

OXIDATIVE METABOLISM AND ABSORPTION SPECTRA OF ANAEROBICALLY GROWN YEAST

MANOMETRIC DATA AND ABSOLUTE ABSORPTION SPECTRA

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TWO FIGURES

Ever since Pasteur's work (1876) it has generally been believed that without oxygen yeast can either grow only to a limited extent, or not at all (Brown, '05; Windisch, '32; White and Munns, '51). The slight growth that was obtained occasionally under anaerobiosis was attributed to the traces of oxygen left in the medium. The careful work of Stier and his collaborators (Stier, Scalf and Brockman, '50; Stier, Scalf and Peter, '50; Andreasen and Stier, '53, '54, '56) finally clarified this problem. They showed (1) that under strict anaerobiosis yeast cells stopped growing entirely when carried for several transfers in a synthetic medium containing all the previously known growth factors; (2) that a culture medium containing vegetable oils (e.g., 0.3% wheat germ oil) and high concentrations (6-7%) of yeast extract permitted anaerobic growth to reach the same final cell density, about 400×10^6 cells/ml, as that reached in aerobic cultures; and (3) finally they were able to identify two new growth factors, required for anaerobic growth only, ergosterol and long-chain unsaturated fatty acids (like oleic acid). In a synthetic medium supplemented with minute amounts of these substances, final cell densities of ca. 200×10^6 cells/ml were ob-

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served under anaerobiosis. The fact that this is only about half of the maximum value that can be obtained in the high-yeast extract medium was interpreted by Andreasen and Stier ('56) as indicating a requirement for a third anaerobic growth factor, as yet unidentified.²

These findings made it now possible to investigate the metabolism and enzymatic composition of yeast cells that were grown anaerobically in cultures permitting rapid and continuous growth, and therefore could validly be compared with that of aerobically grown cells. The fact that the metabolism of yeast cells can vary to a great extent depending on the culture conditions, particularly on the presence or absence of oxygen, was first indicated by the work of Meyerhof ('25), then by Warburg ('27), Trautwein and Wassermann ('30), as well as numerous other workers since. The connection between cytochrome composition and respiratory rate was shown by Euler, Fink, and Hellström ('27), Fink ('32), and Fink and Berwald ('33), who found the three α bands of cytochromes a , b , and c , in baker's yeast, but only two α bands, attributed later to cytochromes a_1 and b_1 , in brewer's yeast, and maintained that the difference between the two kinds of yeast was caused by the availability of oxygen during growth. This view was supported by their finding that a culture with one type of cytochrome spectrum could be converted into the other by growing it with or without aeration. Borei and Sjöden ('43) measured the extracted cytochrome c in yeast cells grown under various conditions of aeration and found that the amount of cytochrome c was proportional to the degree of aeration during growth.

In none of these studies were the cells grown under strict anaerobiosis, which accounts for finding some cytochrome c in all cultures, neither was the possibility excluded that during growth the composition of the population changed through

² Harris ('56) showed that the continuous anaerobic cultivation of yeast in the presence of ergosterol and oleic acid was possible for as many as 173 generations, and that the frequency of respiratory-deficient mutants did not increase in the absence of oxygen (Harris, M.; J. Cell. and Comp. Physiol., 48: 95-112).

mutation and selection. These objections were met by the studies of Ephrussi and Slonimski ('50), who used adequate techniques to ensure anaerobiosis during the growth of the cultures, and observed the transition from the anaerobic to aerobic cytochrome spectrum under non-growing conditions. Their spectroscopic results confirmed previous observations on brewer's yeast, and established the presence of two pigments in anaerobically grown yeast, called provisionally cytochromes a_1 and b_1 , in contrast to aerobically grown yeast which is known to have cytochromes a , a_3 , b , and c (review by Smith, '54), as well as cytochrome c_1 (Lindenmayer and Estabrook, '58). Ephrussi and Slonimski ('50), and Slonimski ('53, '55) have also studied the anaerobically grown yeast cells with regard to their oxygen uptake, as well as the activities of several enzymes involved in their oxidative metabolism, and found in these cells very low oxidase and dehydrogenase activities, except for the malic, isocitric and alcohol dehydrogenases.

In the present work use was made of two methods that have not been previously applied to the question of oxidative metabolism and the corresponding enzymatic constitution of anaerobically grown yeast. One of the techniques was the use of ergosterol and oleic acid to make rapid and continuous anaerobic growth possible. The other method, by which quantitative data were obtained about the absorption intensities of the varying pigments present in these cells was the sensitive spectrophotometry developed by Chance (cf. review by Chance, '54). In this paper the absolute absorption spectra of anaerobically, as well as of aerobically grown cells, are presented together with the manometric data on these cells, representing the rates of oxygen consumption, and of CO_2 evolution in the presence and absence of oxygen. On the basis of these results, the oxidative enzymes and the Pasteur effect of the anaerobically grown yeast are discussed. Further papers in this series will deal with the enzymatic activities and the oxidized minus reduced difference spectra of this organism.

METHODS AND MATERIALS

Organism. Cells of *Saccharomyces cerevisiae*, strain LK2G12, a diploid strain, were used, a culture of which was originally received from Dr. S. Spiegelman, University of Illinois.

Media. The basic medium was adopted from the one used in the laboratory of Dr. S. Spiegelman and had the following composition:

40	gm	glucose (Difco Bacto-Dextrose)
5	gm	Difco Bacto-Peptone
2.5	gm	Difco Yeast Extract
6	gm	(NH ₄) ₂ SO ₄
2	gm	KH ₂ PO ₄
0.34	gm	CaCl ₂ ·2H ₂ O
0.50	gm	MgSO ₄ ·7H ₂ O
6	ml	Na lactate, 60%
1000	ml	distilled water

This medium was used for aerobic cultures, while for anaerobic cultures it was supplemented the following way: (1) The glucose concentration was raised from 4% to 10%; and (2) 10 ml of an ergosterol and oleic acid stock solution was added to 1000 ml of the basic medium. The stock solution had the composition (after Andreasen and Stier, '54):

100	mg	ergosterol
22	ml	mono-oleate sulfate ester (Tween 80, Atlas Powder Co., Wilmington, Del.)
28	ml	ethyl alcohol

This mixture was boiled until the ergosterol dissolved, then made up to 50 ml again with ethyl alcohol. The stock solution must be made up fresh at weekly intervals, as ergosterol seems to decompose quite rapidly in solution.

Another growth medium was also employed in a few experiments, following Stier, Scalf, and Peter ('50). This medium consisted of:

10%	glucose
0.5%	KH ₂ PO ₄
7%	yeast extract
0.3%	wheat germ oil
0.03%	Tween 20

and will be referred to as the "high yeast extract — wheat germ oil medium."

In the latter medium the Tween 20 is added as an emulsifying agent, while in the former anaerobic medium Tween 80 serves as a source of oleic acid (Tween 20 is a mono-laurate, while Tween 80 is a mono-oleate sulfate ester, lauric acid being a saturated and oleic acid an unsaturated fatty acid).

Culture techniques. The aerobic cultures were set up by placing 70 ml medium in 250-ml Erlenmeyer flasks and shaking them on a rotary shaker at a rate of 160–180 cycles per minute at 26°C.

In the case of anaerobic cultures the main problem is the exclusion of traces of oxygen from the culture flask. The following procedure has proved satisfactory. In a one-liter suction flask 500 ml of the supplemented medium is placed, and the flask is closed at the top with a rubber stopper through which a glass tube is extended to the bottom of the flask for the gas inlet. The side outlet of the flask is connected with a rubber tube to a water trap consisting of a small stoppered bottle in which two glass tubes are inserted, one extending down to the bottom and the other a short way below the stopper. This whole assembly is autoclaved for 20 minutes at 15 lbs. pressure. After autoclaving high purity nitrogen gas is bubbled through it immediately. The purpose of placing the medium under nitrogen while still hot is to prevent oxygen from being dissolved in it. The nitrogen is passed through a cotton filter, and the connection of the nitrogen tank with the flask is through a short piece of rubber tubing that can be clamped off at the end of the gassing period. The medium is left to cool down while being bubbled with nitrogen, then the inoculum is introduced by taking the stopper out for a moment. Gassing is continued after this for 15 minutes. Then the rubber tubing at the gas inlet is clamped off, but the flask remains open for the evolution of CO₂ through the water trap. The medium is stirred during incubation (at 26°C) either with a magnetic stirrer or by placing the culture flask and the

water trap on a reciprocating shaker with a slow rate of shaking.

Inocula were obtained from two-day-old cultures in standing Erlenmeyer flasks containing 50 ml basic medium. These cultures were transferred every other day, and since they were open to the air but not shaken, the cells were of an intermediate type between aerobically and anaerobically grown ones. The inoculum size was 0.5 ml to 500 ml medium. It is important not to use too large an inoculum for the anaerobic cultures, since it is desirable to let the cells grow for at least 7 or 8 generations before harvesting in order to be sure of having cells with a true anaerobic enzyme composition.

Manometric techniques. Standard Warburg manometric procedures were followed as described by Umbreit, Burris and Stauffer ('49). CO₂ determinations were done following the direct method of Warburg. The Warburg vessels were of approximately 15 ml volume. The shaking rate was 130 cycles per minute with a 5 cm arc. The bath temperature was 26.4°C. For the measurements the vessels were set up with the following contents: 0.3 ml of cell suspension of a cell density of approximately 10⁸ cells/ml (the cells were washed twice in distilled water and were suspended in M/15 KH₂PO₄ solution), and 0.2 ml of 20% glucose solution in the side arm. After all other reagents had been added, the final reaction volume in each vessel was made up to 3.0 ml with M/15 KH₂PO₄ solution (pH 4.5), so that the final cell density was 20–40 × 10⁶ cells/ml and the final glucose concentration was 1.33% or 74 mM. The centerwell contained either 0.2 ml 30% KOH solution or the same volume of M/15 KH₂PO₄. For measuring the endogenous rates of O₂ uptake or CO₂ output, no glucose was added to the vessels and ten times as many cells were used. It usually took 40 minutes to set up an experiment, from the start of centrifugation of the yeast cultures to the tipping of the contents of the side arms. Before tipping 5 minutes temperature equilibration was allowed. The first reading was taken 10 minutes after tipping. The rate values were calculated from the readings between 10 and 40 minutes

after tipping, during which interval the rates stayed approximately constant. Cell counts were made at each experiment on the yeast suspension using a Spencer bright line haemocytometer. The results were expressed in units of μl gas/hr. \times cell.

Spectroscopic techniques. In order to determine quantitatively the light absorption of intact yeast cells in the visible spectrum region, one must use either high sensitivity spectrophotometers (i.e., with photomultipliers) or spectrophotographic methods, because of the high scattering power of a cell suspension and the low concentration of the absorbing pigments. Both of these methods were used in the course of work. During the first part of the experiments a spectrophotographic method was worked out that consisted of placing 0.8-mm thick layers of frozen yeast paste in front of the slit of a Cornu-type spectrograph, and then obtaining optical density curves of the yeast cells by comparing the density of the photographed yeast spectra at each wavelength with a series of photographed spectra of known density neutral filters. This method was described in detail previously (Lindenmayer, '55), and was similar to that reported by Euler, Hellström, and Günther ('39), and by Chaix and Fromageot ('42).

In the latter part of this work it became possible to use the sensitive spectrophotometric equipment at the Johnson Research Foundation that offered great advantages over the previous method both in higher accuracy and speed of measurement. The results presented here were all obtained with this latter procedure. A split-beam sensitive spectrophotometer was used that was devised by Yang and Legallais on the basis of previous designs by Chance (Chance, '51; Yang and Legallais, '54; Yang, '54). The yeast suspensions for these measurements were prepared by washing the cells in distilled water, making up a cell suspension of 450 Klett density, then centrifuging 20 ml of this suspension and resuspending the cells in 2 ml buffer. This final suspension has ca. 10^8 cells/ml. To obtain the absorption spectra of the cells in the reduced state, one cuvette (1 cm diameter) of the split-beam spectro-

photometer contained 2.0 ml cell suspension reduced with a few crystals of solid $\text{Na}_2\text{S}_2\text{O}_4$, while in the other cuvette several layers of oiled filter paper were placed to balance the light scattering of the yeast cells. The absorption curves were corrected to a base line that connected the portions of the curve without absorption peaks. Spectra obtained this way are referred to as the absolute absorption spectra, as distinguished from the difference spectra where both cuvettes contain cells.

RESULTS

Growth data. Anaerobic growth was observed in three different media, and the cell yield was measured in the stationary phase of each culture. The unsupplemented basic medium was used to determine the yield of aerobic growth. The results are shown in table 1.

TABLE 1
Final cell densities under various culture conditions

ATMOSPHERE		MEDIUM	FINAL CELL DENSITY
			<i>10⁸ cells/ml</i>
Aerobic	Basic		350
Anaerobic	Basic		4
Anaerobic		Basic supplemented with ergosterol and oleic acid	250
Anaerobic		High yeast extract — wheat germ oil medium	380

It is evident from these data that anaerobic growth was very limited in the basic medium. In this case the cell count was made 4 days after starting the culture, while in the other media the rapidly-growing phase was over and final cell densities were reached in less than 24 hours (the generation time for the aerobic culture was found to be 2.5 hrs. at 26°). The highest anaerobic yield was obtained in the 7% yeast extract — 0.3% wheat germ oil medium, in agreement with the findings of Andreasen and Stier ('56). Throughout this work the ergosterol-oleate supplemented basic medium was used for anaerobic cultures, rather than the high yeast extract — wheat germ oil medium, although the latter gave considerably higher yields. The reason for this is that including such

large amounts of yeast extract in the medium would raise the level of amino acids so high that they might also become a major source of carbon in competition with glucose. Such a shift in major nutrients would, in turn, influence the enzymatic composition of the cells to an extent that any comparison with the results of other studies, where conventional media were used, would be impossible. On the other hand, no defined micronutrient is yet known that would raise the anaerobic cell yield to the aerobic levels when added together with ergosterol and oleic acid.

Manometric data on aerobically grown cells. Measurements were performed at three growth stages of the aerobic cultures in the basic medium, the stages being characterized by the time elapsed from inoculation to the harvest of the cells, as well as by the cell densities. The first two measurements were within the exponential phase, and the third in the stationary phase. In each case, the rates of oxygen uptake were measured, as well as the rates of CO₂ output in air and in nitrogen atmosphere; furthermore, these rates were determined both in the presence and in the absence of glucose in the Warburg vessels. Thus both the "exogenous" and the "endogenous" metabolic rates were determined. The data obtained in the presence of exogenously supplied glucose were utilized for the calculation of the Meyerhof Quotients as well, furnishing information about the operation of the Pasteur effect in the cells. The following formula expresses a definition of the Meyerhof Quotient (Meyerhof, '21; Warburg, '26):

$$\text{M.Q.} = \frac{\text{fermentative cleavage products in N}_2 - \text{fermentative cleavage products in air}}{\text{oxygen consumption}}$$

The rate of fermentative CO₂ production will serve for our purposes as a measure for the appearance of fermentative cleavage products. The rate of fermentative CO₂ production in air ($Q_{\text{CO}_2 \text{ ferm.}}^{\text{air}}$) is obtained by subtracting the rate of oxygen uptake (Q_{O_2}) from the rate of total CO₂ production in air ($Q_{\text{CO}_2}^{\text{air}}$):

$$Q_{\text{CO}_2 \text{ ferm.}}^{\text{air}} = Q_{\text{CO}_2}^{\text{air}} - Q_{\text{O}_2}$$

assuming thereby that the Respiratory Quotient is unity under the conditions employed. Thus the Meyerhof Quotient is computed by the formula:

$$\text{M.Q.} = \frac{Q_{\text{CO}_2}^{\text{N}_2} - Q_{\text{CO}_2}^{\text{air ferm.}}}{Q_{\text{O}_2}}$$

The validity of using this quotient as an evidence for the operation of the Pasteur effect is discussed further below.

The results of these measurements are shown in table 2. The "exogenous" respiratory rate, i.e., the rate in the presence of exogenously supplied glucose, is more than doubled from the exponential to the stationary phase, while the exogenous fermentative rates, $Q_{\text{CO}_2}^{\text{air ferm.}}$ and $Q_{\text{CO}_2}^{\text{N}_2}$ decline with the age of the culture. This agrees with the findings of Slonimski ('53, p. 154) and of Ephrussi et al. ('56), who proposed explanations for this change in respiratory and fermentative activities during the development of a population.

The endogenous rate of respiration, on the other hand, shows a tenfold increase from the exponential to the stationary phase, which is very likely connected with the accumulation of endogenous reserves after rapid growth has ceased. The endogenous R.Q. remains near the value of one in cells at all stages, corresponding to the well-established fact that aerobically grown yeast cells are unable to ferment their endogenous carbohydrate reserves (Stier and Stannard, '36; Spiegelman and Nozawa, '45).

The values for the M.Q. were between 1.1 and 2.1. Since the criterion for the existence of the Pasteur effect in an organism is an M.Q. higher than $\frac{1}{3}$, all three of the cell types can be considered as having normal Pasteur effect.

Absorption spectra of aerobically grown cells. Absolute absorption spectra were obtained with a sensitive recording spectrophotometer as described under the methods. The cells were in the early stationary phase, harvested after 24 hours of growth. The first spectrum (indicated by the solid line in figure 1) was taken of the washed cell suspension after glucose was added to it (to a concentration of ca. 0.01 M), and

TABLE 2
Respiratory and fermentative rates of cells grown aerobically in basic medium, at various stages of growth

TIME AFTER INOCULATION	NUMBER OF CELLS PER ML MEDIUM	WITH EXOGENOUS GLUCOSE			WITHOUT EXOGENOUS GLUCOSE		
		$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	$Q_{CO_2}^{N_2}$	$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	$Q_{CO_2}^{N_2}$
hrs.							
16.0	3.8×10^6	0.49	5.1	4.6	—	—	—
21.5	16.3×10^6	0.50	5.5	5.0	0.07	0.05	0
40.7	333×10^6	1.15	3.5	2.4	0.56	0.52	0

Q values in units of $10^{-6} \times \mu\text{l gas/hr.} \times \text{cell.}$

the cell suspension became anaerobic. The second spectrum was obtained after adding a few crystals of a reducing agent, sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), to the previous suspension (dashed line). The difference between the two spectra should be due to the pigments that can be reduced "chemically" but not "enzymatically," i.e., which are not reduced when the cells are without oxygen, but only in contact with a strong reducing agent that can enter the cells.

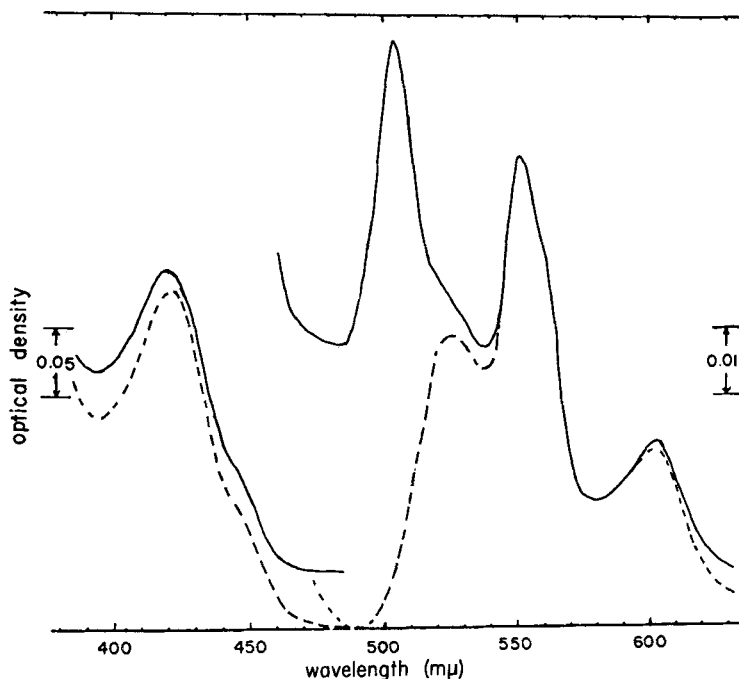


Fig. 1 The absolute absorption spectrum of *aerobically grown* yeast cells, stationary phase. Cell density: 10^8 cells/ml. Solid line: cells + glucose. Dashed line: cells + glucose + sodium dithionite. Optical density scale is different above and below $480 \mu\mu$, as indicated.

Both curves exhibit more or less pronounced absorption maxima at the following wave lengths: 420, 445, 525, 551 and $605 \mu\mu$, of which only the $551 \mu\mu$ one is sharply defined. The curve taken in the presence of glucose alone shows in addition to these a large and sharp maximum at $503 \mu\mu$, as well as a

very broad shoulder between 450 and 550 $m\mu$. All of the absorption maxima are easily identifiable in terms of the known cytochrome and flavin spectra (cf. Smith, '54), except the 503 $m\mu$ peak. Briefly, the 605 $m\mu$ peak is chiefly attributed to the α band of cytochrome *a*; the one at 560 $m\mu$ to the α band of cytochrome *b*; the one at 551 $m\mu$ to the overlapping α bands of cytochromes *c*₁ and *c*; the absorption around 525 $m\mu$ to the β bands of cytochromes *b*, *c*₁, and *c*; the 445 $m\mu$ peak chiefly to the γ band of cytochrome *a*₃; and the one at 420 $m\mu$ to the γ bands of cytochromes *b*, *c*₁, and *c*. Yeast cytochrome *c* peroxidase absorbs in the reduced state at 437 $m\mu$, and in the oxidized state at 410 $m\mu$ (Abrams, Altschul and Hogness, '40), but these bands could not be identified here because of the absorption of the other pigments in the same region. The absorption in the broad interval between 450 and 550 $m\mu$ is due to the oxidized flavins present in the cells, which disappear upon the addition of a reducing agent.

The pigment that absorbs at 503 $m\mu$ has not been observed before, although many spectroscopic studies have been reported on baker's yeast. The main reason for this failure on the part of previous observers may have been the fact that this pigment cannot usually be found in starved commercial baker's yeast. On the other hand, we have observed it in fresh cultures of a number of various laboratory strains of baker's and brewer's yeasts. As will be seen further below, it is also present in the anaerobically grown cells. As figure 1 shows, this absorption band disappears in the presence of sodium dithionite, but no other absorption band has been found to appear paralleling this change.

Manometric data on anaerobically grown cells. Cells, grown in the ergosterol-oleate supplemented basic medium and harvested in the exponential or in the stationary phase, were used for the Warburg measurements, which were performed the same way as in the case of aerobically grown cells.

The results are presented in table 3. The rate of oxygen uptake in the presence of exogenously supplied glucose was in either phase less than 10% of the corresponding aerobically

TABLE 3

Respiratory and fermentative rates of cells grown anaerobically in supplemented basic medium, at various stages of growth

TIME AFTER INOCULATION	WITH EXOGENOUS GLUCOSE			WITHOUT EXOGENOUS GLUCOSE		
	$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	$Q_{CO_2}^{air, N_2 term.}$	$Q_{U_2}^{air}$	$Q_{CO_2}^{air}$	$Q_{CO_2}^{air, N_2 term.}$
<i>hrs.</i>						
16	0.05	10.4	10.35	0.06	0.12	0.06
28	0.09	6.0	5.9	0.11	0.19	0.08

Q values in units of $10^{-4} \times \mu\text{l gas/hr.} \times \text{cell.}$

grown cells. The Q_{O_2} of exponential phase cells was again about half of that of the stationary phase ones. The exogenous fermentative rates, on the other hand, were about twice as high as those found in aerobically grown cells, but a decline can be seen from the exponential to the stationary phase, as in the aerobic cultures. The M.Q.'s, calculated on the basis of low respiratory rates, cannot be considered very accurate; it is still significant, however, that their values are higher (between 3–5) than are those observed for aerobically grown cells.

It is interesting to note that in these cells the rate of endogenous respiration is approximately the same as the exogenous rate. This fact was investigated in more detail, paying particular attention to some of the experimental difficulties. Using the manometric method for oxygen and CO_2 determinations, the difficulty arises when one is trying to establish accurately the very low rates of oxygen uptake in cells that are liberating at the same time large amounts of CO_2 , which is the case with anaerobically grown yeast cells in the presence of exogenous glucose. The usual method of placing a paper wick in 20% KOH solution in the centerwell is not satisfactory in this instance; the liberated CO_2 is not absorbed rapidly enough, and therefore the manometric readings do not express the true rate of oxygen disappearance. This situation was corrected by raising the KOH concentration to 30%, and by placing 0.2 ml KOH solution and paper wicks in the two sidearms of the Warburg vessels in addition to the centerwell. In this way results were obtained that were essentially similar to those presented in table 3, and that agreed well, furthermore, with oxygen uptake measurements in the same kinds of cells by a polarized platinum electrode. Thus, it has been confirmed by various methods that the anaerobically grown cells had practically identical rates of oxygen uptake in the presence and absence of exogenous glucose, and this was found to be true even after the anaerobically grown cells have been aerated for a few hours in various media, but, of course, was not true for aerobically grown cells. It seems

that we are dealing here with cells in which the very weak respiratory pathway is saturated with the endogenously available substrates.

Another interesting aspect must be pointed out with regard to the endogenous rates shown in table 3, and that is the appearance of endogenous fermentation in these cells, both in air and nitrogen atmosphere. As mentioned above, endogenous fermentation has been reported by several workers to be entirely absent in baker's yeast, i.e., aerobically grown baker's yeast. Only Fales ('51) observed some endogenous fermentation in yeast cells during the first hour after removal from their growth medium, and attributed it to the presence of a fermentable substrate in the cells that is rapidly exhausted and is not identical with the substrate of respiration. Endogenous fermentation can also be induced in yeast cells by dinitrophenol (Rothstein and Berke, '52), and by azide (Brady and Duggan, '54), thus seemingly the uncoupling of phosphorylation makes endogenous fermentation possible without involving the necessity for a special substrate. Whatever the case may be, there is a significant difference in this regard between the aerobically and anaerobically grown cells, that could be exploited for further work on this subject.

Absorption spectra of anaerobically grown cells. The absorption curves, shown in figure 2, were obtained using early stationary phase cells grown anaerobically and harvested the same way as above. The dashed curve was taken in the presence of sodium dithionite, in addition to glucose, and shows a weak maximum at around 590 $m\mu$, a well-defined one at 556 $m\mu$, another weak one at about 530 $m\mu$, and finally a large peak in the Soret region at 425 $m\mu$ and a shoulder around 445 $m\mu$. The curve represented with the solid line was taken in the presence of glucose alone, and it shows two absorption bands in addition to the ones exhibited by the other curve. One is the large peak at 503 $m\mu$, and the other a broad absorption region between 450 and 550 $m\mu$.

The 590 and 556 $m\mu$ bands are identical with those that were found in brewer's yeast by Fink ('32), and designated

as the α bands of cytochrome a_1 and b_1 by Ephrussi and Slonimski ('50), and Chin ('50), while the 530 $m\mu$ maximum was attributed by them to the β band of cytochrome b_1 . The absorption spectrum of the anaerobically grown yeast in the Soret region has not been investigated previously. The two

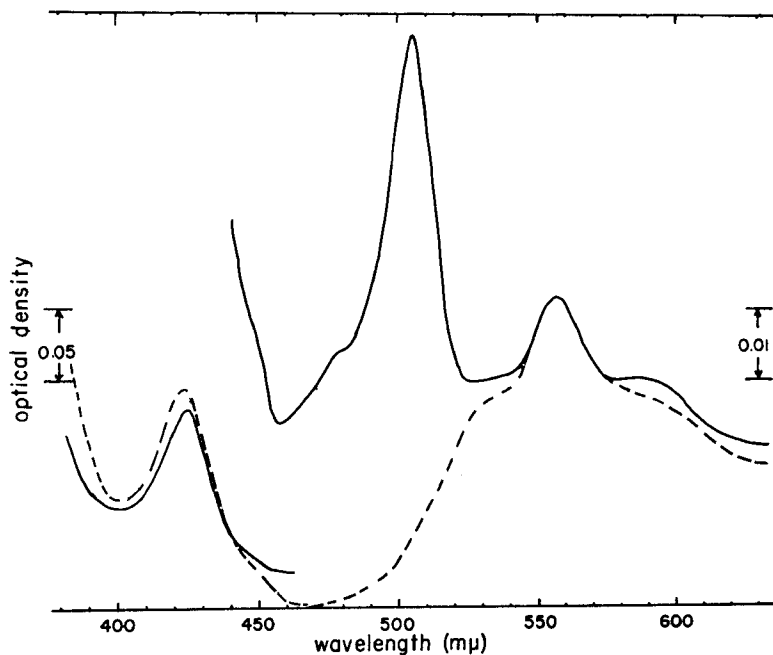


Fig. 2 The absolute absorption spectrum of *anaerobically grown* yeast cells, stationary phase. Cell density: 10^8 cells/ml. Solid line: cells + glucose. Dashed line: cells + glucose + sodium dithionite. Optical density scale different above and below 480 $m\mu$, as indicated.

maxima in this region, at 445 and 425 $m\mu$, can be regarded as the γ peaks of cytochromes a_1 and b_1 , but this view requires further tests, particularly by a study of the behavior of these peaks in difference spectra (cf. following paper in this series).

The absorption bands that are missing in the cells reduced with sodium dithionite are the same two as those in the aerobically grown cells. The absorption between 450 and

550 $m\mu$ is again due to the oxidized flavins. It seems, however, that their contribution is considerably smaller than in the aerobically grown cells. The sharp band at 503 $m\mu$ has about the same intensity in anaerobically as in aerobically grown cells judging from the curves presented here, but this should by no means be considered to be the rule. The absorption intensity of this unidentified pigment varies greatly with the age of the culture and the subsequent treatment of the cells; it can entirely disappear under prolonged starvation, or its concentration can be built up much higher than that shown in the figures by aeration in glucose solution.

DISCUSSION

The study of the anaerobically grown yeast with respect to the oxidative processes and the enzymes involved is an interesting field not because of the role these processes play during growth but because of the potentialities these enzymes lend to the cells. In the course of anaerobic growth obviously no reaction or reaction sequence can take place that involves molecular oxygen. In these cells the presence of compounds that can combine with oxygen can be important, however, because it enables the cells to carry out oxidative metabolism as soon as oxygen becomes available, and also because these compounds can serve as the inducers or the precursors for the formation of the efficient oxidative systems that are found in the aerobically grown cells. Among the various oxidases of yeast, attention has been given by several investigators primarily to the origin of the cytochrome system, and thus the role of the cytochrome-like pigments in the anaerobically grown cells has become a subject of interest.

When exposed to oxygen, the cells raised under strict anaerobiosis exhibit immediately a low but measurable and reproducible rate of oxygen uptake. This rate, as the results indicate, is higher in cells harvested in the stationary phase than in the ones harvested in the exponential phase, and it is not affected by an exogenous supply of glucose. The ques-

tion is what are the enzymes that mediate the uptake of oxygen. It is possible that the low level of oxygen consumption of these cells, 10^{-7} $\mu\text{l O}_2/\text{hr.} \times \text{cell}$, or in other terms $10 \mu\text{l O}_2/\text{hr.} \times \text{mg dry weight}$, is due to the activity of flavin enzymes or to some other oxidase and not to a heme enzyme. The fact, however, that several hemoproteins can be detected spectroscopically in these cells presents the possibility that they are the enzymes responsible for the utilization of oxygen.

The heme pigments that were provisionally named cytochromes a_1 and b_1 by Ephrussi and Slonimski ('50) and Chin ('50) should be considered first. The absorption maxima at 590 and 556 $m\mu$ were attributed to the α bands of these pigments, the maximum at 530 $m\mu$ to the β bands, while the 445 and 425 $m\mu$ peaks shown in figure 2 can now be attributed to their γ bands (the 425 $m\mu$ peak includes very probably the absorption of another pigment as well). These bands are present when the cells are in the reduced state and, as will be shown in a following paper, they disappear when the cells are vigorously aerated. The oxidation and reduction of the cytochromes a_1 and b_1 are thus closely connected with the presence or absence of oxygen in the cells. This means either that one or both of them can directly combine with oxygen, or that they are oxidized and reduced by another enzyme that combines with oxygen. The first seems to be the more likely one in view of the evidence, presented in the following paper, that no cytochrome a_3 can be detected in these cells.

The absorption maximum at 503 $m\mu$ presents a special problem. It is found both in aerobically and in anaerobically grown cells of the yeast strain used in this work. The concentration, i.e., the intensity of the absorption peak at 503 $m\mu$, is increased considerably when the cells are aerated in the presence of glucose, while it decreases upon prolonged starvation. This peak does not change rapidly with the presence or absence of oxygen in the cell suspension, but it disappears instantaneously from the absorption spectrum when sodium dithionite or potassium cyanide is added to the cells. The

chemical nature of this pigment has not yet been established. It certainly does not belong either to the cytochrome-like or to the flavin-like compounds, and it does not participate directly in the oxidative processes as those pigments do. Further data concerning this pigment will be presented with the difference spectra.

The Pasteur effect, i.e., the suppression of fermentation by respiration, should also be considered in connection with the oxidative metabolism of this organism. As mentioned above, we are using here the Meyerhof Quotient as a criterion of the Pasteur effect, calculated from the "exogenous" rates of the fermentative CO_2 production, in the presence and absence of oxygen, and of the rate of oxygen uptake. This means that we are determining the Pasteur effect by comparing the appearance of the fermentative cleavage products, rather than by the uptake of carbohydrates, between the two conditions. The former procedure has been advocated by Burk ('39), but not by Dixon ('37). Three other assumptions are implicit with regard to the Meyerhof Quotient, these being: (1) that respiration in these cells proceeds with a Respiratory Quotient of unity; (2) that the rates of endogenous respiration and fermentation are negligible in the presence of exogenously supplied glucose; and (3) that equivalent amounts of CO_2 and ethyl alcohol are produced in fermentation. These assumptions are not necessarily fulfilled in all the various cell types studied and under all conditions, but they are in general agreement with the results of numerous studies of yeast metabolism.³

The point that becomes evident from the data presented here is that anaerobically grown cells show consistently higher values for the Meyerhof Quotient than the aerobically grown ones do. The data given in table 3, being representative of a large number of experiments, indicate an average Meyerhof Quotient of around 4 for the anaerobically grown cells.

³ Pasteur effect, as the term is employed here, refers only to the *reversible* effect of respiration on fermentation, and not to changes over a long period of time, which may be due to changes in the enzymatic composition of the cells.

The determination of this quotient in this case is subject to a number of difficulties and errors. The cells have a very low respiratory and a high fermentative rate, and the rate of aerobic fermentation is only 2-5% lower than the anaerobic rate. The problem of measuring the small amounts of oxygen taken up during the same period when large quantities of CO_2 are liberated by the cells, and the answer to it, have been described above. Another source of error could have been the fact that the Warburg vessels used for Q_{O_2} determination did not contain any CO_2 , while the other vessels in the same experiment that are set up without KOH solution do have CO_2 . If the yeast cells can fix or "recycle" CO_2 , the metabolic situation would not be comparable in the two vessels. Therefore, some CO_2 determinations were carried out in the course of this work by Warburg's indirect, or two-vessel method (Umbreit, Burris and Stauffer, '49). The indirect and the direct methods of Warburg gave identical results. Thus the measurements giving high values for the Meyerhof Quotient in the anaerobically grown cells seem to be valid. Previous workers have reported similar results with yeast strains deficient in cytochromes. Stier and Castor ('41) have reported high Meyerhof Quotients in their cyanide induced yeast mutants, while Meyerhof and Davidson ('52) and Slonimski ('53, p. 186) obtained Meyerhof Quotients of 8-15 and around 4, respectively, working with a "petite" strain of yeast.

The observation is, therefore, that yeast cells devoid of the cytochrome system exhibit Meyerhof Quotients of 4 or higher, while the normal, aerobically grown cells give values of about two or lower. If the Pasteur effect, i.e., the slowing down of glycolysis, is brought about by a localized lack of phosphate acceptors when respiration is operating, as it was proposed first by Johnson ('41) and Lynen ('41), then the value of the Meyerhof Quotient will be related to the rates at which phosphorylation proceeds in the various enzyme systems of the cell, and to the rates at which the phosphorylated compounds diffuse from one system to the other, or are used up. When yeast cultures are compared that have entirely different

respiratory systems, i.e., the cytochrome system in the case of aerobically grown cells and an unidentified system in the case of the anaerobically grown cells and the mutant strains, the difference in the phosphorylation and the diffusion rates evidently becomes expressed by the difference in the Meyerhof Quotients.

SUMMARY

Cells of *Saccharomyces cerevisiae*, strain LK2G12, were grown anaerobically in the presence of ergosterol and oleate. Their overall metabolism was studied by measuring manometrically the rates of oxygen consumption, and of carbon dioxide evolution in air and in nitrogen atmosphere. The absorption spectra of the intact cells were obtained with a recording spectrophotometer. These results were compared with corresponding ones for aerobically grown cells. The presence of cytochromes a_1 and b_1 is related to the oxygen uptake and to the Pasteur effect of the anaerobically grown cells. A new pigment, with absorption maximum at 503 m μ , is observed, which is present in the intact cells when aerated in glucose solution, and disappears when dithionite or cyanide is added.

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