# SPECTROPHOTOMETRICAL INVESTIGATIONS ON ENZYME SYSTEMS IN LIVING OBJECTS

## II. CYTOCHROMES $a$ AND $a_3$*

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## INTRODUCTION

In their investigations on heart-muscle preparations, KEILIN AND HARTREE[1] discovered properties of cytochrome $a$ (KEILIN[2]) that according to them indicated the coexistence of two components, $a$ and $a_3$. KEILIN AND HARTREE based their conclusions on direct spectroscopic observations of the response of the cytochrome system to certain inhibitors, mainly carbon monoxide and cyanide. It was shown that the component $a_3$ formed spectroscopically visible complexes with CO and CN similar to those that WARBURG had ascribed to his "Atmungsenzym", whereas the component $a$ behaved indifferently. Several later investigators[3,4,5,6] subjected purified preparations from heart-muscle homogenates to spectrophotometric analysis. The oxidized and reduced spectrum of $a + a_3$ was recorded, but no separation or further characterization of the closely allied compounds was achieved.

In his investigations on the cytochrome system of living roots of cereals, primarily wheat, the author showed that in the absence of oxygen the quotient $\frac{442.5\text{-}460\ \mathrm{m}\mu}{605\text{-}620\ \mathrm{m}\mu}$ attained the value 5.0 (average of seventy spectrograms). This value was maintained irrespective of the presence or absence of CO or CN (*cf.*[3]), but additional bands appeared in the region 585-595 mμ. These observations are not incompatible with KEILIN's conception of a cytochrome $a$, which is insensitive to CO and CN and characterized by a high band at about 605 mμ, and a cytochrome $a_3$, which is characterized by a high band at about 444 mμ and easily reacts with CO and CN.

In addition to measurements of the response to the common inhibitors (CO, CN, F, urethane, antimycin A, etc.), recording of the time course of reduction and reoxidation appeared to be a useful tool for analysis of the complex spectrum of the intact cytochrome system[3,5,7]. Because of the comparatively slow response of the root tissue to sudden changes in the oxygen tension, the author's recording spectrophotometer[8,9] is very suitable for studies of the kinetics of the single cytochromes in these objects. Owing to the direct contact between the single cells and the medium, baker's yeast reacts more rapidly. To meet these requirements, a special technique was developed by means of which the start lags of the single enzymes, including also flavoprotein and diphosphopyridine nucleotide, and their apparent velocity constants could be determined[10]. These experiments yielded conclusive evidence as to the separate activity of cytochrome $a$ and cytochrome $a_3$. It was shown that the apparent velocity constant ($k_{\mathrm{app}} = \frac{c\ \text{(molar concentration)}}{ht\ \text{(time value of 50\% reduction or oxidation)}}$) of reduction (oxidized enzyme →

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e $\xrightarrow{k_{app}}$ reduced enzyme) of cytochrome $a_3$ was 4–5 times higher than that of cytochrome *a*, whereas the velocity of reoxidation was about the same. It was assumed that cytochromes *a* and $a_3$ are present in equimolar quantities. As a consequence of the large difference in velocity, the time-courses of reduction of the two enzymes are fundamentally different, $a_3$ reacting even more rapidly than cytochrome *c*, whereas the complete reduction of cytochrome *a* was delayed until cytochromes $c_1$, *b*, $a_3$, and a part of the flavoprotein, combined with cytochrome *b*, are largely reduced.

## METHODS

Three years ago Prof. KIESE in Marburg kindly sent a preparation of "cytochrome oxidase". A number of spectrograms were immediately recorded from this preparation. The results are shown in Figs. 1, 2, 4, 5, 7. The spectra are very similar to those published by a number of investigators[3,4,5,6].

The construction of the automatic spectrophotometer and suitable vessels for yeast suspension are described in previous communications[8,9,11]. The experimental device for continuous measurements of the state of oxidation and reduction in baker's yeast was recently described[7].

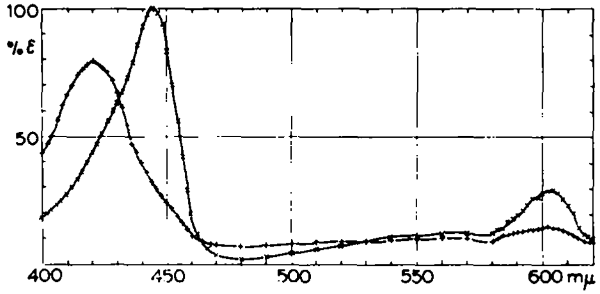

Fig. 1. Oxidized (+——+) and reduced (×——×) spectrum of a heart-muscle preparation (KIESE) of cytochromes ($a$ + $a_3$). Direct spectrograms.

### *Calculations from spectrophotometric measurements*

Owing to the influence of the turbidity of the yeast suspension on the spectral transmission, measurements of the height of the $a_3$ λ-band in a 15% suspension have to be corrected by the factor 1.33. The height of this band was calculated as the distance between the top of the band and the zero line of the difference spectrum, *viz.* 444 minus 462 mμ. The *a* α-band was calculated as 604 minus 620 mμ (see below).

In calculations from difference spectra, due attention must be paid to depressions of the background caused by negative changes of other enzymes (*cf.* Fig. 3). These depressions are calculated in % of the height of the corresponding positive band. The depressions bordering on the γ-bands of cytochromes *c* and *b* correspond to about 40% of the height of the *c* α-band. Simultaneous decolorization of flavoprotein on reduction adds an extra depression to the region of the $a_3$ γ-band. As shown in Fig. 3, the measured height of $a_3$ above the apparent isosbestic level (zero line of the difference spectrum) is only about $\varepsilon = 0.070$, whereas the calculated true value amounts to $0.070 + 0.050$ (= depression by *c* γ and *b* γ) + 0.080 (depression by flavoprotein) = 0.200.

The α-band of cytochrome *c* is practically free of any depression caused by adjacent bands. The band *b* α is slightly depressed by influence from *c* α, the depression of which amounts to about 30% at 575 mμ. This depression distorts the *a* α-band, too, in particular towards the lower wavelengths. Because the depression disappears towards about 610 mμ, measurements of *a* should be made from a base line connecting λ 580 and λ 620 mμ (see Fig. 3).

As previously mentioned, the influence of turbidity is negligible in green and red. The calculation of $a_3/a$ thus gives values 4.4–5.3, or nearly the same as in wheat roots (see above) or in preparations from heart muscle[5]. No unknown pigments are thus interfering with the spectroscopic picture of the bands of $a + a_3$. After prolonged aeration, however, catalase may be formed[12].

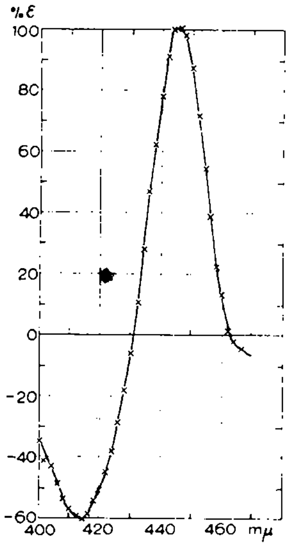

Fig. 2. Difference spectrum (reduced *minus* oxidized) of the Soret band of a heart-muscle preparation (*cf.* Fig. 1).

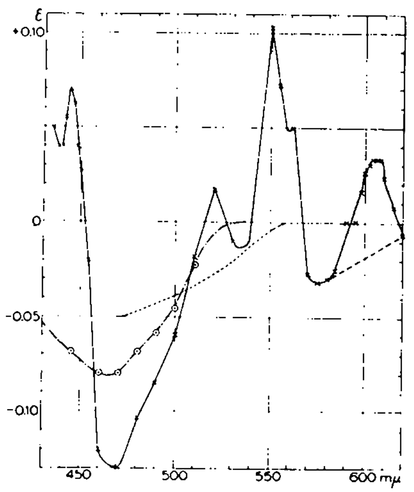

Fig. 3. Spectrum of a 15% suspension of yeast in water (diameter of the circulation vessel 18 mm). The spectrum is computed from measurements of the single bands, recorded by means of a device for alternating reduction and reoxidation[9]. It shows the difference between yeast in $N_2$ and aerated yeast. × —— × observed spectrum. ◉ · · · ◉ calculated difference spectrum of flavoprotein. – – – calculated depression caused by the negative portions of the γ-bands of cytochromes *c*, *b*, and $a_3$ and the *α*-bands of cytochromes *c* and *b*.

## RESULTS

### *Cytochromes* a + $a_3$ *from heart-muscle preparations*

As shown in Fig. 1, the bands of cytochromes *a* and $a_3$ are somewhat broader than those of cytochromes *c* and *b*. The Soret band of the oxidized, greenish looking enzyme lies at 420 mμ, that of the reduced reddish enzyme (dithionite) at 444 mμ. The Soret band of the difference spectrum (Fig. 2) is more slender and its peak is moved toward 445 mμ. Isosbestic points appear at 431 and 462–463 mμ. The 60% depression below the zero line attains its maximum at 414 mμ and thus strongly lowers the band of cytochrome c at 418 mμ.

The α-band of the preparation is asymmetrical. The enlarged image of the band in Fig. 7 shows a slight elevation at about 596 mμ, caused by the α-band of $a_3$. Addition of CO evokes an intensification of the elevation, but the rest of the band remains unchanged (Fig. 4). Subtraction of the bands results in the $a_3 \cdot$CO-band at 590 mμ. Cyanide (buffered at pH 6.5), on the contrary, lowers the elevation at 596 mμ, because

$a_3$ is now partly transferred into $a_3\cdot$CN, the band of which does not completely cover the reduced band (see below). In CO the partial disappearance of the reduced $a_3$-band is noticed as an asymmetry of the $a_3\cdot$CO-band and a small depression below zero.

The reduced pyridine hemochromogen of $a + a_3$ (*cf.*[4]) shows an α-band at 586–588 mμ (Fig. 5). It was observed, however, that the formation of the hemochromogen starts with a comparatively low peak at 583–584 mμ and that this peak then slowly moves to 586–588 mμ simultaneously with an intensification of the extinction. The observation possibly points to a slight difference in the hemes of the two enzymes.

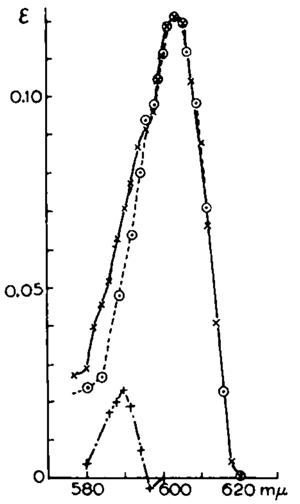

Fig. 4. ×—× α-band of cytochromes ($a + a_3$) in a heart-muscle preparation, treated with CO + dithionite. ⊙—⊙ band of the reduced preparation without CO. +·—·+ calculated $a_3\cdot$CO band.

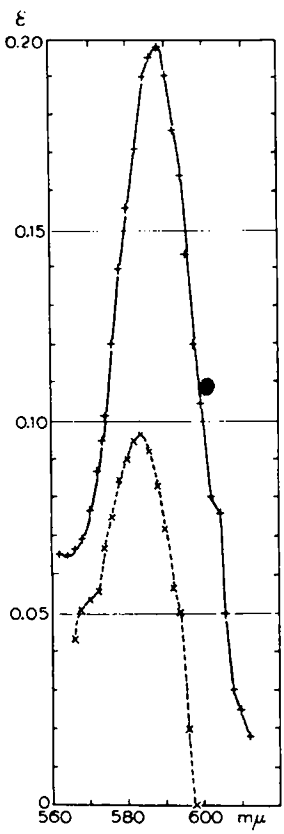

Fig. 5. 0.4 ml heart-muscle preparation, treated with 5 ml pyridine + 3 drops 0.1 *N* NaOH. ×---× spectrum recorded 5 min after the start. +—+ 30 min after the start.

*Cytochromes* a + $a_3$ *in baker's yeast*

***Observations of the time-course of reduction.*** Continued observations at 444 and 604 mμ, performed by aid of the device for automatic alternation between reduction and reoxidation[7], showed that *ht* (= time of 50% reaction, measured from zero time) of reduction of $a_3$ is 1/5 to 1/7 that of $a$ (see Fig. 6). Because *ht* is inversely related to the reaction velocity, this means that $a_3$ is much more rapidly reduced than $a$. Fig. 6 also shows *ht* values of the cytochromes $c$, $c_1$, and $b$. In evaluating these curves, attention should be paid to the interference by adjacent bands (see above). The *ht* values of cytochrome $a_3$ at 444 mμ are somewhat retarded by the coincidence with cytochrome $c$ and flavoprotein, but this still more accentuates the extremely rapid course of the reduction of cytochrome $a_3$. More important is the retarding influence of cytochrome $a$

on the $a_3$ α-band. This is probably the reason why the *ht* values at 598 mμ (the α-band of $a_3$; see below) are not as low as those of the $a_3$ γ-band at 444 mμ. However, the value at 598 minus that at 604 mμ is significant. The response of the small γ-band of cytochrome *a* is completely masked by the adjacent strong γ-band of cytochrome $a_3$, and also by the slower working flavoprotein. The uniform velocity at different points of an uncomplicated band is illustrated by $a_3$, *c*, and *b* (Fig. 6).

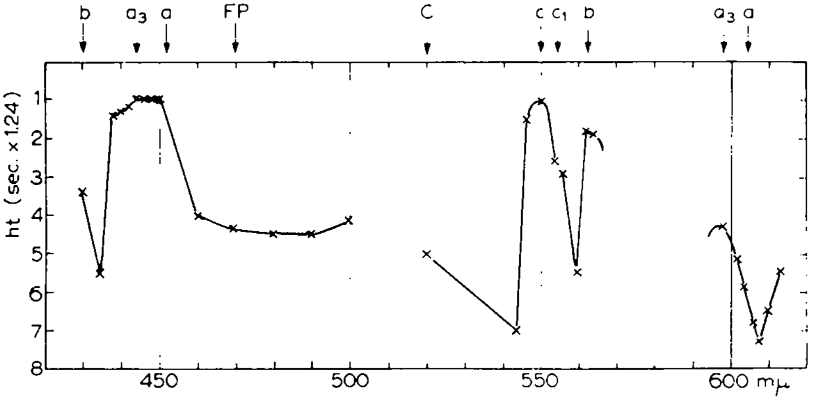

Fig. 6. Measurements of band heights recorded by aid of the device for alternating reduction and reoxidation[9]. *ht* = half-time = time of 50% reduction, in seconds × 1.24. × points of observation.

*The effects of CO, CO + $O_2$, and CN.* Difference spectra were recorded with aerated yeast in distilled water as reference. Reduction of the sample (15% yeast suspension in distilled water) was performed by bubbling through 99.99% $N_2$ (from Aga). The CO compounds were developed by changing the stream of $N_2$ to one of pure CO (from Matheson). Partial oxidation of the remaining quantities of reduced oxidase was performed by changing the gas stream to a mixture of CO and $O_2$. In the experiments with HCN, the stream of air or $N_2$ was conducted through a flask containing KCN + HCl.

The completely reduced spectrum shows an $a_3$ γ-band at 444 mμ, exactly the same as in the heart muscle preparation. As mentioned above, the peak of the difference band is moved toward 445 mμ (see Fig. 2). The top of the *a* α-band was measured on a series of 34 spectrograms. The average position was 604.65 mμ. Like the spectrum of the heart-muscle preparation, the yeast spectrum usually shows an elevation at 597–598 mμ (Fig. 7), or, as an average of 10 spectrograms, 597.7 mμ. For convenient calculation the values are approximated at 604 mμ (*a*) and 598 mμ ($a_3$) respectively. The existence of an $a_3$ α-band was concluded from the response to CO and cyanide.

In ten experiments the height of the *a* α-band amounted to $\varepsilon$ 0.065 in $N_2$ and 0.061 in pure CO. The small difference (6%) may be explained from a slight depression of the *a* α-band caused by the partial disappearance of $a_3$ in CO (see below). The shape of the slope of the band in the direction of the longer wavelengths is identical in $N_2$ and in CO, owing to the complete indifference of cytochrome *a* to CO.

The only significant difference between the $N_2$-spectrum and the CO-spectrum in green and red is an elevation at the shorter wavelengths side of the combined bands of cytochromes *a* and $a_3$ (Fig. 8), caused by the formation of $a_3 \cdot CO$. The band of this

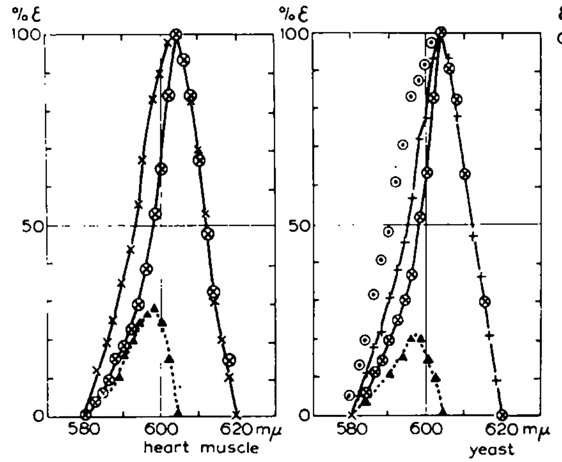

Fig. 7.

Fig. 7. α-Bands of a heart-muscle preparation and of yeast. ×—× and +—+ observed difference spectrum (reduced *minus* oxidized). ⊗—⊗ calculated α-band of cytochrome *a*. ▲---▲ calculated α-band of cytochrome $a_3$.

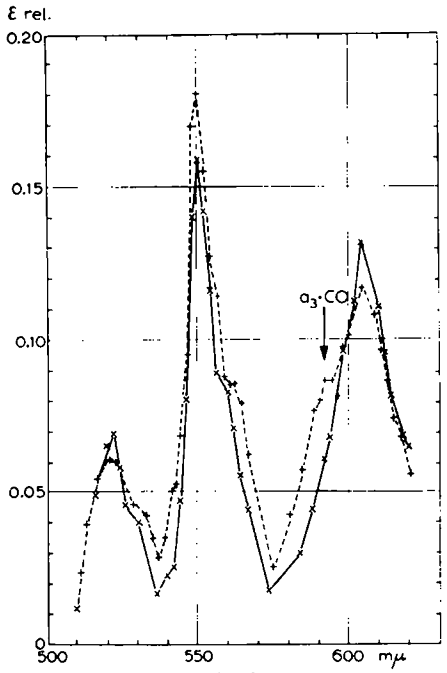

Fig. 8.

Fig. 8. Difference spectrum (reduced *minus* oxidized) of yeast in $N_2$ (×—×) and in pureCO(+---+).

compound appears from a subtraction of the $N_2$-spectrum from the CO-spectrum (Fig. 9). The top of the band is at 590.5–591 mμ. Because a corresponding quantity of reduced $a_3$ disappears simultaneously with the formation of $a_3 \cdot CO$, a depression may be expected at 598 mμ. It is sometimes observed as a slender negative band, but is mostly restricted to a slight asymmetry of the $a_3 \cdot CO$ band (Fig. 9).

The negative band caused by the disappearance of reduced cytochrome $a_3$ is more easily recognizable in the CN-spectrum (Fig 10). $a_3 \cdot CN$ is characterized by a broad band which may appear split up in two, because of this effect of the disappearance of reduced cytochrome $a_3$. The compound $a_3 \cdot CO$ dissociates only very slowly when $O_2$ is introduced. The reoxidation of uncombined cytochrome $a_3$ is furthermore slowed down owing to the relatively enhanced effect of the reducing systems. The difference curve $\varepsilon(CO + O_2) - \varepsilon(CO)$ consequently gives a purer negative spectrum of cytochrome *a*, than is given by simple reoxidation from $N_2$. The resulting negative band, mainly cytochrome *a*, is narrower because its short wavelength side is more excavated than obtained by reoxidation from $N_2$ (see Fig. 7). This "purified" *a* α-band is convenient for calculation of the shape of the $a_3$ α-band. As shown in Fig. 7, the subtraction of the "purified" *a* α-band from the combined band of $a + a_3$ (reduction in $N_2$ or dithionite) results in the $a_3$ α-band with its peak at 598 mμ. It was observed that in yeast the height of the $a_3$ α-band is about 20% that of the *a* α, but in the heart muscle this ratio is about 28%. However, in view of the possible errors of calculation and imperfect purification of preparations, this difference is not significant.

The quantity of reduced $a_3$ which is converted into $a_3 \cdot CO$ may be calculated from the height of the negative band of $a_3$ γ appearing in the difference spectrum $\varepsilon(CO) - \varepsilon(N_2)$ (Fig. 11). At room temperature about 44% of reduced $a_3$ is transformed into

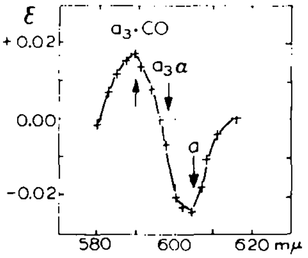

Fig. 9.

Fig. 9. Difference spectrum (reduced *minus* oxidized) of yeast, ε (CO + 20% $O_2$) ε ($N_2$), showing partial oxidation of cytochrome *a* and formation of $a_3$·CO at the expense of reduced cytochrome $a_3$.

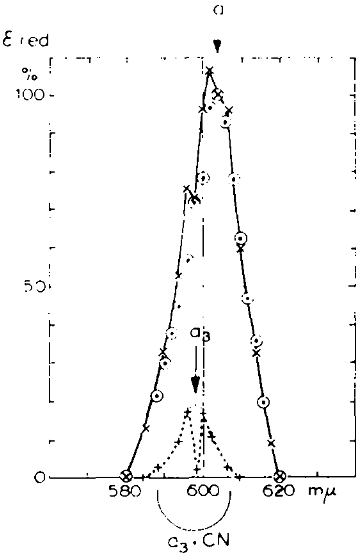

Fig. 10.

Fig. 10. Difference spectrum (reduced *minus* oxidized) of yeast in $N_2$ (◉) and in HCN + air (× ×). + +, ε (HCN + air) — ε ($N_2$), showing the apparent doubling of the $a_3$·CN-band owing to the disappearance of reduced cytochrome $a_3$.

$a_3$·CO. The γ-band of this compound has a peak at 431 mμ (Fig. 11). The CO-band thus coincides with the γ-band of reduced cytochrome *b*, resulting in an apparent increase of this band in the direct spectrum (*cf.*[1]). The $a_3$·CO-band may be determined as the difference between the negative band shown in Fig. 11 and the calculated band of the amount of reduced $a_3$, consumed during the formation of $a_3$·CO. The latter may be calculated from the decrease in (ε 444 mμ minus ε 462 mμ) on going from $N_2$ to CO. As shown in Fig. 11, the observed negative band of *a* + $a_3$ is considerably narrower than the same band appearing from simple reduction (Fig. 1). This is just the effect of the combination with the positive band of $a_3$·CO, as shown in Fig. 11. The top of the observed negative band is moved to 446–447 mμ and it is markedly asymmetrical.

The fact that 44% of cytochrome $a_3$ is converted into $a_3$·CO (in pure CO), whereas the amount of cytochrome *a* remains at its original value results in a corresponding rise of the percentage of cytochrome *a* in the spectrum of reduced (*a* + $a_3$). The negative γ-band will show a cavity on that side where *a* γ has its position, as actually shown in Fig. 11. The cavity on the left side of the negative band corresponds to the positive $a_3$ γ-band, the height of which corresponds to 44% of the total quantity of the enzyme. The *a* γ-band has its top at 453.7 mμ. The relative surplus of cytochrome *a*, as compared with reduction in $N_2$, may also appear as a peak or elevation on the combined (*a* + $a_3$) γ-band (see Fig. 12).

Besides the main bands at 431 and 590 mμ, the CO-spectrum of baker's yeast frequently shows a band at about 420 mμ (see Fig. 11) and sometimes elevations at about 460–470, 540 and 580 mμ. These bands are probably related to a hemoglobin-like pigment (*cf.* [13]). Hemoglobin shows absorption bands at 418, 538–540, and 568–572 mμ[14]. A hemoglobin would possibly serve as an oxygen buffer and explain the fact that reduction does not instantaneously start with removal of oxygen from the surroundings of the cells. However, this possibility is not supported by other observations (see below). Yeast contains peroxidase and sometimes catalase (*cf.*[7, 13]). Calculations from the CN-spectrum (*cf.*[9]) yield a peroxidase concentration which is of a similar, or slightly higher magnitude, than that of cytochrome *c*.

If yeast is transferred from an atmosphere of pure CO to 80% CO + 20% $O_2$, the cytochrome system is only partly reoxidized (all these experiments refer to starving yeast). Compared with the predominantly oxidized system in 80% $N_2$ + 20% $O_2$ (air), cytochromes *c*, $c_1$, and *b* are only about half oxidized (50–59%). Cytochromes *a* and $a_3$ are even less oxidized (see Table I), the latter as a rule more reduced than any other cytochrome.

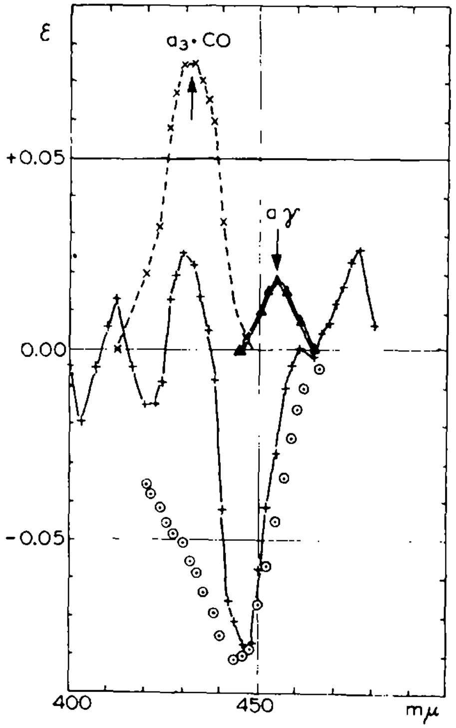

Fig. 11. +—+ Difference spectrum ε (CO) -- ε ($N_2$) in yeast, showing the interference between the positive $a_3$·CO band and the corresponding negative band of reduced cytochrome $a_3$. ◉—◉ calculated negative band of reduced cytochrome $a_3$. ×---× calculated band of $a_3$·CO. ▲=▲ calculated band of cytochrome *a*.

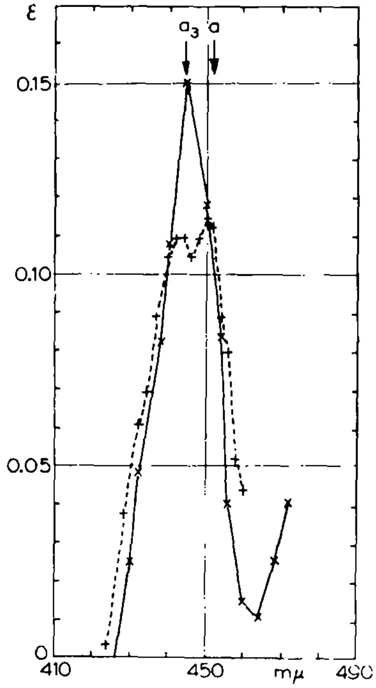

Fig. 12. Soret band of cytochrome $a_3$ of yeast in $N_2$ (×—×) and CO (+---+), showing the relative increase of *a* γ.

TABLE I

REOXIDATION OF THE CYTOCHROMES OF BAKER'S YEAST IN THE PRESENCE OF CO

Extinction of reduced bands. In brackets the percentage oxidation.

| *Extinction in* | $N_2$ | *CO* | *CO* + 5% $O_2$ | *CO* + 10% $O_2$ | *CO* + 20% $O_2$ |
|---|---|---|---|---|---|
| $c_{550}$ | 0.132 | 0.142 | 0.127 (15%) | 0.106 (25%) | 0.058 (59%) |
| $c_{1\ 554}$ | 0.057 | 0.059 | 0.050 (15%) | 0.038 (35%) | 0.028 (53%) |
| $b_{562}$ | 0.073 | 0.073 | 0.057 (24%) | 0.038 (48%) | 0.036 (50%) |
| $a_{604}$ | 0.068 | 0.067 | 0.058 (14%) | 0.046 (31%) | 0.040 (40%) |
| $a_{3\ 444}$ | 0.191 | 0.108 | 0.098 (9%) | 0.090 (17%) | 0.070 (35%) |

N.B. The percentage oxidized refers to that fraction of $a_3$ (about 56%) that does not combine with CO.

The spectrum of $a_3 \cdot CN$ is less distinct than that of $a_3 \cdot CO$. In HCN + $N_2$, about 57% of $a_3$ is converted into the cyanide compound, in HCN + air only 35%.

### *Kinetics of cytochromes* a *and* $a_3$ *as regulators of the respiration*

It has been shown[5,6] that preparations of cytochrome ($a + a_3$) oxidize reduced cytochrome *c*. Observations have also been made of the oxidation-reduction potential states of the combined enzymes[6], but owing to the obvious remarkable difference in the velocity of reduction of *a* and $a_3$[7], no conclusions can be drawn from such group observations as to the behaviour of the single enzymes. The large difference in the apparent velocity constants point to individual reactions of *a* and $a_3$. The obvious similarity in the reactions of $a_3$ and cytochrome *c* point to a close liaison between these two enzymes.

KEILIN AND HARTREE[1] put forward the idea that $a_3$ is identical with the cytochrome *c* oxidase, and no facts brought together by later investigators speak against it. The apparent velocity constants, computed from rapid recording of reduction and reoxidation (see above and[7]), show that cytochrome $a_3$ is reduced a little more rapidly than cytochrome *c*. This means that cytochrome $a_3$ is reduced by cytochrome *c*. The very much slower reduction of *a* shows that this enzyme is probably not reduced by cytochrome *c*. The fact that the respiration is heavily inhibited by CO and HCN, in spite of the inertness of cytochromes *a*, *c*, $c_1$ and *b* and the dehydrogenases to these compounds, obviously rules out cytochrome *a* from any appreciable ability as an oxidase.

In the absence of oxygen, *viz.* when electron transfer from cytochrome *c* oxidase to oxygen is blocked, the reduction of the oxidase obviously runs faster than the reduction of any other cytochrome, because it receives electrons from the whole system, in this case via cytochrome *c*. The function of the oxidase as an exquisite "suction pump for electrons" delays its reoxidation, if the on-reaction, *viz.* the uptake of electrons from the rest of the completely reduced system, runs faster than the off-reaction, *viz.* the delivery of electrons to oxygen (see the scheme below). This is in fact the case in most objects (yeast, roots[7-11]): the apparent velocity constant of oxidation is lower for cytochrome $a_3$ than for cytochromes *c* and *a*[7]. That there is a slight preponderance of the on-reaction, as compared with the off-reaction appears from the fact, too, that the normal oxidation-reduction state of the cytochrome system of a respiring object comprises about 80% oxidation of cytochrome *b* and only 50–60% of cytochrome oxidase[7,8]. This fact throws light upon an important physiological question, namely the controlling effect of the oxygen tension on the intensity of the aerobic respiration. Because this question is intimately linked to the effect of CO and HCN on respiration, a series of experiments was performed with the Warburg apparatus, simultaneously with observations on the state of oxidation-reduction of the cytochromes.

$$\text{Dehydrogenases} \xrightarrow[\text{cytochromes } b,\, c_1\, a,\, c]{\overset{\text{On}}{e}} a_3 \xrightarrow{\overset{\text{Off}}{e}} O_2$$

The experiments yielded a marked difference in the response of washed, starved yeast (in distilled water) and of yeast in 1% glucose. The aerobic respiration runs 4–6 times faster in glucose than in distilled water. *Starved yeast* is only little influenced by variations in the oxygen tension. The respiration in 2% oxygen (+ 98% $N_2$) amounts to 75% of the value in air (20% $O_2$) and 100% $O_2$ has no further accelerating effect.

This result obviously depends upon the slow working dehydrogenation of the starved yeast, caused by lack of fuel (glucose). The off-reaction (see above) of $a_3$ is consequently strongly dominating and the whole cytochrome system is accordingly nearly 100% oxidized.

*Yeast in 1% glucose* is considerably more affected by variations in the oxygen tension. The aerobic respiration rises to 140 in pure oxygen against 100 in air, and goes down to 32 in 2% $O_2$ + 98% $N_2$. The intensity of the respiration varies with the logarithm of the oxygen tension (Fig. 13). The spectroscopic picture of aerated yeast in 1% glucose shows a dominating reduction of the cytochrome system, caused by the intensively working dehydrogenation of glucose. The respiration is now controlled by the off-reaction of $a_3$, *viz.* the velocity of oxidation, and Fig. 13 shows that this reaction closely follows a first order equation. It was previously shown that the over-all reoxidation of the complex system is of first order[11].

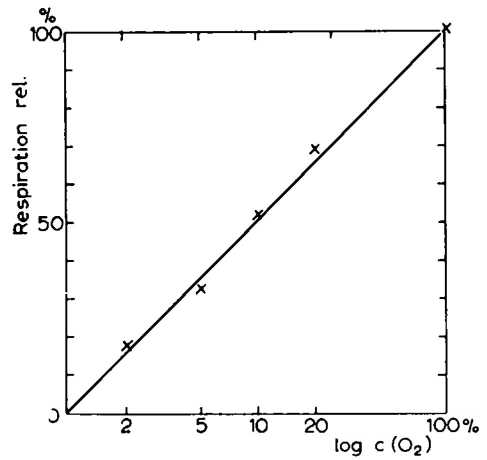

Fig. 13. The relation between oxygen tension ($O_2/(O_2 + N_2) \times 100$) and respiration (measured as $O_2$-consumption in Warburg flasks) of 5% yeast in 1% glucose solution.

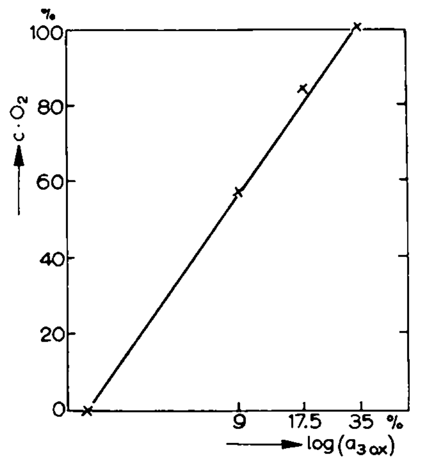

Fig. 14. The relation between the percentage of oxidized $a_3$ and the respiration (as $O_2$ consumption) of 5% yeast in 1% glucose.

The experiments in $O_2 + N_2$ throw light upon the results of experiments with HCN, $NaN_3$, and CO + $O_2$. As mentioned above, CO and HCN only combine with about 40–60% of cytochrome $a_3$ (these figures refer to starved yeast, 0.001–0.002 *M* KCN and 80–100% CO in the atmosphere). About 50% of the cytochrome *c* oxidase is consequently still available for oxidation work. The experiments in Table II now show that this quantity is apparently sufficient for an approximately undisturbed respiration of starved yeast in 0.001 *M* KCN. Even at the high concentration 0.010 *M* KCN or $NaN_3$ about 30% of the respiration of starved yeast is still going on. This result agrees with the above-mentioned experiments with $N_2 + O_2$ and with the conclusion that in starved yeast the on-reaction, *viz.* the production of electrons by the dehydrogenases, serves as the pacemaker.

At the high speed of respiration in yeast fed with glucose, the decrease of the actual concentration of free cytochrome $a_3$ by formation of inhibitor complexes, slows the off-reaction to a level which strongly retards the oxidation as compared with the

TABLE II

RESPIRATION, MEASURED AS $O_2$-CONSUMPTION IN WARBURG-FLASKS, OF YEAST AT VARYING CONCENTRATIONS OF KCN AND $NaN_3$

Volume of the sample 2 ml. Relative values.

| | *A*<br>*5% washed yeast in water* | *B*<br>*2.5% yeast + 1% glucose* |
|---|---|---|
| Control | 100 | 100 |
| + 0.001 *M* KCN | 100 | 27 |
| + 0.003 *M* KCN | 45 | 10 |
| + 0.005 *M* KCN | | 3 |
| + 0.010 *M* KCN | 30 | 2 |
| Control | 100 | 100 |
| + 0.001 *M* $NaN_3$ | 100 | 3 |
| + 0.003 *M* $NaN_3$ | 58 | 2 |
| + 0.010 *M* $NaN_3$ | 28 | 1 |

N.B. Because no compensation was made for HCN absorbed in the central well of the flasks the lower concentrations of KCN yield too high values. They reflect, however, the difference between A and B.

control. The relation between intensity of respiration and actual oxidation of cytochrome $a_3$ (see Table I) was determined in a series of Warburg experiments in CO + varying amounts of $O_2$. Fig. 14 shows a first order relation between the quantities of oxidized $a_3$ and the observed respiration of baker's yeast. The experiments clearly prove the ability of the non-combined $a_3$ to carry on respiration, in spite of the presence of high concentrations of CO. Because hemoglobin is completely inactivated in 80–95% CO the result also speaks against the hypothesis[3] that yeast hemoglobin would participate in the respiration (see above).

A comparison between A and B in Table II shows a 2-5 times higher retarding effect of $CN^-$ and $N_3^-$ upon the respiration of that system which is fed with glucose, as compared with yeast in distilled water. This observation points to an additional effect of the inhibitors $CN^-$ and $N_3^-$ on the system fed with glucose, probably related to phosphorylations.

### *Spectrophotometric properties of cytochromes* a *and* $a_3$

The peculiar spectral picture of cytochrome *a*, *viz.* a very low Soret band, as compared with other known cytochromes, has been frequently stressed in the literature. Our assumption of equimolar amounts of cytochromes *a* and $a_3$ in baker's yeast is a tentative one. It should be noticed that the relative values of the apparent reaction velocity of cytochromes *a* and $a_3$, given in a previous communication[7], are based on the assumption of equimolarity. Other molar relations will result in other values, but they will most probably not alter the conclusions about the considerably slower reduction of cytochrome *a* as compared with $a_3$.

The measured band relations of the two enzymes are shown in Table III. The figures refer to difference spectra. The relation $a_3\text{-}\gamma/a\text{-}\alpha = 4.4\text{–}5.0$ is also valid for wheat roots (see above) and heart-muscle preparations, a fact supporting the idea of a commonly fixed molar relation between cytochromes *a* and $a_3$. This is the main reason why the observed difference in the height of the calculated $a_3$-band between heart muscle and yeast (Fig. 7) should not be stressed too far. The calculation of $a_3$ $\alpha$ is exposed to greater errors than any other band.

TABLE III

MEASURED BANDS OF $a$ AND $a_3$ IN BAKER'S YEAST

The heights are given in direct extinction of 15% yeast in tubes of 18 mm diameter.

A. *Band heights*

| *Bands* | $a_{604}$ | $a_{452}$ | $a_{3\ 598}$ | $a_{3\ 444}$ | $a_3 \cdot CO_{590}$ | $a_3 \cdot CO_{431}$* |
|---|---|---|---|---|---|---|
| *Gas medium* | $N_2$ | $N_2$ | $N_2$ | $N_2$ | $CO$ | $CO$ |
| ε, absolute | 0.065 | 0.065 | 0.013 | 0.270 | 0.024** | 0.282 |
| ε, relative | 5 | 5 | 1 | 21 | 1.8 | 22 |

B. *Band relations*

| *Bands* | $\frac{a-\gamma}{a-\alpha}$ | $\frac{a_3-\gamma}{a_3-\alpha}$ | $\frac{a_3-\gamma}{a-\alpha}$ | $\frac{a_3 \cdot CO-\gamma}{a_3-\gamma}$ | $\frac{a_3 \cdot CO-\alpha}{a_3-\alpha}$ | $\frac{a-\alpha}{a_3-\alpha}$ | $\frac{a_3-\gamma}{a-\gamma}$ |
|---|---|---|---|---|---|---|---|
| Relation | 1.0 | 20.8 | 4.4 | 1.0 | 1.85** | 5.0 | 4.2 |

* Calculated on the total quantity of $a_3$. Actually only 44% is converted into $a_3 \cdot CO$.

** This value is only approximative, owing to the difficulty of measuring the height of $a_3 \cdot CO$.

## SUMMARY

The properties of the cytochromes $a$ and $a_3$ were studied in suspensions of baker's yeast and compared with spectrograms of a heart-muscle preparation. The results corroborate the original findings of KEILIN AND HARTREE on the coexistence of two enzymes and show the identity of cytochrome $a_3$ with cytochrome $c$ oxidase. The individual spectra of the two enzymes were calculated according to the method of optical subtraction, applied to spectrograms from yeast treated with $O_2$, $N_2$, CO, HCN and various mixtures of these gases. Observations of the kinetics of cytochromes $a$ and $a_3$ were made with the aid of alternating reduction and reoxidation, with continuous recording of the band heights. Complete spectrograms were recorded in the automatic spectrophotometer. The role of cytochrome $a_3$ in the aerobic respiration was studied by parallel observations of the oxidation-reduction state and the intensity of oxygen consumption.

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