is based on a figure by Lindegren (1949).



Janssens and Leblanc regarded the large vacuole (1) in

the yeast cell as the nucleus and considered it to consist of a membrane, nucleoplasm and chromatin. They designated a central granule in the vacuole (2) as the nucleolus. Wager and Peniston had a different concept of the nucleus. They thought that it comprised a number of different parts, namely, a body beside the vacuole which they called the nucleolus (3), a peripheral layer of chromatin around this (4), the nuclear vacuole (1), and strands of material (5) inside the vacuole which they considered to be chromatin. Guilliermond's conception of the nucleus was much simpler; he considered that Wager and Peniston's nucleolus (3) was actually the nucleus and that the vacuole in the cell was not part of the nucleus. Kater also considered the body beside the vacuole to be the nucleus, and implied that the situation is the same as that in higher plants. Lindegren's conception of the yeast nucleus resembled that of Wager and Peniston. He regarded their nucleolus (3) as a centrosome surrounded by a layer of heterochromatin (4) associated with a nuclear vacuole (1) which he believed to be part of the nucleus as they did. Wager and Peniston's chromatin network (5) he considered to be chromosomes. In 1951 Lietz described the nucleus as a regular watch-glass shaped object filled with chromatin. He did not treat the vacuole in the cell as part of the nucleus and could find no nucleolus. In 1954 Mundkur described the nucleus of Saccharomyces as "an extra vacuolar optically empty vesicle

having no detectable chromosomes at any period". He also stated that the chromatin particles were evenly dispersed throughout the nucleus. More recently, Ganesan described a similar type of vegetative nucleus. The later workers are more in agreement concerning the structure of the nucleus than were the earlier ones. They do not consider the nucleus to be partly within the vacuole as implied by the above diagram.

Janssens and Leblanc, Wager and Peniston and Guilliermond all described the vegetative division of the yeast cells they studied as amitotic, or at least as not following recognizable mitotic phases. Beams, Zell and Sulkin, working particularly with Saccharomyces cerevisiae, concluded that the nuclear division was amitotic. Socias and Ramirez were undecided whether the nuclear division during budding was amitotic or mitotic. On the other hand, Kater and Lindegren claimed that the nuclear division was typically mitotic. Kater concluded that eight chromosomes were present, and Lindegren set the number at twelve. Lietz stated that the vegetative division in Saccharomyces cerevisiae was an intranuclear mitosis and that the diploid number of chromosomes was six. More recently Mundkur described amitotic nuclear division in yeast. His observations indicated that the nucleus formed an hour glass shape and that final division occurred by completion of the medial constriction. In 1959 Ganesan, after an extensive

cytological study of Saccharomyces concluded that the vegetative division showed all the stages of mitosis.

This survey of the conclusions drawn by those who have studied the nuclear cytology of vegetative yeast cells shows that much disagreement exists in the field.

With reference to the cytology of sporulating cells of Saccharomyces cerevisiae, work by Guilliermond, Winge and Ganesan has shown that the nucleus divides once and then the two daughter nuclei divide again so that there are four nuclei present in the yeast cell before ascospores form. Although it has been shown genetically by both Winge and Lindegren (1949) that reduction division must take place during sporulation since each spore is haploid, it is not known whether meiosis in the classical sense does occur. The cytological events that take place during sporulation of Saccharomyces have been studied very little. Lindegren (1952) has proposed a mechanism for reduction division in Saccharomyces which involves the appearance of chromosomes within the vacuole followed by a "true" meiosis. This was not observed by Winge or Ganesan.

#### Physiological Studies

A number of physiological factors have been shown to influence the amount of sporulation that occurs in sporogenous strains of Saccharomyces.

(a) Presporulation Nutrition

E. Chr. Hansen found that only vigorous, young cells

would sporulate well, and this showed that sporulation depends a good deal on previous growth conditions. Other workers have since confirmed this observation. Special presporulation media were recommended by Lindegren and Lindegren, and by Adams and Kleyn. Tremaine and Miller (1954), using a chemically defined presporulation medium, found that the vitamin content of the presporulation medium influenced the yield of asci in the sporulation medium. They also noted, in another paper (1956), that nitrogen starved cells sporulated very poorly when placed in an acetate sporulation medium. In 1932 Ochmann had previously made a similar observation with cells sporulating on wooden blocks. If a nitrogen source was added to the sporulation medium, sporulation of nitrogen starved cells was enhanced in many instances (Tremaine and Miller, 1956).

(b) Oxygen Supply

Hansen was also the first to realize that aeration plays an essential part in sporulation. In 1902 Barker experimentally confirmed Hansen's observation. Maneval found that yeast cakes have spores only on the outside where the cells are in contact with the air. Lindegren and Hamilton also found spores only in the outer layer in cross sections of yeast colonies. Stantial (1935) found a large increase in the number of asci formed when she agitated yeast suspensions in a liquid sporulation medium in a rocker tube instead of allowing them to stand. Adams and Miller found that sporulation was reduced by a low oxygen ten-



sion. Miller and Halpern found that fluoroacetate inhibited sporulation, and since the citric acid cycle is known to be important in bakers' yeast (Nossal, Nickerson and Schultz, Kalnitsky and Barron) fluoroacetate inhibition of sporulation would indicate that the ability of the citric acid cycle to function has some influence on the spore-forming capacities of the cell. Miller and Halpern also observed inhibition of sporulation by cyanide. As the cytochrome system is very sensitive to cyanide (Pett), this is further evidence that oxygen must be actively consumed for sporulation to occur.

(c) Chemical Stimulants of Sporulation

De Seynes suggested that yeast cells form spores when well-fed cells suddenly encounter conditions unfavourable for growth. This opinion has often been held since. Research on chemical stimulants of sporulation has shown that while usually yeast cells will not sporulate in buffer alone without a carbon source, they will sporulate if a small amount of carbohydrate is added to the buffer solution. Work by Saito, Stantial (1935), Miller et al. (1955), Miller and Halpern, and Miller (1957) have shown that certain carbohydrates, for example, glucose, mannose and maltose, actually promote sporulation, although if they are added to the sporulation medium in too great a concentration, sporulation can be inhibited (Miller 1957). Stantial was the first to show that a carbon source other than carbohydrate, i.e. acetate, stimulated sporulation. Such studies are evidence that complete starvation may prevent

sporulation as well as growth.

(d) Chemical Inhibitors of Sporulation

Saito, besides noting the stimulating effects of carbohydrate, also found that ammonia or compounds yielding ammonia were detrimental to sporulation. Miller (1959) made a study of the effects of certain nitrogen compounds on sporulation and found that substances such as ammonium sulphate and certain amino acids were strong inhibitors of sporulation. He concluded that this inhibition by amino acids was not owing to the promotion of growth, since glycine, which inhibits sporulation, did not support the growth of the yeast cells under study. Saito made similar findings, and noted further that while small amounts of carbohydrate had a stimulating effect upon sporulation, higher concentrations inhibited sporulation. Stantial (1935) also found this inhibiting effect towards sporulation when she used over 0.1% glucose in the sporulation medium. Research by Miller *et al.* (1955) has confirmed this observation. At this point it is worth stressing that carbohydrates and nitrogen compounds, the major nutrients for growth, actually inhibit sporulation when added to the sporulation medium in concentrations favourable for growth. There are other factors known to affect sporulation but only those points pertinent to this study have been discussed here.

(e) Manometric Studies of Sporulation

The first workers to compare the respiration of growing and sporulating yeast cells were Scheiber et al. and Miller et al. (1957, 1959). They studied the changes that occurred when yeast cells were transferred from growth (presporulation) medium to sporulation medium and found that, while the respiratory quotient of growing yeast cells respiring glucose was invariably greater than 1 (usually about 1.8 or 2), after the yeast had been in sporulation medium for a number of hours it was lower than this, although it never became less than 1. Cells suspended in buffer alone, where no sporulation occurred, showed no similar decline in respiratory quotient. In the present study the work of Miller et al. (1957, 1959) on respiratory variations in sporulating yeasts was continued and an attempt was made to correlate the results with observed intra-cellular morphological changes during sporulation.

Objectives

Miller, in a recent paper (1959), discussed the change-over from equational division in the vegetative yeast cell to reduction division in the sporulating cell. He stated that "the question of why a nucleus sometimes divides by one method and sometimes by the other is a fundamental problem in biology". By studying the physiological and cytological changes that occur during sporulation of Saccharomyces cerevisiae it may be

possible to gain some insight into the basic causes underlying this vital biological phenomenon.

Yeast cells can be readily induced to sporulate, and do not form complicated fruiting bodies as do most fungi. They are unicellular organisms, easily cultured in the laboratory, and can be made to change from one type of nuclear division to the other by manipulation of the environment. It was the aim of this present work to take advantage of these features of the organism, (1) to contribute to the knowledge of the cytology of reduction division in yeast, (2) to study certain aspects of the physiology of the sporulation process in Saccharomyces cerevisiae, and (3) to attempt to correlate physiological changes with cytological changes occurring simultaneously during the sporulation process. It was hoped that a contribution would thereby be made toward understanding the factors that cause a vegetative cell to undergo reduction division.

## MATERIALS AND METHODS

### Yeast Culture

The yeast used throughout this series of experiments was a strain of Saccharomyces cerevisiae designated as F493. This strain had been isolated from Fleischmann's packaged yeast and has been maintained in the microbiology laboratory, Department of Biology, McMaster University for twelve years. It has been frequently used in yeast sporulation studies undertaken in this laboratory.

### Stock Cultures

The yeast was maintained on slants of Wickerham's Yeast Nitrogen Base (Difco) containing 3% of agar. The list of ingredients in this chemically defined medium is given below. The carbohydrate source, glucose to give a final concentration of 2%, was added separately. The medium was sterilized by gradually heating in the autoclave to 120<sup>o</sup> C. and then cooling to room temperature. Stock cultures were grown on this medium at room temperature and transferred to fresh slants every four or five days.

### Buffer Solution

In all the experiments the ingredients of the pre-sporulation and sporulation media were dissolved in pH5 <sup>M</sup>20 phthalate buffer. The buffer was made up in three litre lots and was stored in the refrigerator in 200 ml. volumes after

sterilization by autoclaving at 120° C. for 15 minutes.

### Presporulation Culture

The presporulation (growth) medium used in these experiments consisted of Wickerham's Yeast Nitrogen Base (Difco) dissolved in buffer at such a concentration as to make a stock solution ten times the final strength which is shown below. This medium lacks a carbon source which was supplied by adding glucose, also at ten times the desired final strength. This concentrated solution of Yeast Nitrogen Base plus glucose was filter-sterilized by passage through a Seitz filter funnel containing a Krueger type E filter pad. The ingredients per litre of the Yeast Nitrogen Base when used at its recommended strength are as follows:

Nitrogen Source	
Ammonium Sulphate	5 gm.
Amino Acids	
l-Histidine Monohydrochloride	10 mgm.
dl-Methionine	20 mgm.
dl-Tryptophan	20 mgm.
Vitamins	
Biotin	2 µgm.
Calcium Pantothenate	400 µgm.
Folic Acid	2 µgm.
Inositol	2000 µgm.
Niacin	400 µgm.
p-Aminobenzoic Acid	200 µgm.
Pyridoxine Hydrochloride	400 µgm.
Riboflavin	200 µgm.
Thiamine Hydrochloride	400 µgm.
Compounds supplying trace elements	
Boric Acid	500 µgm.
Copper Sulphate	40 µgm.
Potassium Iodide	100 µgm.
Ferric Chloride	200 µgm.

Manganese Sulphate	400	µgm.
Sodium Molybdate	200	µgm.
Zinc Sulphate	400	µgm.

## Salts

Potassium Phosphate Monobasic	1	gm.
Magnesium Sulphate	0.5	gm.
Sodium Chloride	0.1	gm.
Calcium Chloride	0.1	gm.

When the concentrated Yeast Nitrogen Base plus glucose was diluted to the final desired strength the glucose content was 2%. This is four times the sugar content recommended by Wickerham for taxonomic work and the purpose in increasing the strength was to insure that cells harvested from this medium would not be carbohydrate-starved. It is known that carbohydrate starvation is detrimental to sporulation (Adams). An additional reason for increasing the concentration of glucose was to make certain that enough cells would be available for sporulation cultures. The presporulation cultures were prepared by mixing, in a sterilized graduated cylinder, 10 ml. of the concentrated Yeast Nitrogen Base plus glucose and 90 ml. of sterilized buffer. This was poured into a sterilized 200 ml. round bottomed flask. An L-shaped piece of glass tubing, plugged at the top end with cotton wool and inserted through a cotton plug in the neck of the flask, had also been sterilized with the flask. After the flask was inoculated with a loopful of yeast cells from the stock culture, the glass tube was re-inserted into the flask so that the open end of the tube was about 5 mm. from the bottom. The flask with its bubbling tube was then placed in an incubator at 27° C. A sterilized rubber

tube was attached to the upper end of the bubbling tube, and sterile, moist air was forced through the medium at such a pressure that a steady stream of bubbles came from the end of the bubbling tube. This stream not only kept the cultures aerated, but it also prevented the cells from settling to the bottom. The volume of air passed through a typical culture per minutes was approximately 0.3 litres, and was supplied by an aquarium pump (Skylark Number 80880). It first passed through a tube of loosely packed cotton wool six inches in length to remove dust particles and micro-organisms. It then passed through a flask of distilled water in the incubator to bring the air up to incubator temperature. Also, this practise, by raising the humidity of the air, prevented evaporation of the aerated cultures. The air was sometimes distributed to a number of flasks at a time by a manifold. The air supply system, including the tube containing cotton wool, the water flask, glass and rubber tubing, and the manifold had all been sterilized by autoclaving at 120° C. for 15 minutes.

Yeast cells were grown in this medium for 30-40 hours before they were harvested for use in preparation of sporulation cultures. At this time there were usually 30 million cells per ml. in the medium. The cells were first removed from the presporulation medium by centrifugation in sterilized 50 ml. centrifuge tubes, and washed by centrifugation three times with sterile buffer. They were then resuspended in approximately 30 ml. of buffer. A 1 ml. sample was removed from this last suspension for counting and



was diluted ten times. A drop from this diluted suspension was examined with an AO Spencer Bright-Line counting chamber. The central square millimeter ruled on the counting chamber is divided into 25 small squares. Yeast cells were counted in five of these squares, as recommended for red blood cell counting, and the following formula was applied:

$$\text{No. cells per ml.} = \frac{\text{No. cells counted} \times \text{dilution} \times 400,000}{8}$$

The number of yeast cells per ml. in the 30 ml. of buffer was thus ascertained an appropriate amount could be included in the sporulation cultures when they were prepared (below).

#### Sporulation Culture

Except where otherwise stated, the sporulation medium consisted of phthalate buffer to which were added the same minerals and trace elements, and in the same concentrations, as contained in the Yeast Nitrogen Base (See Pages 14, 15). All of the vitamins present in the Yeast Nitrogen Base with the exception of p-aminobenzoic acid, folic acid and riboflavin were included in the sporulation medium. Also omitted from the sporulation medium were l-histidine monohydrochloride, dl-methionine, dl-tryptophan, and ammonium sulphate. The minerals and trace elements were dissolved in buffer to prepare a stock solution of twenty times the final desired strength. They were sterilized in the autoclave and kept in the refrigerator.

The six vitamins were dissolved at concentrations of one hundred times the desired final strength and filter-sterilized. 1% sodium acetate (anhydrous) was the carbon source used in the sporulation medium except where otherwise stated. Dihydroxyacetone was also used as a carbon source for some sporulation experiments at concentrations of 0.5% or 1%. To avoid possible deleterious effects of heat sterilization, the sodium acetate and dihydroxyacetone were not sterilized, but were weighed out on sterilized filter paper and added directly to 200 ml. round bottomed flasks. The buffer and the solutions of vitamins and minerals were then placed in the flasks.

A figure of 10 million cells per ml. was arbitrarily decided upon as the population density of yeast cells to be used in the sporulation medium. Miller, Calvin and Tremaine had found that in pharmaceutical bottles, where the oxygen available to the cells came from the air above a still medium, 4 million cells per ml. was an optimum concentration. Since in the present study the sporulation medium was aerated by bubbling, it was felt that a higher number of cells per ml. could be employed without diminishing sporulation yields, and this was found to be so.

The final volume of the contents of the 200 ml. round bottomed flasks was 100 ml. This comprised 5 ml. of the stock solution of mineral salts and compounds supplying trace elements, 1 ml. of the stock solution of vitamins, and a sufficient volume

of the cell suspension in buffer from the centrifuge tube (above) to give, when made up to 100 ml. with additional buffer, a cell population density of 10 million cells per ml. The flasks prepared in this way were then placed in the incubator at 27° C. and aerated in the same manner as the presporulation cultures. Samples were withdrawn at varying intervals for use in respiration experiments and cytological study.

As a check on the sporulation of the cells in each experiment, at the end of three days (72 hours) or sometimes after five days (120 hours) samples were removed from the sporulation medium and mounted on microscope slides without staining. Using the oil immersion objective, 300 cells from every culture were examined at random and the number of cells that contained 1, 2, 3 and 4 spores was recorded, as well as the number of cells that had not sporulated. The percentage of sporulated cells (asci) was thus obtained.

#### Cytological Techniques

##### (a) Fixation

Sporulating or growing yeast cells were prepared for fixation in the following manner. A drop of Haupt's adhesive\* was placed in the centre of a 22 mm. square grease-free cover

\* 1 gm. of pure finely divided gelatin is dissolved in 100 ml. distilled water at 30° C. Two gm. phenol crystals and 15 ml. c. p. glycerin are added. (Johannsen; Plant Microtechnique). In the modification employed in this study, sufficient formalin was added to the adhesive to make a 12% solution of formaldehyde.

glass lying flat on a glass plate. To this drop of adhesive was added a drop of cell suspension and the mixture was allowed to stand for a minute or two to allow the yeast cells to settle to the bottom of the drop. The cells were then fixed.

The fixative was always freshly prepared and two types were used. The principal one was acetic acid-alcohol, which is a modified Carnoy fixative employed by Robinow. It consists of one part absolute ethanol and three parts glacial acetic acid. The other was Helly's fixative, which contains the following ingredients:

Potassium dichromate, 3% aqueous	50 ml.
Mercuric chloride, saturated aqueous solution	50 ml.
Sodium sulphate	1 gm.
Immediately before use 1 ml. of formalin is added to each 20 ml. of solution. (Gurr; Biological Staining Methods)	

The cover glasses were carefully lowered into a petri dish containing 20 ml. of either fixative so that the liquid flowed smoothly over the side bearing the cells. When this was done the cells then adhered evenly to the upper surface of the cover glass. The cells were exposed for ten minutes to the acetic acid-alcohol or twenty minutes to Helly's fixative. The cover glasses were then stored until needed in "Columbia" staining jars<sup>\*</sup> containing either 50% or 70% ethanol.

(b) Giemsa Stain

The cell-bearing cover glasses were removed from the

\* Small glass jar holding 1 to 8 22 mm. square cover slips, first used at Columbia University, New York.

ethanol and washed in several changes of distilled water in Columbia staining jars. They were then placed in one of these jars containing 1N HCl maintained at  $60 \pm 0.5^{\circ}$  C. in a water bath for 10 - 14 minutes. The time of hydrolysis was varied, since it was found that sporulating cells had to be hydrolysed longer than vegetative cells. After hydrolysis the cells were washed in cold tap water and placed in the Giemsa stain. This was always freshly prepared by adding 9 or 10 drops of Gurr's R66 Giemsa solution to 9 - 10 ml. of Gurr's Giemsa buffer, pH 6.8, in a Columbia staining jar. The cover glasses with the cells attached were allowed to remain in the staining solution from 15 minutes to one hour, were washed briefly, and mounted in tap water on a grease-free slide. The edges of the cover glass were sealed to the slide with melted candle wax. Mounts prepared in this manner were suitable for study for up to two weeks. The nuclei of the yeast cells in these preparations were stained an intense red and the cytoplasm was a faint blue.

(c) Fat Stain

In order to detect the presence of fat, the yeast cells were treated for twenty minutes in Helly's fixative, preserved in 50% alcohol, and stained for 15 - 20 minutes in a 1% solution of Sudan Black B (British Drug Houses) in 70% alcohol. The cells were then washed and mounted in tap water, or in some cases, washed in 70% alcohol and mounted permanently in Gurr's

Neutral Mounting Medium. The latter method occasionally caused some shrinkage or collapse of the yeast cells and the better preparations were those mounted in tap water. The fat appeared as blue-black globules in the cytoplasm.

(d) Glycogen Stain

Each preparation for the detection of glycogen was made by mixing a drop of cell suspension with a drop of Lugol's Iodine. This contains the following ingredients:

Iodine	1 gm.
Potassium iodide	2 gm.
Water	100 ml.

Iodine and iodide are added to about 25 ml. water and when they have dissolved, the remainder of the water is added. (Gurr; Biological Staining Methods).

A clean cover glass was then applied and its edges were sealed to the slide with melted candle wax. In some cases the cells were first fixed on cover glasses in the manner described above, using Helly's fixative, and then mounted in Lugol's Iodine. The glycogen was visible as reddish-brown patches in the cytoplasm.

(e) Mitochondrial Stain

Attempts were made to stain mitochondria using the method recommended by Lee. The yeast cells were first fixed in Helly's solution, rinsed briefly in 50% alcohol, and then mounted in a 1:5000 aqueous solution of Janus green. When the stain is employed in this manner, mitochondria should appear as very dense green dots or rods against a light green background.

### Photography

The yeast cells stained by these methods were photographed on Ilford FP3 9x 12 cm. cut film using a Zeiss Model W microscope with a Bausch & Lomb apochromatic objective (Numerical Aperture 1.3), and a 12.5x K Zeiss eyepiece. Koehler illumination was employed throughout, as described by Shillaber (Page 93 ). The light source was a Reichert Point-o-Light Lamp and the light was filtered through a Wratten B No. 58 filter in conjunction with a Bausch & Lomb 0.7 neutral density filter. All photographs were taken at a magnification of 1100x. This was checked by photographing a stage micrometer scale. They were enlarged three times during printing and the total magnification of the cells in the figures is thus 3300x.

### Techniques used in Respiration Experiments

The Warburg apparatus used for respiration studies was a Bronwill Series 5 UV. Each manometer was calibrated with two reaction flasks, using a syringe type manometer calibrator, as described in "Manometric Techniques" by Umbreit, Burris and Stauffer (Page 47). The 15 ml. reaction flasks had a centre well and single side arm. During all experiments the water bath of the Warburg apparatus was kept at 27.00 <sup>0</sup>.01 C. In preparing the yeast for the respiration experiments, a certain volume of suspension was removed from either a presporulation or sporulation culture. The cells were separated from the medium by centrifugation, following which they were resuspended

in buffer and again centrifuged. They were washed in this manner two more times and then were suspended in sufficient buffer to make a final population density of 12 million cells per ml. Of this suspension 2.5 ml. was added to each reaction flask. One-half ml. of buffer in which substrate was dissolved was placed in the side arm and when this was mixed with the contents of the reaction flask, the final volume of suspension in the flask was 3 ml., and the final population density was 10 million cells per ml. In cases where no substrate was to be employed (endogenous respiration studies), 0.5 ml. of buffer was added directly to the 2.5 ml. of suspension already in the flask. The final concentration of substrate in the manometer flasks was 1% except in the experiments with dihydroxyacetone when it was 0.5%. It should be mentioned that when sodium acetate was added to the  $20 \text{ M}$  phthalate buffer of pH5 in a concentration of 1%, it increased the pH to  $5.4 \pm 0.1$ . Thus in the reaction flasks in which this compound served as respiratory substrate the pH of the suspension was somewhat higher than in the flasks to which glucose or dihydroxyacetone were added. The direct method for measuring carbon dioxide production described in "Manometric Techniques" (Page 28) was followed. This requires two flasks for each determination. Both flasks contain the same ingredients, except that one has 0.3 ml. of 30% KOH in the centre well and the other has 0.3 ml. of buffer. Thus the total volume of liquid in each flask was always 3.3 ml.



To aid in the absorption of carbon dioxide by KOH, a small rectangle of filter paper was folded accordion-like and was slipped into the well containing the KOH. The rectangle of paper was of such a size that it protruded a millimeter or two above the top of the centre well.

After the flasks had been prepared they were attached to their matched manometers and allowed to equilibrate in the water bath of the Warburg for twenty minutes. A thermobarometer was always included in each set of experiments to enable corrections to be made for changes in barometric pressure or air temperature. After equilibration, the fluid in the manometers on the closed side was adjusted until it reached the reference point (150 mark) on the manometer. The reading of the fluid on the open side of the manometer was then taken and the stop cocks were closed. Readings were made every fifteen minutes for two or two and a half hours. In two experiments readings were made every seven minutes. When substrate was to be added, it was usually tipped into the reaction flask from the side arm at the end of the third reading, one-half hour after the experiment had begun.

Since the final volume of liquid in each flask in all the experiments was 3.3 ml., the oxygen constant ( $K_{O_2}$ ) and carbon dioxide constant ( $K_{CO_2}$ ) of each flask with its appropriate manometer could be calculated and be used throughout the whole series of experiments. When oxygen uptake was to be

calculated for a given interval, the difference between the reading on the manometer bearing the flask containing KOH was taken at the beginning of the interval and at the end, corrected for changes in air pressure and temperature, if any, and then multiplied by the particular  $K_{O_2}$  for the flask and manometer. The uptake of oxygen in microlitres by the yeast cells in the given time interval was thus obtained. The calculations for obtaining  $CO_2$  production in microlitres involved comparing the readings obtained with the two flasks, one with 0.3 ml. of 30% KOH in the centre well and the other with 0.3 ml. of buffer, using a formula derived by Umbreit, Burris and Stauffer in "Manometric Techniques" (Page 28).

In all the respiration experiments control flasks lacking respiratory substrate were included. Thus a measurement of the endogenous respiration of the cells was obtained.

## EXPERIMENTAL

### Growth and Sporulation in Aerated Cultures

It was realized that large numbers of sporulating cells would be required for the cytological and physiological experiments that were planned. Attention was therefore given to the development of cultural methods that would answer this need.

Cells grown for 30 - 40 hours in 100 ml. volumes of aerated presporulation medium were found to sporulate very readily. Although almost as many cells were present in presporulation cultures grown in this manner for only 18-24 hours, it was found that sporulation cultures prepared from these cells gave low spore yields.

It had been decided to use 10 million cells per ml. in aerated sporulation cultures. Neither this number of cells nor the aeration method had been used previously with this strain of yeast. Therefore, it was necessary to determine the concentrations of sodium acetate or dihydroxyacetone that would give optimum sporulation under these circumstances. In an attempt to attain still higher spore yields the effect of some further modifications of the sporulation culture was determined. The results obtained from these experiments are shown in Table I. Four concentrations of sodium acetate were compared (0.1, 0.5, 1.0 and 2.0%) and it was found that the highest spore yields were obtained with a 1.0% concentration of sodium acetate.

With dihydroxyacetone as the carbon source the greatest spore development occurred at concentrations of 0.5% and 1.0%. In these experiments with sodium acetate and dihydroxyacetone, vitamins, minerals and trace elements were included, as described in the preparation of sporulation medium in Materials and Methods. In order to determine whether the inclusion of these substances increased or decreased spore yields, the effect of omitting either the vitamins or the minerals and trace elements or both was investigated. The carbon source used in these experiments was 1.0% sodium acetate, the concentration that gave the highest spore yields. As shown in the lower portion of Table I, the inclusion of the vitamins in the sporulation medium increased spore yields but inclusion of minerals and trace elements had no apparent effect. In the absence of the carbon source no sporulation occurred. In other words, the vitamins, minerals and trace elements alone did not support sporulation.

Previous work in this laboratory (Miller, 1957) with the same yeast strain using non-aerated sporulation cultures in pharmaceutical bottles gave spore yields of 50-60% but with the present method, the yields were raised to 75-80%.

#### Cytological Studies

Striking morphological changes occur when a vegetative yeast cell becomes a multispored ascus. Included in these changes are the cessation of equational nuclear division and

commencement of reductional division. A careful study was made of the changes in cell size, nuclear condition, and in content of glycogen and fat.

Morphological Changes Observed with Unfixed Unstained Cells

The normal vegetative cell of Saccharomyces cerevisiae is usually round or oval with or without a bud near one pole. Measurements with a Leitz micrometer eyepiece disc were taken at random of 200 cells each from (a) 30 hour vegetative yeast cells and (b) cells that had been 5 and 10 hours in acetate sporulation medium. The average length of the vegetative cells was  $5.4 \pm 0.8^*$  microns, and the average width  $4.8 \pm 0.8$  microns. The average length and width of the 5 hour cells was  $6.0 \pm 0.9$  and  $5.3 \pm 0.8$  microns. The 10 hour cells were found to be  $6.2 \pm 0.8$  microns in length and  $5.4 \pm 0.8$  microns in width. It can be noted that average lengths and widths of the 5 and 10 hour sporulating cells is greater than the corresponding averages for growing cells. However, when the standard deviation is taken into consideration, it is questionable whether the differences are statistically significant. Visual inspection of the cell populations gave the impression that there was a definite increase in average size after 5 hours in the sporulation medium. In the vegetative populations no cells as long as 8 or more microns were found. However, with 5 hour cells 9% were measured as 8 microns in length and 1% as 9 microns. With the 10 hour cells the corresponding figures

\* Standard deviation

were 11% and 1%. These measurements show that at least a part of the population did increase in size after as little as 5 hours in acetate sporulation medium. After 20 hours in the acetate sporulation medium most of the cells contained spores which would affect the shape of the cell and further measurements were not taken.

The cytoplasm of vegetative cells was fairly homogeneous, sometimes containing a large vacuole. After about 5 hours in the sporulation medium, tiny granules began to appear in the cytoplasm of the enlarged round cells. These granules increased in size and shortly before spores began to form they had become quite noticeable in the cell and were scattered around in the cytoplasm. Active Brownian movement precluded photography of these particles. Granules that may correspond to these were observed in sporulating Saccharomyces cerevisiae cells by Lindegren (1949), who considered them to be mitochondria.

Observations on the Structure and Behaviour of the Nucleus  
 .....

(a) Vegetative Cells

The picture of the vegetative nucleus of Saccharomyces cerevisiae obtained by the present method was that of a mass of chromatin, often reticulate, of varying shape and size that could be located anywhere in the cell, except within the vacuole. This is illustrated in Plate I, Nos. 1 - 4. Plate I, Numbers 1 and 3 in particular illustrate well the

reticulate feature of the nucleus. During vegetative division, the dividing nucleus was usually found in the isthmus joining the mother cell with the bud. Before division occurred, the chromatin formed a compact, intensely staining mass (Plate I, Nos. 4, 5). During division the nucleus normally appeared as an elongated structure, one end of which was located in the bud and the other in the parent yeast cell (Plate I, Nos. 6 - 9). No distinctive chromosome configurations such as are seen during mitosis in higher organisms were noted at any time during the division (Plate I, Nos. 6, 7, 8). When the nucleus had finished dividing, one daughter nucleus was located in the bud and the other in the mother cell (Plate I, Nos. 9, 10).

An important question is: are chromosomes visible during vegetative division of the yeast nucleus? Numbers 7 and 8 in Plate I show paired densely-staining bodies entering the buds and there is a suggestion of this in Number 6, Plate I. In Number 8, Plate I there is evidence of other paired bodies following behind the leading pair. If these are the yeast chromosomes, their manner of separation is different from that characteristic of higher organisms. In a great many instances the nuclear material during division was seen to consist of two rows of more or less distinct bodies lying parallel to the direction of division. This is particularly well illustrated in Plate I, No. 6.

(b) Sporulating Cells

When the cells had been in the sporulation medium with acetate as the carbon source for 5 hours, the nucleus was seen to have changed in shape in a few of the cells. It had expanded to a larger size and had become much more diffuse than the usual vegetative nucleus. This is illustrated in Plate I, Nos. 11, 12. Photography of the lightly stained outer portion of the nucleus was difficult and, therefore, the nucleus was actually larger than it appears in the photographs. In certain instances chromosome-like bodies could be distinguished in the diffuse nuclear material (Plate I, No. 13).

At 10 to 12 hours the nuclei in more of the cells had entered into this diffuse state (Plate II, Nos. 14, 15). Number 15 shows the diffuse nature of the nucleus particularly well. Chromosome-like bodies could be observed in many of the nuclei and were becoming more distinct (Plate II, Nos. 16, 17). Separation into daughter nuclei was not evident in any of the cells at this time.

Fifteen hours after the cells had been placed in the sporulation medium, quite distinct chromosome-like bodies could often be observed. In one particularly clear photograph (Plate II, No. 18), a count was made which indicated that six, or perhaps six pairs, of these bodies were present. At this time, the first nuclear divisions were evident (Plate II, Nos. 19 - 22) and some of the cells already contained two nuclei. In rare instances the second nuclear division could be seen in progress



(Plate II, Nos. 22, 23; Plate III, No. 24). It can be seen in Plate II, Nos. 22, 23; Plate III, No. 24 that the separating nuclear material contains dense areas which are not paired but appear to be moving apart as single rows of three. In some of the rows the three bodies could not be clearly distinguished. In a few of the cells at this time four small but brightly stained nuclei were present (Plate III, Nos. 25 - 27). In Plate III, Nos. 25, 26 the shape of some of the nuclei strongly suggests that they are made up from the union of three separate bodies. In Plate III, No. 26 the small stained dot in the lower part of the cell may be a body, similar to those that occur in rows in the dividing nuclei, but which has not yet joined the other two.

After 20 hours or more in the acetate sporulation medium, 50-60% of the cells had developed into spore-containing asci, and the spores were in various stages of maturation. The nuclei in some of the spores appeared to have enlarged and to have become lobate (Plate III, Nos. 28, 29). Not all of the cells formed four spores; in certain cases there were only one, two or three spores formed and the remaining nuclei could sometimes be seen in the portions of cytoplasm between the spores and the ascus wall (Plate III, Nos. 30-32). A spore containing more than one nucleus was never seen. The nuclei of many of the non-sporulated cells were still in the vegetative condition (Plate III, No. 33). Sometimes vegeta-

tive division was visible (Plate III, No. 34).

Repeated experiments showed that after three days, at which time 75 - 80% of the cells contained spores, the majority of the non-sporulated cells still contained a vegetative type nucleus. Very few cells at this time still had the diffuse nucleus. Stains were also done with cells that had been in the sporulation medium for four and five days and no change was evident from that observed in cells from 3 day old sporulation cultures. Since cells with their nuclei in the diffuse state were very rarely found in 3 - 5 day sporulation cultures, it suggests that if the yeast cell nucleus attains the diffuse state, the cell usually forms spores.

Examination of cells from sporulation medium with dihydroxyacetone as the carbon source demonstrated that the nuclear changes were the same as those observed with cells from acetate sporulation medium, except that they occurred more slowly. At 20 hours the nuclei of the cells appeared in much the same condition as the nuclei observed in cells that had been 10 hours in acetate sporulation medium. It was mentioned in the previous section that after 20 hours in the acetate sporulation medium 50 - 60% of the cells contained spores, but few or none could be seen in cells after a similar length of time in dihydroxyacetone sporulation medium.

As mentioned in the Introduction, ammonium sulphate and glucose are two strong inhibitors of sporulation. It seemed of

importance to determine whether these compounds inhibit sporulation by preventing reduction division. Eight ml. of cell suspension with a population density of 4 million per ml. were placed in 180 ml. pharmaceutical bottles. The buffer in which the suspension was prepared contained 0.2% acetate and 0.1% ammonium sulphate. There was only 7% sporulation in this medium after 48 hours incubation at 27° C. Of the non-sporulated cells many contained diffuse or expanded nuclei. These cells had the general appearance of cells that had been 10 hours in acetate sporulation medium lacking ammonium sulphate. A similar experiment was done using 1.0% glucose in place of the ammonium sulphate. No spores were found in this environment and the nuclei of the cells were similar to those seen in cells from growth medium. The glucose seemed to prevent the nuclei of the cells from entering the diffuse state.

#### Glycogen Stain.

##### (a) Vegetative Cells

When vegetative cells were mounted in Lugol's iodine solution, the cytoplasm of the cells became uniformly stained a golden yellow and the reddish-brown colour, characteristic of glycogen, was not visible.

##### (b) Sporulating Cells

After 5 hours in sporulation medium containing acetate as the carbon source, reddish-brown patches were visible in the cytoplasm (Plate IV, No. 35) and these had become more extensive after 10 and 15 hours in most cells (Plate IV, Nos. 36, 37).

In cells from dihydroxyacetone sporulation medium these areas were barely visible after 10 hours and were not clearly evident until nearly 20 hours had elapsed. With the acetate medium, after 20 hours, when 50-60% of the cells contained spores and other spores were appearing, the areas of glycogen deposition had become less abundant in cells where the cytoplasm was beginning to round up in a manner indicative of spore formation. In the cells containing spores the glycogen had almost all disappeared except for a small patch in an occasional spore.

Cells that had formed spores in dihydroxyacetone sporulation medium also lacked evidence of glycogen, except for small amounts seen in occasional spores.

Cells maintained in buffer without a carbon source for up to 30 hours, showed no visible evidence of glycogen formation.

Since ammonium sulphate inhibits spore formation in acetate but not in dihydroxyacetone, it seemed important to compare its effect on glycogen formation in these two substrates. The effect with acetate was studied as part of the experiment described on Page 35 in which the effect of ammonium sulphate on reduction division was observed. Cells exposed to buffer containing 0.2% acetate and 0.1% ammonium sulphate for 10 or 20 hours showed little evidence of glycogen formation.

When cells were placed in buffer containing 0.2% dihydroxyacetone and 0.25% ammonium sulphate, even at 15 hours there was a small amount of glycogen present and at 20 hours glycogen was visible in many of the cells (Plate IV, No. 38). After 3 days sporulation in this medium was about 26% compared to 58% in the control. Thus ammonium sulphate evidently decreased glycogen deposition in acetate sporulation medium but not in dihydroxyacetone sporulation medium.

Concentrations of glucose of the order of 0.1% or higher are also known to inhibit spore formation and the possibility was investigated that it might influence glycogen formation. It was found that cells aerated for only 10 hours in a sporulation culture containing 0.5% glucose in addition to 1% acetate possessed very extensive deposits of glycogen, although after 3 days no spores were found. This observation indicates that inhibition of sporulation by glucose is not due to interference with glycogen formation.

#### Fat Stain

Vegetative cells and cells from acetate sporulation medium were stained with Sudan Black B in order to determine whether sporulating cells accumulate fat. In vegetative (0 hour) cells, several small, darkly stained globules of fat could be seen in some of the cells (Plate IV, No. 39), but the amount of fat in these cells was never found to be extensive. After 5 hours in the sporulation medium, a number of cells showed an increase in

fat content (Plate IV, No. 40), although there were also cells in which no fat was visible. At 10 hours many more of the cells showed evidence of fat accumulation and in some of them large globules of fat could be seen in the cytoplasm (Plate IV, Nos. 41, 42). There were, however, still a number of cells that showed no evidence of fat. Fat deposits were very extensive in many of the 15 and 20 hour cells (Plate IV, Nos. 43, 44) and in the cells where the cytoplasm was starting to round up before spore formation, masses of fat could be seen beside the maturing spores (Plate IV, No. 45; Plate V, No. 46). The fat can be seen both inside and outside the maturing spores. Where the spores had become more clearly defined, fat deposits could be seen surrounding the spores. At 20 hours and beyond little fat could be seen in any of the asci outside the spores but usually each spore contained a small globule of fat (Plate V, No. 48). Cells suspended in buffer without a carbon source were tested for the presence of fat and even after 42 hours only a small amount could be seen in a few of the cells (Plate V, No. 47). These resembled cells from growth medium in this respect. Most of the cells lacked fat after 42 hours. In general, there was an overall decrease in the amount of fat present in the cells.

#### Mitochondrial Stain.

Janus Green stain was used in an attempt to determine whether the granules observed to appear in unfixed, unstained

cells during sporulation possessed mitochondrial characteristics. The results obtained were inconclusive since the cytoplasm for the most part stained evenly, although in a few 10 hour cells from acetate sporulation medium some darkly stained bodies could be seen (Plate V, No. 49). Also at 20 hours some of the cells appeared to contain more of these bodies (Plate V, Nos. 50, 51). These results are not sufficiently consistent to warrant a conclusion concerning the presence or absence of mitochondria.

#### Attempts to Detect Dipicolinic Acid

In 1953 Powell found that spores of Bacillus megatherium contained dipicolinic acid (pyridine-2,6-dicarboxylic acid), a substance that had not been previously found in nature. It occurred only in the spores and was not present in vegetative bacterial cells. Since ascospore formation in yeast has certain physiological features in common with endospore formation in bacteria (Miller, 1959), it seemed important to determine whether the ascospores contain dipicolinic acid. The colourimetric technique for determining dipicolinic acid developed by Janssen, et al. was used. Spores are first autoclaved in a small amount of distilled water to release the dipicolinic acid. They are then centrifuged out, and the supernatant tested with a reagent that indicates, by the development of a yellow colour, the amount of dipicolinic acid present. Janssen et al. found that the method would detect as little as 30  $\mu\text{gm.}$  of dipicolinic

acid per ml. of supernatant prepared from bacterial spores.

The yeast cells were grown and sporulated in aerated flasks as described in Materials and Methods. The cells were removed from 100 ml. volumes of highly sporulated (75-80%) three day old sporulation cultures containing 10 million cells per ml. The dry weight of the cells and spores obtained from 100 ml. of sporulation culture was found to be approximately 100 mgm. Six experiments were done in which cells from 100, 200 or 300 ml. volumes of sporulation culture were autoclaved in 4 ml. of distilled water. In each experiment the supernatant was always removed by centrifuging out the cells and was tested with the colour reagent of Janssen et al. This supernatant was compared in a Bausch and Lomb colourimeter with a cuvette containing distilled water and the colour reagent without spore supernatant. No colour development was detected in any of these experiments. This indicates that if dipicolinic acid was present the amount was too small to be detected by this method.

In an attempt to improve further the sensitivity of this test a chromatographic technique was employed. Two ml. of supernatant prepared as above with the yeast cells and spores from a 100 ml. sporulation culture was spotted on chromatographic paper beside 1 ml. of a control containing a known amount (6 mgm. per litre) of dipicolinic acid in distilled water. This compound was prepared for the purpose by oxidation of pyridine-2,6-dialdehyde using the method of Mathes et al. The lower end of the



4 x 12 inch piece of chromatographic paper (Whatman No. 1) was dipped in a solvent containing 10 volumes ethyl ether, 3 volumes formic acid (98%), 2 volumes water, and was run for 12 hours in a glass tank. The front had moved about 10 inches in this time. The paper was then dried in air and sprayed with the colour reagent of Janssen et al. A strong yellow spot was obtained with the control. The dipicolinic acid had moved approximately 7 inches from the point where it had been applied to the paper. There was no colour development with the yeast spore supernatant. This experiment was done three times with identical results. These results support the conclusion of the experiments done with the colourimeter.

### Respiration Studies

#### Respiration in the presence of substrates

##### (a) Glucose as a Respiratory Substrate

Both vegetative cells and cells from acetate sporulation medium responded in a similar manner when they were supplied with 1% glucose solution in the reaction flasks. After an initial short lag, oxygen uptake and carbon dioxide production assumed a high constant rate. These features are illustrated for vegetative cells in Figures 1 - 4. It can be noted in these figures that the rate of oxygen uptake was always less than the rate of carbon dioxide production.

Results with cells that had been 5 hours in acetate sporulation medium are shown in Figures 11 and 13. The rates

of carbon dioxide production and oxygen uptake show no evident difference from those observed with cells from growth medium. Cells that had been in acetate sporulation medium for 10 hours showed high rates of carbon dioxide production and oxygen uptake in two experiments (Figs. 12, 15), however, in the third experiment (Fig. 14) the rates were less. High rates of gas exchange were also found with cells that had been 15 hours in acetate sporulation medium (Figs. 16, 17). Three experiments were done with cells that had been 19 hours in acetate sporulation medium (Figs. 21-23) and one experiment each with cells after 42 and 43 hours in this medium (Figs. 30, 31). In these five experiments there was some indication of a decline in respiratory activity as compared with vegetative cells but glucose utilization was still active. Cells that had been 117 and 139 hours (Figs. 38, 39) in acetate sporulation medium seemed not to have declined obviously with respect to the utilization of glucose as a respiratory substrate compared with 42 and 43 hour sporulating cells.

In Table II are shown the respiratory rates obtained during the foregoing experiments. The values shown were obtained by measuring the slopes of the steep portion of the graphs after glucose had been added and a more or less steady state reached. The data indicate that with prolonged exposure to acetate sporulation medium the rates of carbon dioxide production and oxygen uptake declined.

Dihydroxyacetone (0.5 and 1%) was also used as a carbon source in sporulation cultures. The ability of sporulating cells from these cultures to consume oxygen and produce carbon dioxide with glucose as substrate was studied. In order to avoid lengthy description, the rates of gas exchange of cells from dihydroxyacetone sporulation medium are shown in Table III. These rates were obtained from Figures 25, 26, 27, 34, 35, 36, 40 and 42, by the same method as was used in calculating the rates in Table II. Concerning Table III, the values shown suggest that a decline in the gas exchange of the cells results from prolonged exposure to dihydroxyacetone sporulation medium but the data are insufficient to allow a definite conclusion to be drawn.

As usual, cells that had been aerated in buffer without a carbon source did not sporulate. Such cells showed an active oxygen uptake and production of carbon dioxide after 19 hours (Fig. 29), 44 hours (Fig. 37) and 113 hours (Fig. 43).

(b) Acetate as Respiratory Substrate

In these experiments acetate was generally used as the carbon source in the sporulation medium. Studies were undertaken to see if the ability of the cells to take up oxygen and produce carbon dioxide in the presence of acetate underwent any marked variations as sporulation proceeded. Vegetative cells (Fig. 9) and cells that had been sporulating for 18 hours (Fig. 24) seemed equally active in respiratory ability. However,

40 hour cells (Fig. 32) from acetate sporulation medium appeared less active.

(c) Dihydroxyacetone as Respiratory Substrate

The rates of oxygen uptake and carbon dioxide production of cells using 0.5% dihydroxyacetone as a substrate were determined with cells from growth medium (Fig. 10) and after the following times in 0.5% dihydroxyacetone sporulation medium: 20 hours (Fig. 28), 43 hours (Fig. 33) and 139 hours (Fig. 41). Inspection of these graphs shows that ability of cells to respire in the presence of dihydroxyacetone is apparently not affected by prolonged exposure to it. The rate of gas exchange of cells exposed to this substrate was always lower than that of cells supplied with glucose or acetate. Perhaps the fact that the amount of dihydroxyacetone used in these experiments was 0.5%, half that of the glucose and sodium acetate concentration, could partly account for this difference but it appears improbable that this is the sole explanation because sometimes the rate of gas exchange with the dihydroxyacetone was only about a quarter of that observed with glucose or sodium acetate.

(d) Maltose, Galactose and Trehalose as Respiratory Substrates

It is known that some yeasts cannot instantly utilize such sugars as maltose or galactose but can do so after they have been exposed to these substrates for a certain length of time, that is, these sugars induce the formation of enzymes enabling the cells to utilize them (Rhoades). It seemed of

interest to compare the ability of vegetative and sporulating cells to promote induced enzyme synthesis using these two sugars, since bacteriologists have shown that sporulating bacteria lose the ability to form induced enzymes (Hardwick and Foster).

When maltose and galactose were used as respiratory substrates, no evidence of an adaptation lag was observed, indicating that this yeast is able to rapidly utilize these substrates without the need to form induced enzymes. This meant that the original purpose of the experiment could not be realized but, nevertheless, information has been obtained about the respiration of these two substrates by vegetative and sporulating cells. It was observed (Figs. 6 and 18) that both vegetative and sporulating cells utilized galactose well. With maltose (Figs. 7 and 19) the rate of gas exchange of vegetative and sporulating cells was also similar but definitely lower than the rate with galactose.

Trehalose is stored by yeast cells in addition to glycogen (Stewart, Richtmyer and Hudson). The ability of sporulating and vegetative yeast cells to utilize this carbohydrate was also compared. Only the oxygen uptake of the cells was measured but here the rate of oxygen uptake of both vegetative and sporulating cells was very low as compared to that observed with glucose, scarcely higher than that of yeast cells respiring endogenously (Figs. 8, 20). There is no indication of a difference between the sporulating and vegetative cells in this respect.

### Endogenous Respiration

In all the experiments run in the Warburg, a control sample of vegetative (0 hour) cells or sporulating cells was run simultaneously in buffer without a carbon source. For clarity, these results are described separately.

#### (a) Vegetative Cells

As can be seen from Figures 1 - 5, the endogenous respiration of vegetative cells was always very low in comparison to that of cells utilizing glucose. Further examples of the low endogenous respiration of cells from growth medium are shown in other Figures (6, 7, 8, 9, 10).

#### (b) Cells from Acetate Sporulation Medium

There was no observed difference between the endogenous respiration of vegetative cells (Figs. 1 - 5) and cells that had been 5 hours in sporulation medium containing sodium acetate as a carbon source (Figs. 11, 13). After 10 hours in this sporulation medium, a definite increase in the rate of endogenous respiration could be noted (Figs. 12, 14, 15). In fact, in one experiment, the initial rate of oxygen uptake in endogenous respiration was almost as great as that usually observed with vegetative cells respiring glucose (Fig. 12). This increase in the initial rate of endogenous respiration over that observed in vegetative cells was also evident in cells that had been sporulating in acetate for the following times: 15 hours (Figs. 16, 17), 18 hours (Fig. 24) and 19 hours (Figs. 21 -

23). The increase in the rate of endogenous respiration at 40 hours (Fig. 32), 42 hours (Fig. 30) and 43 hours (Fig. 31) is less obvious, with the possible exception of Figure 32. Cells that had been in the sporulation medium for longer times (117 and 139 hours (Figs. 38, 39)) had an initial rate of endogenous respiration similar to that observed with cells from growth medium.

(c) Cells from Dihydroxyacetone Sporulation Medium

The rate of endogenous respiration of cells sporulating in 0.5 or 1% dihydroxyacetone increased above that shown by the vegetative cells but this was not seen until the cells had been sporulating for 43 hours (Fig. 36) and 45 hours (Fig. 35) in the 1% dihydroxyacetone sporulation medium. Cells sporulating in 0.5% dihydroxyacetone do not seem to show this after a similar length of time (Figs. 33, 34). It was mentioned above that cells sporulating in acetate medium for 117 hours (Fig. 38) and 139 hours (Fig. 39) showed a decline in their rate of endogenous respiration. A decline of endogenous respiration was not observed with cells from dihydroxyacetone medium; even after the cells had been in this medium for 139, 141 and 142 hours (Figs. 41, 42, 40).

(d) Cells from Buffer without a Carbon Source

It has been noted (Table I) that cells suspended in phthalate buffer alone without substrate did not sporulate. It seemed of interest to determine whether such cells would show a

rise in endogenous respiration. When the endogenous respiration of cells suspended in buffer for 19 hours (Fig. 29) was tested, there was no distinct difference found between the respiration rates of these cells and that of vegetative (0 hour) cells. The oxygen uptake and carbon dioxide production after 44 hours (Fig. 37) in buffer and after 113 hours (Fig. 43) seemed even lower than that of the original vegetative cells.

#### Respiratory Quotients of Vegetative and Sporulating Cells

To the physiologist the Respiratory Quotient (R. Q.) of a respiring cell or organism is the ratio of the volume of carbon dioxide expired to the volume of oxygen consumed. A theoretical R. Q. can be calculated for the oxidation of various foodstuffs. For carbohydrates it is 1, for fat about 0.7. An R. Q. of 1 for a carbohydrate such as glucose is taken to mean that it is completely oxidized to carbon dioxide and water. If, however, the glucose is partly fermented to alcohol, then more carbon dioxide will be produced than oxygen consumed and the R. Q. will become greater than 1. Comparing the R. Q. of vegetative and sporulating cells using glucose as a substrate would perhaps indicate whether there was any change in the relative ability of these cells to ferment and respire glucose. The rates of carbon dioxide production and oxygen uptake used in calculating the R. Q. of cells supplied with glucose, sodium acetate or dihydroxyacetone were determined by fitting the best straight line to the portion of the graph



where the rates of gas exchange had become steady following addition of the substrate. The R. Q. values for endogenous respiration were determined from the total oxygen uptake and carbon dioxide production obtained during the experiment. It was thought best to obtain the values in this way because the rates of gas exchange during endogenous respiration did not remain steady but tended to decrease during the course of the experiments. The Respiratory Quotients are shown in Table IV.

Yeast cells utilizing glucose as a substrate were invariably found to have an R. Q. greater than 1. There was no evidence from the results (Table IV) that prolonged exposure to acetate or dihydroxyacetone sporulation medium affected the Respiratory Quotient. This was also true of cells exposed to buffer without added carbon source.

Yeast cells, whether from growth or sporulation medium, gave R. Q. values approximating unity when supplied with acetate. One would expect the complete oxidation of this substance to carbon dioxide and water to result in an R. Q. of 1.

When dihydroxyacetone was the substrate in the reaction flasks the R. Q. values were also very close to unity, indicating that fermentation did not occur and that it was degraded by oxidation.

Yeast cells respiring endogenously had a variable Respiratory Quotient. Since the net gas exchange was always small with such cells, experimental error should have a greater

influence on the measurement of the R. Q. than with cells utilizing various substrates. Exposure of the yeast cells to sporulation medium had no consistent effect on the R. Q. as far as could be determined.

## DISCUSSION

### Methods for Inducing Sporulation

For many years, yeast cells have been induced to sporulate by growing them on rich media, and then spreading the cells in thin layers over a moist substratum possessing little or no nutritive value. Reess in 1870 introduced slices of vegetables such as carrots or beets for this purpose, and 2 years later Engel recommended gypsum blocks. Although these methods are found to be reasonably reliable for producing spores, the spore yield is uncertain and the results are not consistently reproducible. This is to be expected since the conditions found over the surface of the substratum are not uniform. Even though the cells may be spread as thinly as possible on the block or vegetable slice, the thickness of the layer will vary. Some cells will receive more oxygen than others, as well as varying amounts of nutrient, according to whether they are in the upper or lower layers of cells. Furthermore, by-products of metabolism will diffuse away less readily from the thicker areas. Because of these uncertainties, this method has serious disadvantages if exacting control of the sporulation environment is to be attained and careful physiological study made of the phenomenon of sporulation in yeasts.

When Stantial (1928) introduced the technique of growing cells in a nutrient solution, washing them, and then suspending

them in a liquid sporulation medium containing glucose and acetate in a rocker tube, she achieved much better environmental control. Tremaine and Miller (1956), and Miller and Halpern prepared both presporulation and sporulation media from chemically defined constituents. In the present work their method was improved on by keeping the cells well aerated and in suspension by bubbling air through the cultures.

Of all the previous methods developed for sporulating yeast, that of Pazonyi (1954) is the most similar to that employed in the present study. Pazonyi grew his cells for four or five days in continually agitated flasks containing must with a sugar content of 12%. The cells were then washed in distilled water and transferred to large sporulation flasks containing 2 to 2.5 litres of distilled water with 0.04% glucose, 0.14% sodium acetate and traces of peptone. Pazonyi does not state the cell population density in the sporulation medium, but his statements indicate that it was equivalent to that finally reached in his growth medium. Four or five days were required in order to obtain good sporulation and the yields were variable (50-90%). The method used in the present study has certain differences from Pazonyi's. (1) The ingredients of the presporulation (growth) medium are known. (2) The sporulation medium contained a constant number of cells (10 million per ml.) and only one carbon source at a time. (3) Certain vitamins, minerals and trace elements were included

and the peptone was omitted.

The present method is considered to be an improvement over Pazonyi's because more consistent spore yields were obtained (75-80%) and spore formation was more rapid. Probably the spore yields were more constant because the cell population density and nutritive conditions did not vary. The presence of vitamins, which were found to increase spore yields, possibly contributed to the high uniform yields obtained. With reference to studies such as the present, it is advantageous to have results that are as reproducible as possible. Furthermore, an important goal of this work was to investigate factors leading to the cessation of equational division and the beginning of reduction division. By keeping the ingredients of the growth and sporulation media similar there were fewer variables to consider when cells are transferred from one environment to the other.

For certain types of biochemical and physiological studies it would be desirable to obtain populations composed entirely of sporulated cells. No one has reported a sporulation medium or method which would give this result. However, a phenomenon observed using the present sporulation technique may lead to the achievement of this goal. It was found that after three or four days in the aerated sporulation medium, a layer of cells could be noted adhering to the bottom and sides of the flask and did not come free even when the flask was vigorously shaken. When this layer was examined, it was found to be composed almost entirely

of sporulated cells. Presumably the ascus walls contained some substance which caused them to stick to the walls of the vessel and to each other. The phenomenon was not investigated further but it is suggested that additional study may lead to a useful method of obtaining large quantities of spores entirely free from non-sporulated cells.

### Cytological Studies

The cytoplasm of vegetative cells of Saccharomyces cerevisiae contained a nucleus whose division occurred by constriction without any obvious mitotic configurations. No evidence of nuclear material in the vacuole was ever found and the observations of the present work therefore do not support the Lindegren concept of the yeast nucleus.

The usual site for nuclear division during budding was in the isthmus joining the mother cell with the bud. Nuclear division at this location in budding yeast cells has been often observed, but a new feature described in the present work is the tendency of the dividing nuclear material to advance into the bud as paired bodies in two rows parallel with the isthmus of the bud. Two drawings by Lietz showing the nuclear division of vegetative cells of Zygosaccharomyces priorianus (now included in Saccharomyces by Lodder and Kreger-Van Rij) (Plate 4, drawings e and g) are suggestive of this type of division but he did not state that he had seen such paired bodies. Ganesan shows a single very small body entering the bud ahead of the

the dividing nucleus but he regards this as a centrosome and makes no mention of paired bodies entering the bud. However, his Figure 15, Plate II does suggest paired masses following behind the "centrosome". No body corresponding to Ganesan's centrosome was observed in this study.

It is possible to interpret the rows of paired bodies in two ways. Either each densely-staining body is a chromosome or each row is a chromosome with densely-stained regions along its length. If the former suggestion is correct, then the configurations observed do not conform to the standard mitotic anaphase. If the second is true, then the configurations seen could be considered as a normal mitotic anaphase.

The observations on the nuclear division in sporulating cells support the view that the nuclear material during division is organized into rows of three bodies. Each haploid nucleus seems to be formed from a single row of three densely-staining masses.

The most recent and most detailed description of the reduction division has been given by Ganesan. He states that the nucleus of the sporulating cell passes through the normal stages of meiotic division but mentions that it is difficult to observe the details of the prophase of this division. In the main the present observations are in agreement with Ganesan's but there are certain points of difference. It was found that the nucleus as seen at the beginning of reduction division became

larger and less distinct than indicated by Ganesan in what he considered to be early prophase. Nothing corresponding to Ganesan's metaphase was found. During the first and second reduction division the separating nuclear material was seen to consist of rows of three bodies and each row coalesced to form a nucleus. Ganesan considered that the haploid number of chromosomes was four but these bodies and their manner of separation were not clearly illustrated.

It should be pointed out that in meiosis, as it occurs in higher organisms, the chromatin in a pre-meiotic nucleus is in a diffuse thread-like state and during meiotic prophase a gradual condensation of the chromatinic material occurs until distinct chromosomes appear. With yeast, on the contrary, the vegetative nucleus appears compact and then becomes more diffuse in the early stages of reduction division before definite chromosome-like bodies appear. For this reason, and the failure to see clearly recognizable meiotic configurations, one should be very cautious about applying the term "meiosis" in the usually understood sense to the type of reduction division observed in yeast.

#### Correlation of Physiological and Cytological Studies

Profound changes occur in the cytoplasm of the yeast cell during sporulation and one might expect that these would be accompanied by changes in respiratory and fermentative abilities. However, the measurements made during this investigation gave no



indication that sporulating cells (i.e. up to 19 hours in sporulation medium) differed from vegetative cells in their ability to consume glucose, galactose, maltose, dihydroxyacetone or acetate. There appeared to be no difference in the Respiratory Quotient values obtained from either cells from growth medium or sporulation medium when glucose was the substrate added to the reaction flasks. This is in apparent contradiction to the findings of Miller et al. (1959), who observed that the R. Q. in sporulating cells gradually declined from that of vegetative cells. However, these workers used much lower concentrations of glucose (0.06%) in their reaction flasks and cells supplied with small amounts of glucose may show this effect more so than cells supplied with an excess. Aldous, Fisher and Stern have shown that fermentation was reduced relatively more than aerobic respiration by lowering the concentrations of glucose.

In experiments using glucose as a substrate, the rate of oxygen uptake and carbon dioxide production of cells 75-80% of which had sporulated, was less than that of vegetative cells but was nevertheless high. This would lead to the conclusion that the ascospores themselves can respire actively. Miller et al. (1959) separated, to a large degree, the sporulated cells from the non-sporulated cells in a three day old sporulation culture. They found no evidence of difference in the respiration between the two types of cells and the oxygen uptake was about half the average observed with growing cells. However,

their sporulation medium consisted only of buffer and acetate without vitamins and mineral salts. The present work approached the problem in a different manner, comparing the respiration of a highly sporulated (e.g. 40 hr.) culture with that of fresh vegetative cells. There was some lessening of oxygen uptake and carbon dioxide production but this may have been partly owing to autolysis of some of the non-sporulated cells. Further evidence to indicate that sporulation does not greatly interfere with respiratory ability can be obtained from the observation that gas exchange rates of cells from phthalate buffer without a carbon source, where no sporulation occurred, was approximately the same as that of sporulated cells. The fact that both the present investigation and that of Miller et al. (1959), although using somewhat different approaches and different sporulation media, came to similar conclusions is good evidence that with this strain of yeast, sporulated cells are capable of a rate of respiration not much below that of vegetative cells.

This is in contrast to the results obtained by workers studying bacterial spores, the respiration of which is virtually negligible (Spencer and Powell, Foster). Bacterial spores have been found to contain a substance, dipicolinic acid, that is not present in the vegetative bacterial cells (Powell). This substance has been thought to act as a binding agent for particular enzymes in bacterial spores and to account for their very low respiration (Foster). The attempts to detect dipicolinic

acid in yeast spores were not successful. From the sensitivity of the chromatographic spot test employed, as determined with known amounts of dipicolinic acid, it can be calculated that if dipicolinic acid does occur in yeast spores, it comprises less than 0.01% of the dry weight. This would be much less, about one-thousandth, of that shown to occur in bacterial spores. It is suggested that this accounts for the greater respiratory activity observed with yeast spores.

Although sporulating cells did not appear to differ from vegetative cells in their ability to utilize external substrates, the endogenous respiration of cells taken from sporulation medium at successive intervals gave evidence of an increase of over that observed with vegetative cells. (Incidentally, a marked increase in the rate of endogenous respiration was observed by Tinelli in Bacillus megatherium as the cells entered the prespore stage). With acetate sporulation medium this was not noticed after five hours but was visible after ten hours, whereas in dihydroxyacetone sporulation medium it did not become evident until the cells had been in the medium for at least twenty hours. This could be interpreted to mean that as a result of exposure of the cells to the carbon source in the sporulation medium some internal substrate has become available for respiration that the vegetative cells lacked. The three most likely such substances would be trehalose, glycogen and fat. Cells in acetate and dihydroxyacetone sporulation

media were found to accumulate both glycogen and fat. An increase in the glycogen and fat content was observed earlier in cells from acetate sporulation medium than in cells from dihydroxyacetone sporulation medium. Since the increase in the rate of endogenous respiration was also observed earlier in cells from the former medium it can be suggested that the additional amounts of one or both of these substances in the cell contribute to the increase observed in the rates of endogenous respiration. The Respiratory Quotients obtained from the endogenously respiring cells are not considered to be sufficiently reliable because of inaccuracies in measurement to be used as an indication of the type of internal substrate, whether carbohydrate or fat, respired by the cells. The impression was gained that fat is consumed very slowly since the fat content of cells after five days in buffer without a carbon source did not greatly diminish below the amount initially present. Therefore, it is more probable that the main substrate that promotes the increase in endogenous respiration is glycogen. This is in agreement with the finding that the endogenous respiration rates in starved yeasts depends on their glycogen reserves (Trevelyan). No tests were made for the presence of trehalose in the present studies and it may be possible that the utilization of this carbohydrate contributes to the endogenous respiration rate.

During the formation of spore walls nearly all of the glycogen

and most of the fat seemed to disappear from the cells. It is doubtful whether respiration of these substrates would account entirely for their rapid decrease. A small globule of the fat and occasionally a patch of glycogen was visible in the spores but the remainder of the fat and the glycogen may have become associated with the newly formed spore walls in such a form as to be no longer stainable. No chemical study has ever been made of the composition of the yeast spore wall.

The nuclei of cells from growth medium did not show any of the phases of reduction division. There were two important constituents of growth medium that were lacking in the sporulation medium, namely, glucose and ammonium sulphate. The present experiments have shown that 1% glucose in a medium otherwise suitable for sporulation causes the nuclei to remain in the state characteristic of vegetative cells. The presence of as little as 0.1% ammonium sulphate in an acetate sporulation medium also prevents reduction division although the nuclei in many of the cells achieve the diffuse state. The presence of glucose and ammonium sulphate in growth medium can therefore account for the lack of reduction division and hence of sporulation in this environment. On the other hand, spores commonly appear in old cultures when the concentration of glucose and ammonium sulphate has been greatly reduced by metabolism and growth.

A further observation was that cells suspended in buffer alone or in buffer plus vitamins, minerals and trace elements did not show any of the phases of reduction division. When a carbon source, such as acetate was added in an appropriate concentration reduction division did occur. It is not known whether the carbon source provides energy or is used in synthesis of spore materials or both. An investigation of this point might yield information of importance in understanding the factors influencing reduction division of yeast.

## SUMMARY

1. A method has been developed for sporulation of yeast cells in 100 ml. volumes of liquid culture containing a large cell population density (10 million per ml.). Consistently high spore yields (75-80%) were obtained. The method is recommended for studies on the respiration or chemical composition of sporulating yeast.
2. The nucleus of the vegetative cell was found to be a compact or sometimes reticulate mass. During vegetative nuclear division two parallel rows of three densely-staining bodies entered the bud and two similar rows remained in the mother cell.
3. During sporulation the nucleus first became very diffuse and difficult to stain. Chromosome-like bodies then appeared and the first and second nuclear divisions occurred. Each of the four haploid nuclei seemed to be formed from the condensation of a single row of three densely-staining bodies similar to those observed during budding.
4. The cells from growth medium contained some fat but no visible glycogen. In the sporulation medium both these substances became much more abundant in the cells, but during the formation of spore walls nearly all of the glycogen and most of the fat seemed to disappear.
5. The oxygen uptake and carbon dioxide production of sporulating cells utilizing glucose, acetate or dihydroxyacetone in

respirometer reaction flasks resembled that of vegetative cells. The only distinct difference between the respiratory activity of growing and sporulating cells was a rise in endogenous respiration noted in the latter.

6. Spores respired glucose, acetate and dihydroxyacetone somewhat less readily than did vegetative cells, but a strong respiration nevertheless did occur. This behaviour is in contrast to that observed by bacteriologists with bacterial spores which have negligible respiration. Dipicolinic acid is considered largely responsible for the low respiratory ability of bacterial spores. This substance could not be detected in yeast spores by the method employed and its absence may account for the relatively high respiration of yeast spores as compared to bacterial spores.



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PLATE I

Numbers 1 - 10 Vegetative cells, fixed in acetic  
acid alcohol, HCl Giemsa

Numbers 11 - 13 5 hour sporulating cells, fixed in  
acetic acid alcohol, HCl Giemsa

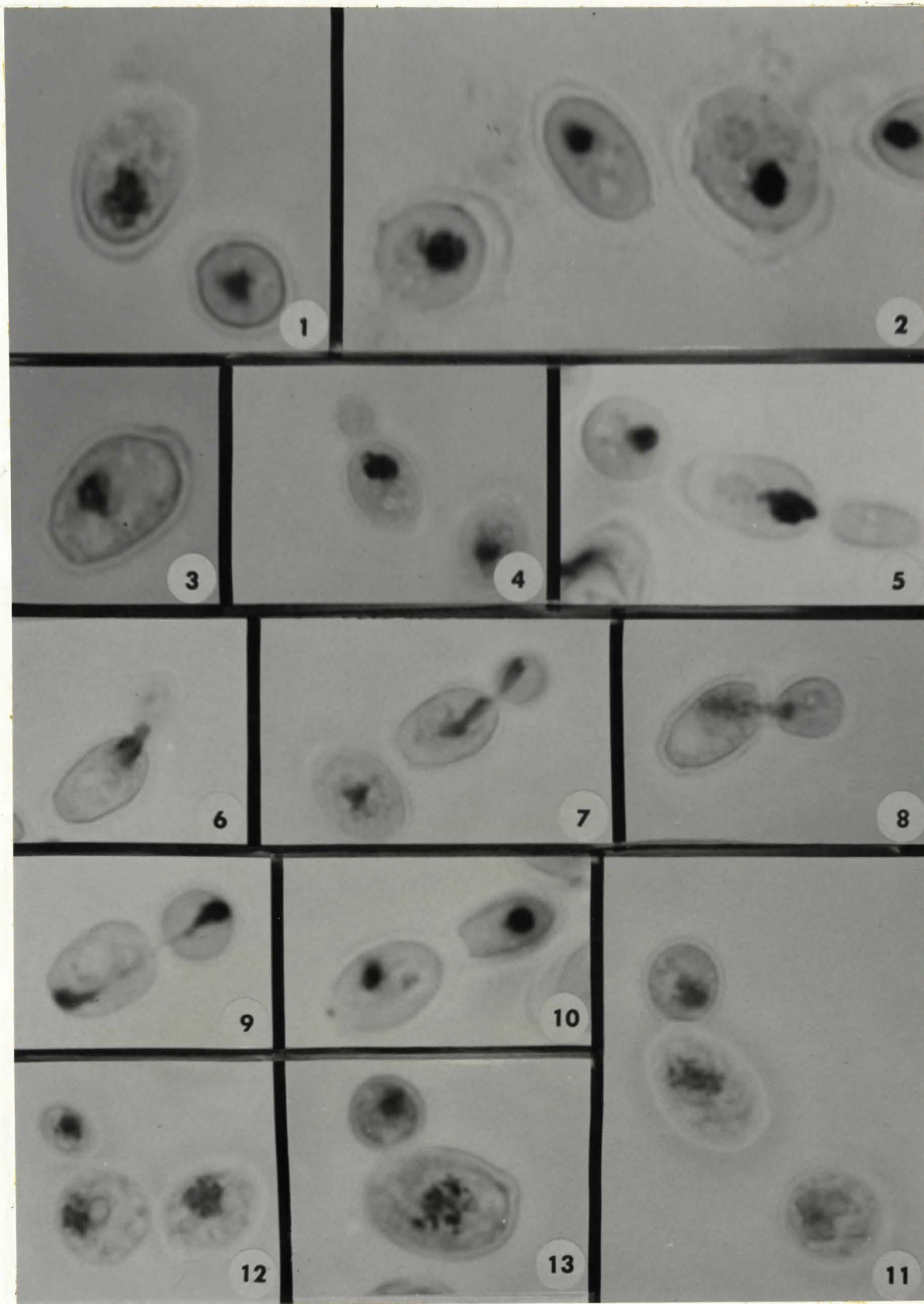


PLATE II

- Numbers 14 - 15 10 hour sporulating cells, fixed  
in acetic acid alcohol, HCl Giemsa
- Numbers 16 - 17 10 hour sporulating cells, fixed  
in acetic acid alcohol, HCl Giemsa
- Numbers 18 - 23 15 - 20 hour sporulating cells,  
fixed in acetic acid alcohol,  
HCl Giemsa, squashed preparations



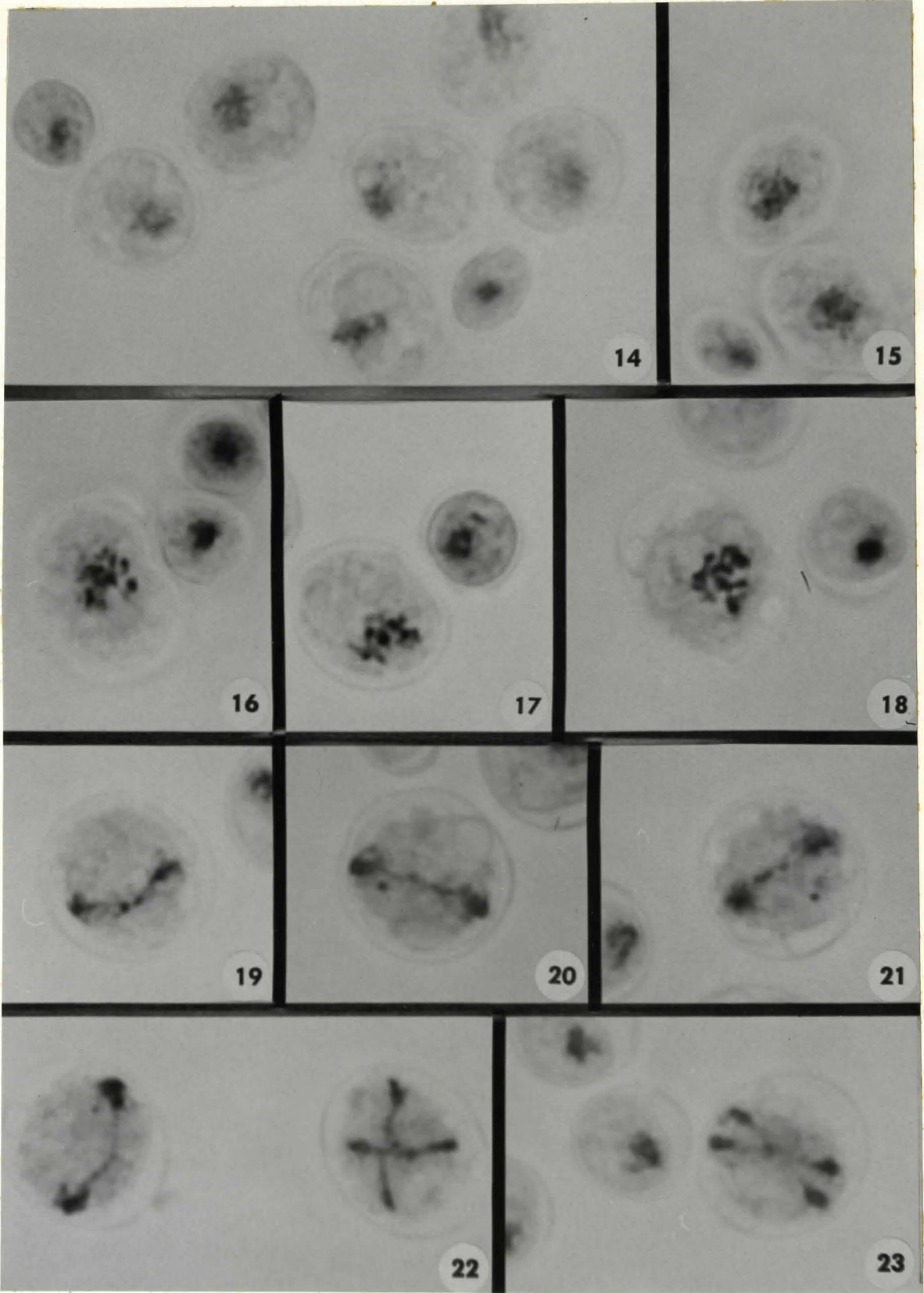


PLATE III

- Numbers 24 - 27 15 - 20 hour sporulating cells,  
fixed in acetic acid alcohol,  
HCl Giemsa
- Numbers 28 - 34 20 hour plus spores, fixed in  
acetic acid alcohol, HCl Giemsa.

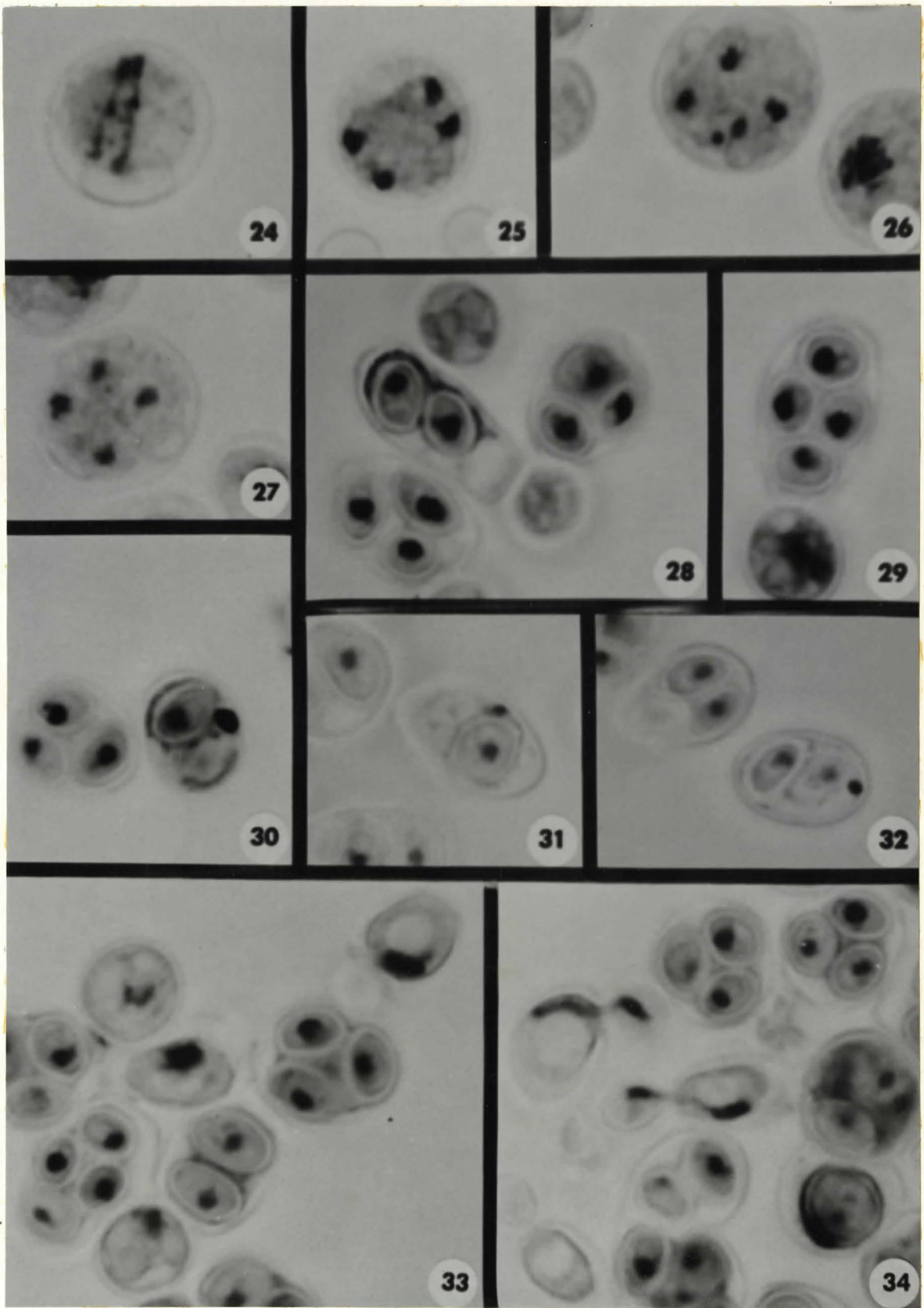


PLATE IV

- Number 35 5 hour sporulating cells, unfixed,  
mounted in Lugol's Iodine
- Numbers 36 - 37 10 - 15 hour sporulating cells,  
fixed in Helly's fixative, mounted  
in Lugol's Iodine
- Number 38 20 hour sporulating cells from  
dihydroxyacetone and ammonium  
sulphate sporulation medium, Helly's  
fixative, mounted in Lugol's Iodine
- Number 39 Vegetative cells, Helly's fixative,  
stained with Sudan Black B
- Number 40 5 hour sporulating cells, Helly's  
fixative, stained with Sudan Black B
- Numbers 41 - 42 10 hour sporulating cells, Helly's  
fixative, stained with Sudan Black B
- Numbers 43 - 45 15 - 20 hour sporulating cells,  
Helly's fixative, stained with  
Sudan Black B

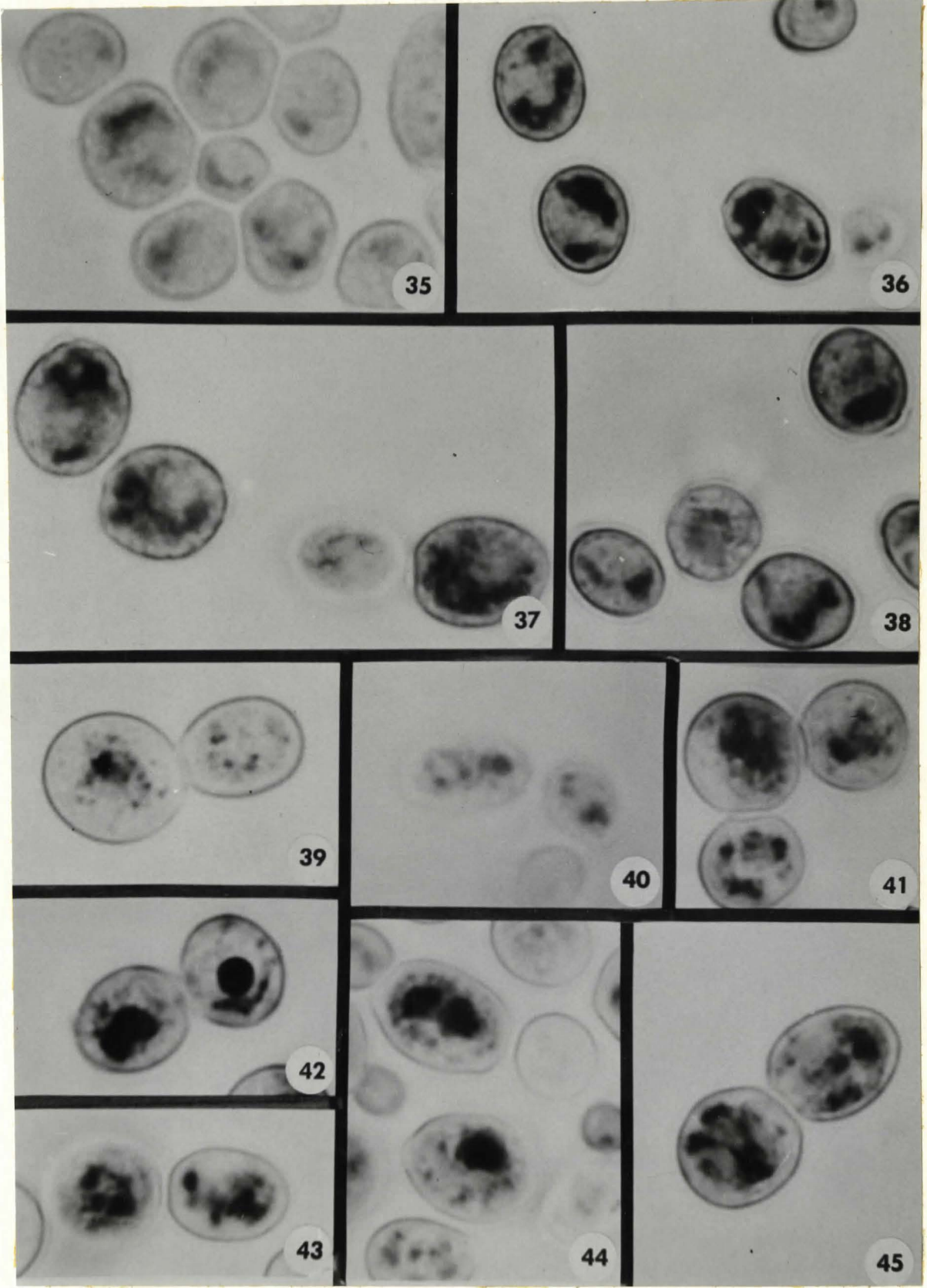


PLATE V

- Number 46 15 - 20 hour sporulating cells,  
Helly's fixative, stained with Sudan  
Black B
- Number 47 Cells after three days in buffer,  
Helly's fixative, stained with Sudan  
Black B
- Number 48 Spores from 20 hour sporulation  
culture, Helly's fixative, stained  
with Sudan Black B
- Number 49 10 hour sporulating cells, Helly's  
fixative, Janus Green
- Numbers 50 - 51 20 hour sporulating cells, Helly's  
fixative, Janus Green

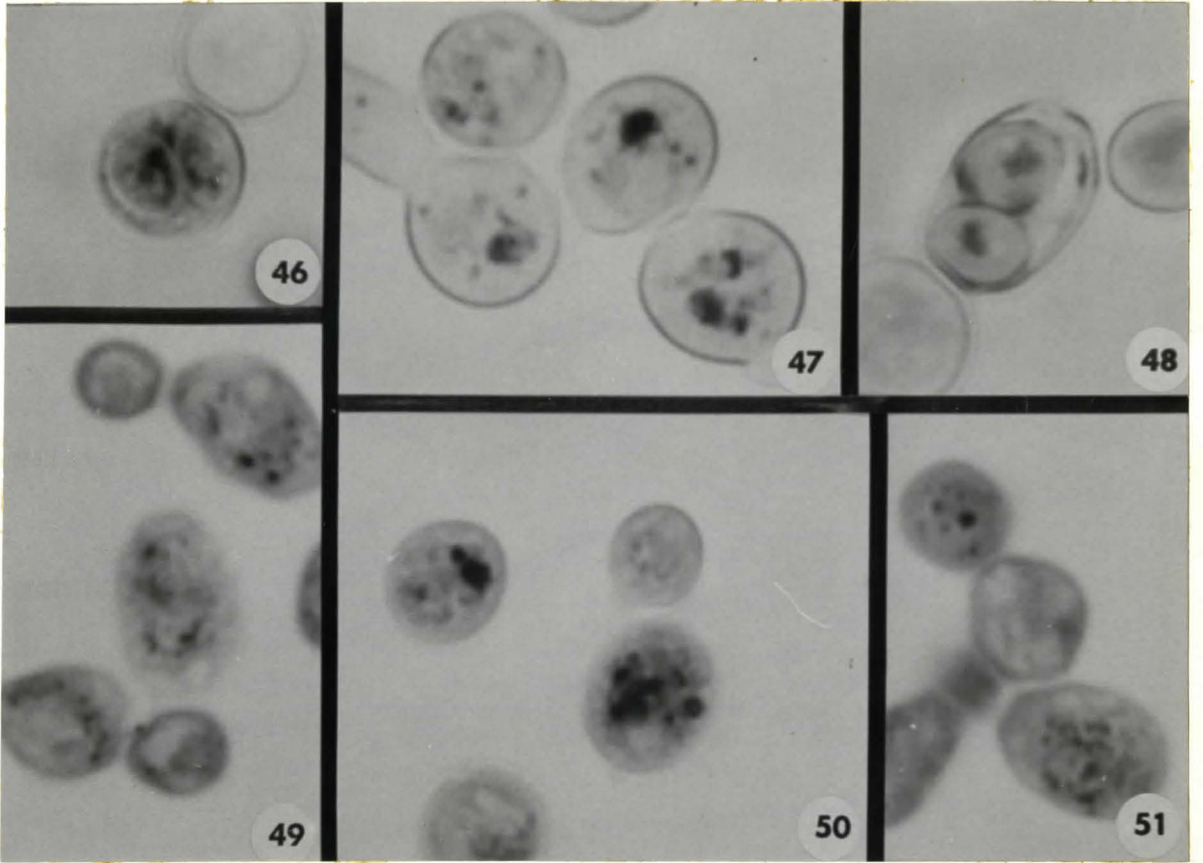


TABLE I

Sporulation obtained with various combinations  
of substances in the sporulation medium

Carbon Source	Vitamins	Minerals and trace elements	Percent sporulation after 3 or 4 days
<hr/>			
Sodium acetate			
0.1%	+	+	5 <sup>*</sup> , 5, 0
0.5%	+	+	56, 68, 65
1.0%	+	+	78, 75, 80
2.0%	+	+	40, 45
<hr/>			
Dihydroxy-acetone			
0.1%	+	+	0
0.5%	+	+	60, 62
1.0%	+	+	60, 65
2.0%	+	+	30
<hr/>			
1% acetate			
" "	-	+	42, 40
" "	+	-	78, 75
" "	-	-	45
None	+	+	0
<hr/>			

\* Each figure is the result of an individual experiment



TABLE II  
 GAS EXCHANGE RATES OF CELLS FROM GROWTH MEDIUM AND ACETATE  
 SPORULATION MEDIUM WITH GLUCOSE AS SUBSTRATE

	Hours in acetate sporulation medium								
	0	5	10	15	19	42	43	117	139
CO <sub>2</sub> produc- tion, $\mu$ l. per hr.	240(1)*	268(11)	295(12)	295(16)	176(21)	117(30)	111(31)	100(38)	142(39)
	213(2)	268(13)	104(14)	142(17)	154(22)				
	213(3)		330(15)		162(23)				
	370(4)								
O <sub>2</sub> uptake, $\mu$ l. per hr.	142(1)	162(11)	82(14)	162(16)	117(21)	70(30)	64(31)	46(38)	55(39)
	148(2)	154(13)	196(15)	92(17)	82(22)				
	92(3)				132(23)				

\* Numbers in brackets refer to the Figure (graph) from which this value was obtained.

TABLE III  
 GAS EXCHANGE RATES OF CELLS FROM GROWTH MEDIUM AND DIHYDROXY-  
 ACETONE SPORULATION MEDIUM WITH GLUCOSE AS SUBSTRATE

O#	Hours in dihydroxyacetone sporulation medium					
	21	43	44	45	141	142
CO <sub>2</sub> pro- duction μl. per hr.	183(25)*	213(36)	142(34)	220(35)	154(42)	132(40)
	295(26)					
	295(27)					
O <sub>2</sub> uptake μl. per hr.	86(25)	132(36)	64(34)	104(35)	80(42)	88(40)
	157(26)					
	157(27)					

# See Table II

\*Numbers in brackets refer to the Figure (graph) from which this value was obtained.

TABLE IV  
RESPIRATORY QUOTIENTS OF YEAST CELLS FROM GROWTH AND  
SPORULATION MEDIA

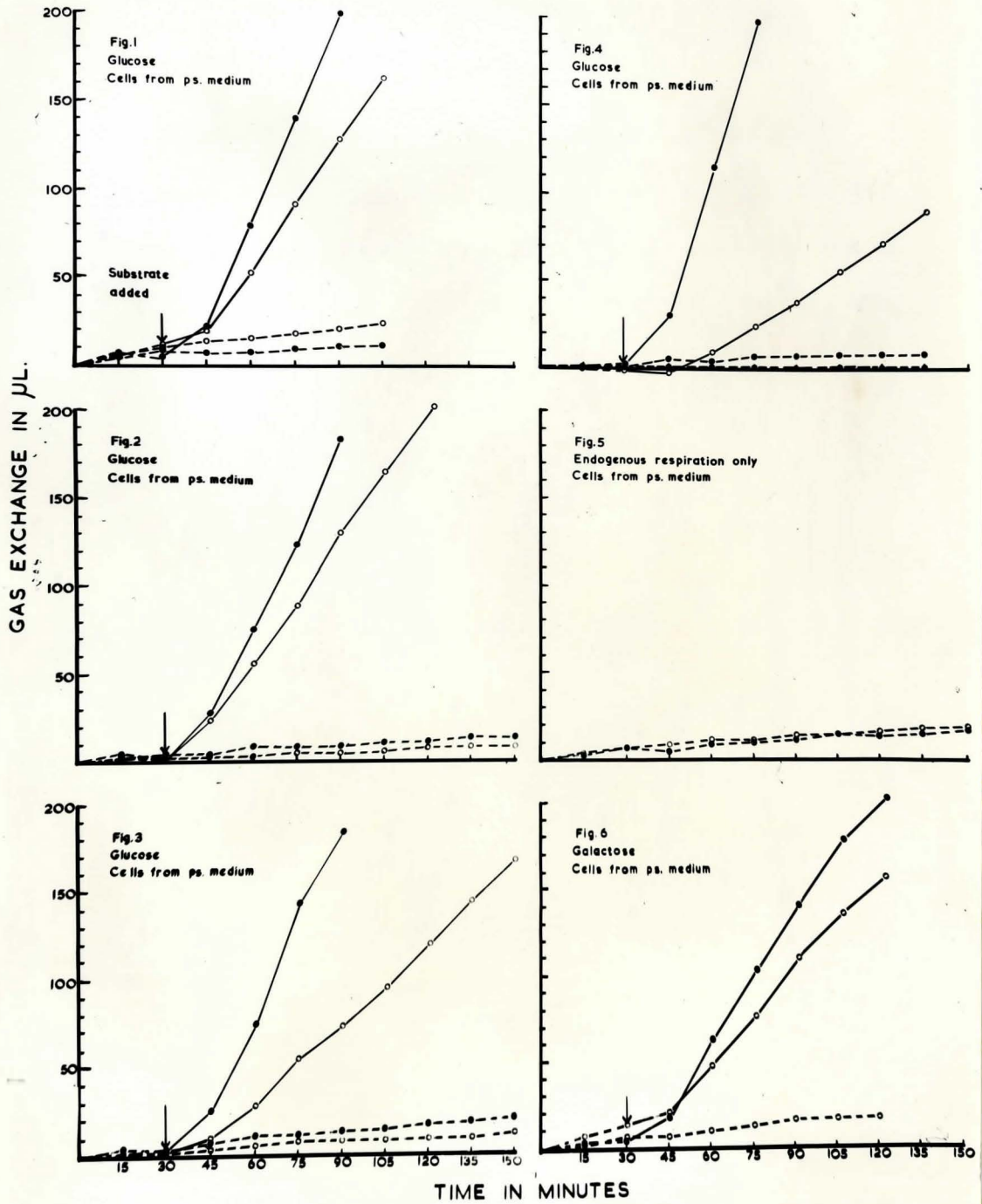
Sporulation substrate	Substrate added to reaction flask	Hours in sporulation medium						
		0 <sup>*</sup>	5	10	15	18-21	40-45	100+
NONE	None#	0.83				0.66	1.7	
	Glucose					1.6	1.4	2.0
SODIUM ACETATE	None#	1.3, 0.54	1.0, 1.2	0.92, 0.95 1.1	0.85, 0.98	0.92, 1.0, 0.62	0.91, 1.6, 0.71	0.76, 0.93
	Glucose	5.2, 1.7	1.7, 1.7	1.3, 1.7, 2.0	1.8, 1.5	1.5, 1.9, 1.2	1.7, 1.7	2.2, 2.6
	Acetate	0.98				0.96	0.97	
1% DHA.	None#	1.7				1.4, 1.1	1.0, 1.2	0.89
	Glucose	1.4				1.9, 1.9	1.6, 2.1	1.9
0.5% DHA.	None#	1.5, 1.7				1.4, 1.6	1.2, 1.1	1.1, 0.82
	Glucose	2.3				2.1	2.2	1.5
	DHA.	1.1				1.1	1.0	0.97

DHA. - Dihydroxyacetone  
# = Cells respiring endogenous substrate only  
<sup>\*</sup> = Cells directly from presporulation medium

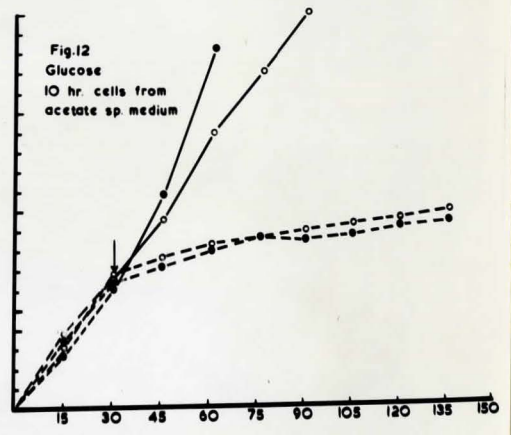
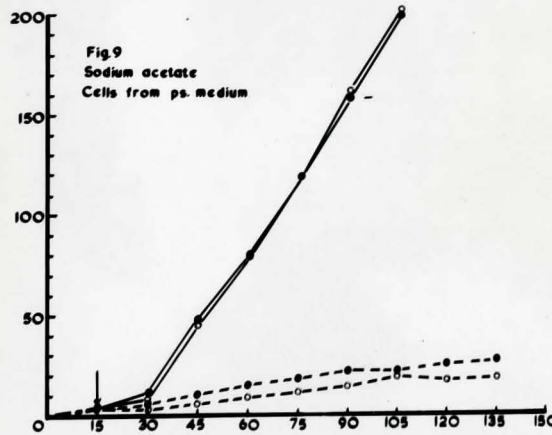
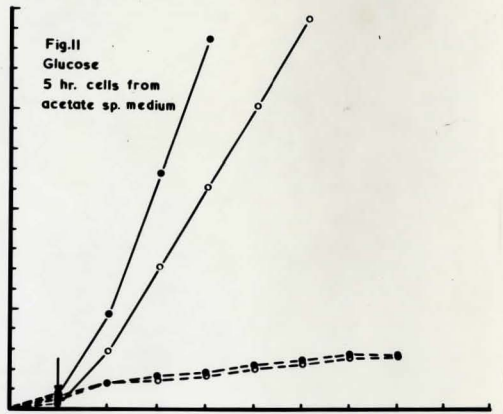
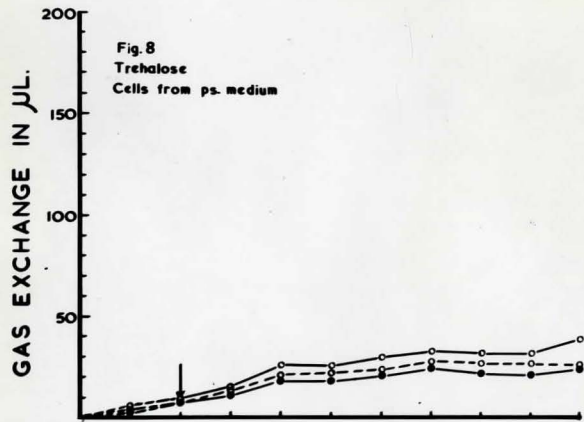
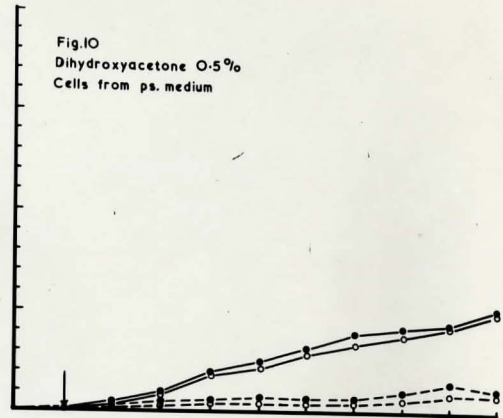
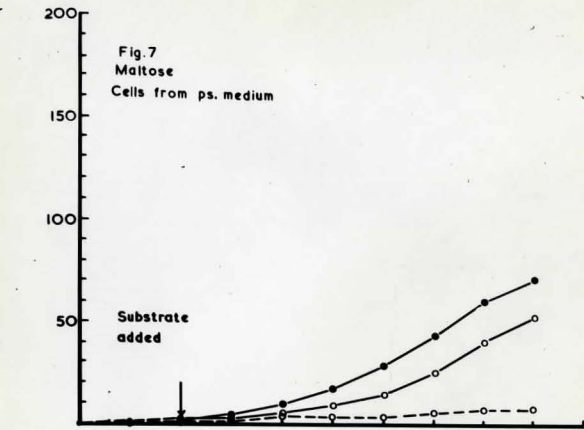
EXPLANATION OF FIGS. 1 - 43

Substance mentioned in upper left hand corner of each figure is the substrate added to the reaction flask at the time indicated by the arrow.

- ps.           - presporulation medium (vegetative cells)
- sp.           - sporulation medium
- - carbon dioxide production
- - oxygen uptake
- - carbon dioxide production of cells  
                  respiring a substrate
- - oxygen uptake of cells respiring a  
                  substrate
- — — ●     - carbon dioxide production of endog-  
                  enously respiring cells
- — — ○     - oxygen uptake of endogenously respir-  
                  ing cells

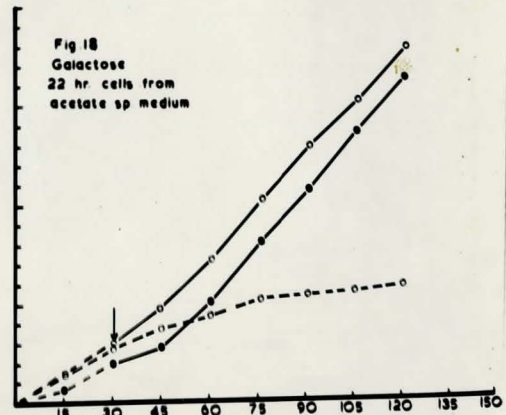
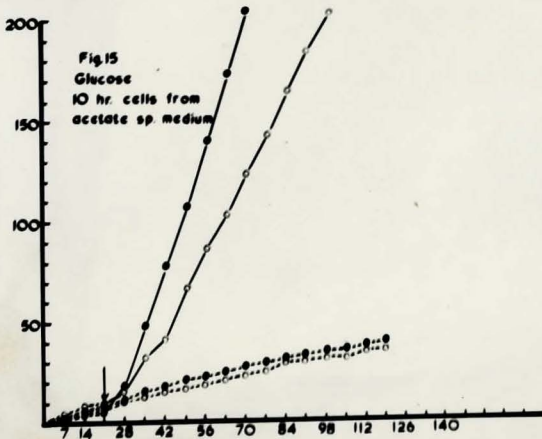
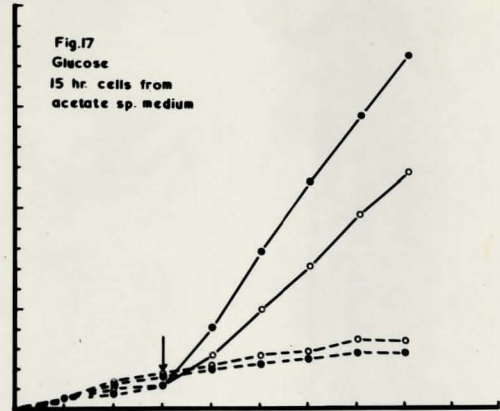
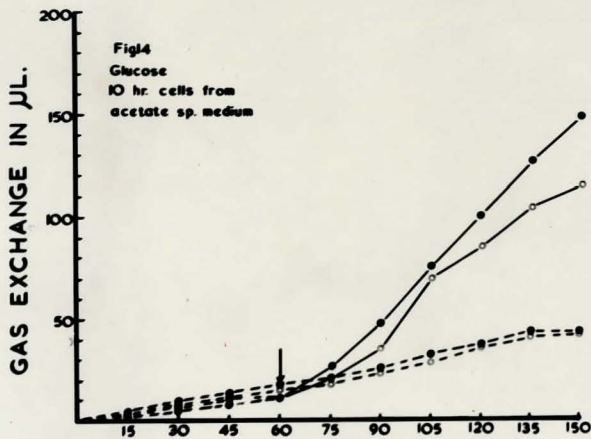
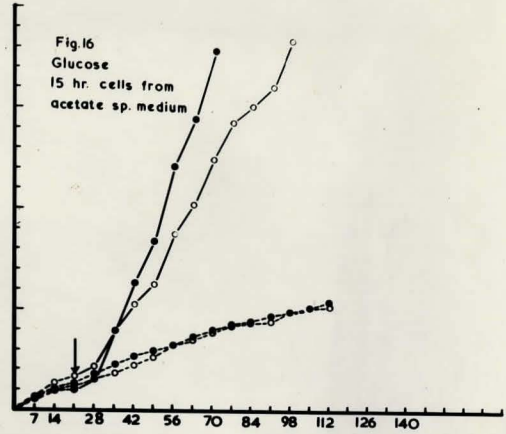
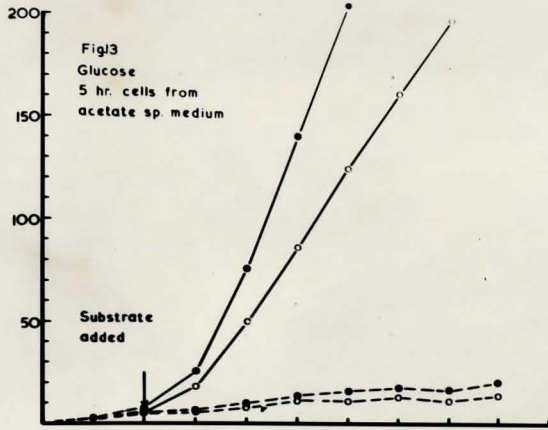


Figs. 1 - 6

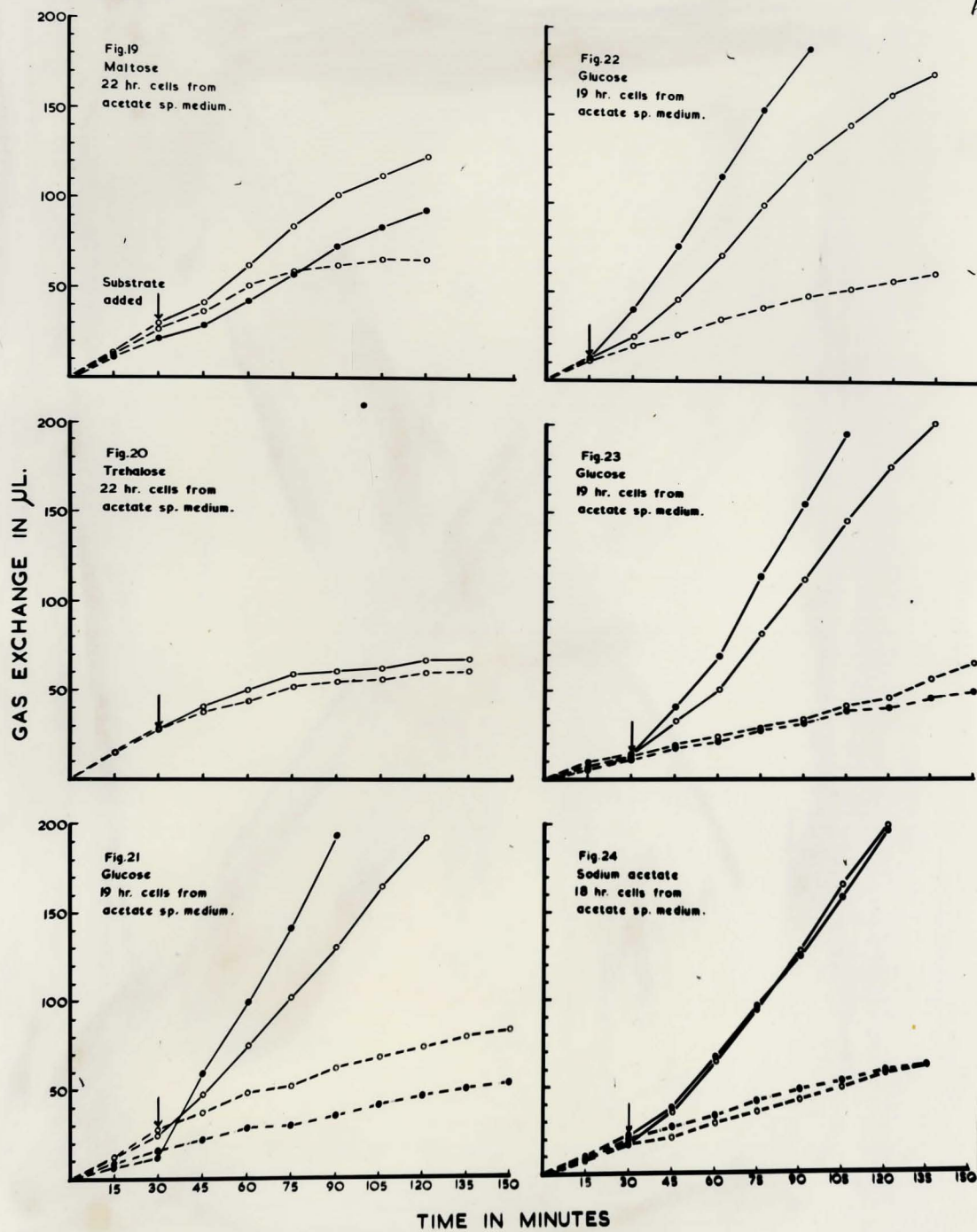


TIME IN MINUTES

Figs. 7 - 12

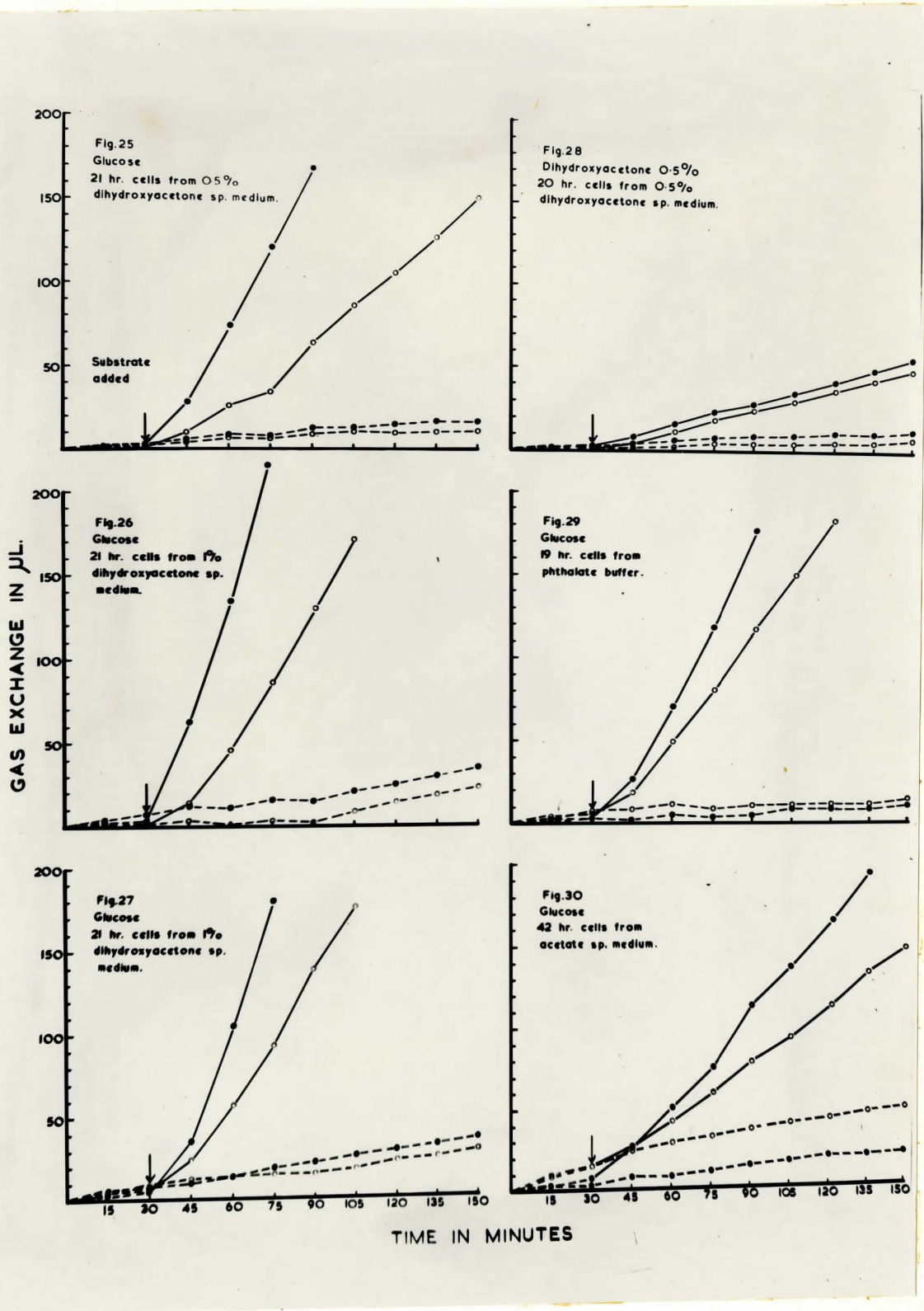


TIME IN MINUTES

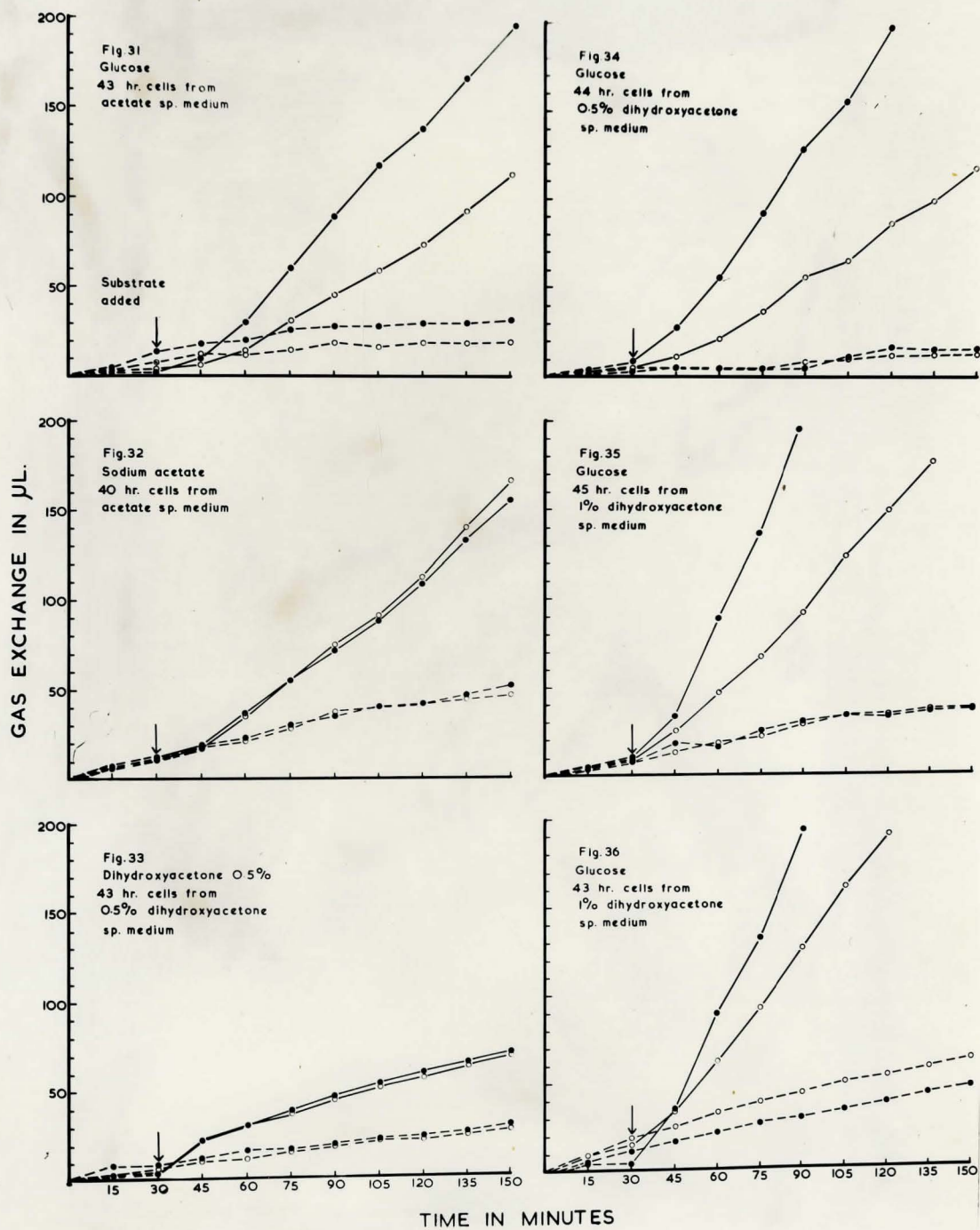


Figs. 19 - 24

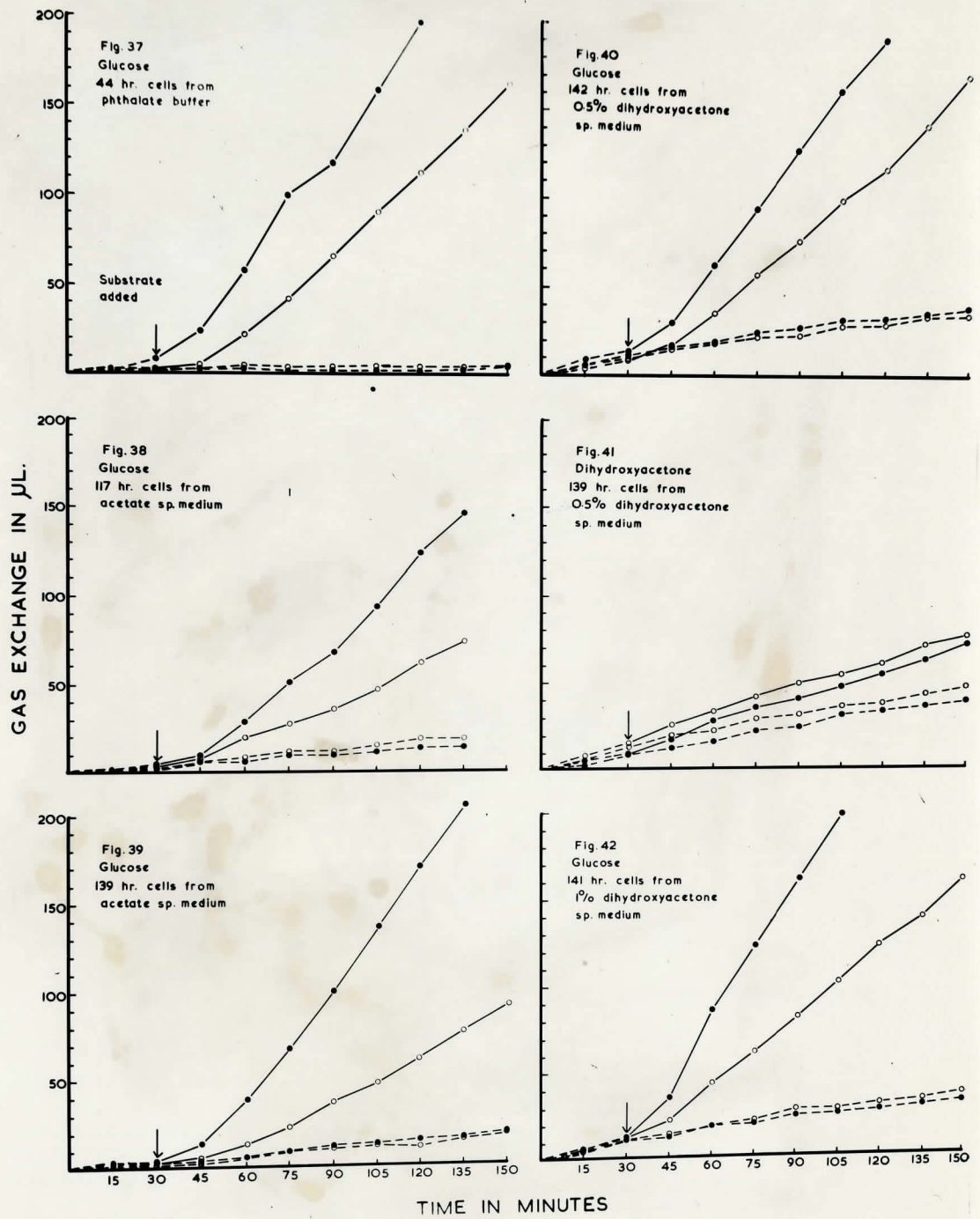




Figs. 25 - 30



Figs. 31 - 36



Figs. 37 - 42

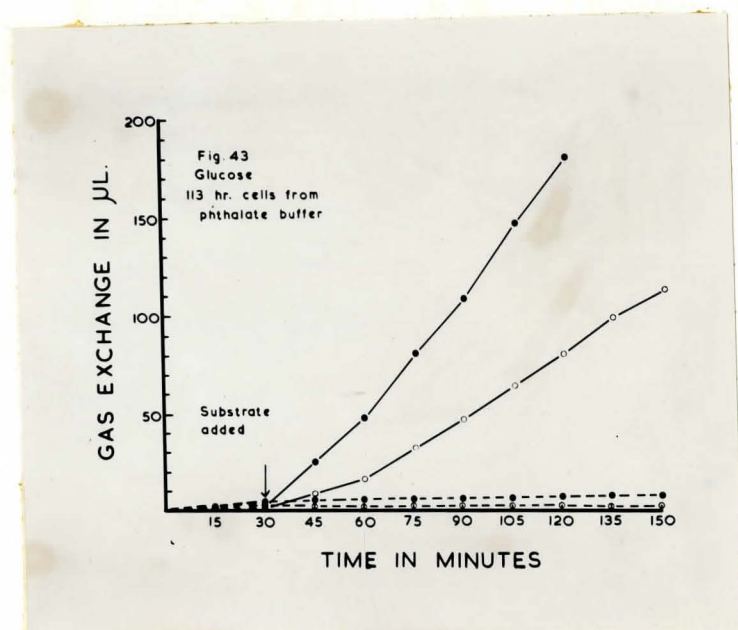


Fig. 43