New Spectrophotometric Methods for Investigation of the Respiratorical Enzymes of Yeast

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Modern photo multiplier tubes have made it possible to observe the state of oxidation-reduction of respiratorical enzymes in living objects on the basis of accurate measurements of the extinction in the whole range of the spectrum. Leading principles for the construction of a new recording spectrophotometer, especially built for this purpose, are briefly presented. A survey is also given of the corrections by means of which the obstacles arising from optical interference of the pigments and from the turbidity of the object may be eliminated. A special technique has been elaborated for the observation of rapid changes in the steady states of the respiratorical enzymes of yeast.

The results regarding observed start lags and apparent velocity constants of reduction and reoxidation elucidate the order of activation of the following enzymes: cytochromes a_1 , a , c , c_1 , and b , flavo-protein (FP), diphosphopyridine nucleotide (DPN) and its combination with alcohol dehydrogenase (ADH). Besides of its far-going inactivation of cytochrome b antimycin A is shown to reduce the operating activities of cytochrome c_1 , FP and DPN to about 50 % but leave the group $a + a_1$ and c nearly unaffected. Cytochrome b and one fraction of the total FP are closely cooperating in equimolar quantities.

EXPERIMENTAL TECHNIQUE AND PROCEDURE

A new recording spectrophotometer. Sensitive recording photometers were introduced only a few years ago^{1,2}. The instruments constructed by the author are primarily built for the purpose of *accurate measurements of the extinction in distinct wave-length intervals*. The figures for the reference and the sample are separately recorded. The new construction eliminates the inertia of the recording millivoltmeter and secures the attainment of precise wave-length positions, a fact of fundamental importance for accurate calculations of extinction values. One of the author's instruments is shown in Fig. 1. It automatically plots extinction values of reference beam and sample for a series of wave-lengths, the intervals of which may be chosen at will. Sufficient for most purposes are intervals of 2 $m\mu$. A record in 2 $m\mu$ intervals of the range

Fig. 1. Scheme of the recording spectrophotometer. A grating monochromator (Bausch and Lomb 60 cm), B screen with triangular opening for adjusting the intensity of the in-going light beam, C gear, D balancing motor for adjustment of the position of B, E potentiometer, balancing against the photo cell, F light source (6 volt bulb or hydrogen lamp), G recording millivoltmeter, H toothed wheel (one cog = 1 $m\mu$) steering the wave-length drum, I device for moving H in distinct intervals, K cam wheel driven by a motor of constant speed, the wheel at will making one turn in 2, 4, or 6 seconds, L lever transmitting the movement of the lower cam of K to I and N (transmission by means of steel cables), N sliding object holder facing the out-going beam of the monochromator and alternately exposing the tube with the reference substance and the tube with the sample to the beam, O photo multiplier tube, P relay for switching over the current from O alternately to G or D, the relay being put in action by the high cam of K. — At one revolution of K in 4 seconds the schedule of operating is the following: 0—0.8 sec. high cam of K closing the circuit through P and bringing O in connection with D (adjustment of the light intensity to a fixed value), 0.8—2.4 sec. relay put out of action and O connected to G (recording of the reference), 2.4—4.0 sec. lever L lifted by the low cam of K, thus moving N to the left (recording of the sample), 4.0 sec. L falling down again thereby releasing the tension of I and instantaneously moving the wheel H one step.

between 400 and 620 $m\mu$ takes 3 to 6 minutes, a single band may be recorded in 10—20 seconds. A high degree of accuracy is attained, enabling optical resolution of bands lying 3—4 $m\mu$ apart (cf. Refs.^{3,4}).

Rapid recording of bands. Complete records of larger spectral regions may be used for the study of more slowly changing steady states. For a more incisive analysis of the kinetics, however, still more rapidly working procedures must be applied. Some bands, e. g. the α -band of cytochrome c, may be measured from the difference in extinction between 550 and 546 $m\mu$, which in the case of a difference spectrum (ϵ reduced- ϵ oxidized, cf. Fig. 4) corresponds to approximately 50 % of the total height. Some authors prefer to compute such measurements by means of a device implying rapid oscillation between the two wave-lengths². According to my experience with yeast constant

Fig. 2. Direct records of the top of the main bands of the cytochromes a_3 (444 $m\mu$), a (604 $m\mu$), c (550 $m\mu$), b (562 $m\mu$), and flavoprotein (460 $m\mu$), at alternating reduction in N_2 and reoxidation in air.

recording of the top of the band, viz. in the case of cytochrome c at 550 $m\mu$, serves the same purpose. With a sensitive and rapid millivoltmeter, e.g. Speedomax G supplied with A. C. 60 periods, changes in time intervals of ca. 0.5 seconds are within the reach of the operator (see Fig. 2).

Corrections for interfering bands. Accurate knowledge of the variations of the background of the spectrum constitutes the basis of all measurements. Baker's yeast, fortunately, lacks pigments which are interfering with the respiratorical enzymes, but the spectra of the different cytochromes and FP are partly overlapping, a fact necessitating a number of corrections. There is little hope for reliable results unless the spectral behaviour of the chosen object is thoroughly known. For successful calculation the observed spectrum has to be carefully compared with spectra of known preparations of the single enzymes, which must be recorded on the same instrument, because variations in dispersion and slit width may to some extent affect the shape of the bands. Good preparations of riboflavin, DPN and ADH are commercially available. Preparations of peroxidase and cytochrome c have been kindly afforded by Professor Keilin and Dr. Hill in Cambridge. Professor Kiese in Marburg kindly sent a preparation of cytochrome oxidase and recommended a method for the preparation of cytochrome b from beef heart. The spectrum of this preparation was carefully compared with the spectrum of the same cyto-

Fig. 3. Direct spectrograms (with filter paper as reference substance) of 15 % aerated yeast in a 17 mm wide quartz tube. $\times$ --- $\times$ aqua destillata, +---+ 0.1 M malonate pH 5.85. O---O 4 μ g antimycin A. About 30 % of cytochrome b remains reduced in aerated water or malonate, together with a few percent reduced cytochrome c. A slight curvature at 554 m μ in antimycin A marks the partial reduction of cytochrome c_1 .

chrome appearing after treatment of yeast with antimycin A. It was shown that the shape of the γ - and α -bands of reduced cytochrome b within ca. 5 % coincide with the shape of the corresponding bands of cytochrome c, but that oxidized cytochrome b, contrary to cytochrome c, shows low β - and α -bands, too, which are displaced a few m μ towards blue in comparison to the reduced bands (*cf.* Ref.⁴). This fact is able to complicate the *direct spectrum* (recorded with filter paper as reference substance; see Refs.^{1,3}) the twobanded weak spectrum of which (in the region 520—570 m μ) is a combination between about 70 % oxidized and 30 % reduced cytochrome b, together with a few percent of reduced cytochrome c (see Fig. 3). A similar weak two-banded spectrum also appears in roots of higher plants as will be shown in a separate publication.

Cytochromes c_1 and b_2 . These more recently studied enzymes are present in baker's yeast⁴. Cytochrome c_1 has its α -band at 554 and its γ -band at 422 m μ . Its concentration is about 50 % of that of cytochrome c. This cytochrome takes part in the normal aerobic respiration. Cytochrome b_2 has its α -band at 556 m μ and it is closely connected to an approximately equimolar quantity of FP^{5,6}. This cytochrome does not participate in the aerobic respiration. Its reduced bands appear after treatment with lactate, primarily in the presence of nitrogen^{4,6}.

The procedure of corrections. Following the recording of the purified enzymes or coenzymes in intervals of 2 m μ the extinction values are conveniently

Fig. 4. Difference curves of preparations of the cytochromes c, b and a ($a_b + a$), and flavo-protein (riboflavin). The reference contains the oxidized and the sample the reduced enzyme. The extinction values are calculated for the purpose of optical analysis, viz. the top of the bands set at 100. See the text.

calculated in percent of the main bands (see Fig. 4). Calculations on spectrograms of living objects ought to be made both from the *direct spectrum* (with strips of filter paper as reference substance^{1,3,4}) and from the *difference spectrum* (e. g., oxidized yeast as standard and O_2 -free yeast as sample). The difference spectrum is extended on both sides of the zero abscissa which is traversed at the isosbestic points (Fig. 4). The difference spectrum is more convenient to handle and it ought to be mentioned that corrections of records of single band tops (see above) have to be made according to the figures of the difference spectrum (Figs. 5, 6).

Corrections for turbidity. This question has been treated in some detail in a previous communication⁴. The turbidity, e. g. of a suspension of yeast, generally lowers the apparent molar extinction of bands in blue and ultra-violet, whereas bands in green and red are far less influenced. The scattering of the light may be to some extent calculated⁷, but the only accurate method is here direct determination of the depression of the bands of added pigments. Different objects may behave rather differently. In a suspension of 15 % yeast

Fig. 5. Example of optical analysis of a spectrogram, according to the procedure mentioned in the text. $\times$ — $\times$ record of a 15 % yeast suspension aerated with nitrogen. $\bullet$ — $\bullet$ spectrum of cytochrome b calculated from the difference 562—570 $m\mu$. +—+—+ spectrum of cytochrome c calculated from the difference 550—546 $m\mu$ minus the value of b at these wave-lengths. $\circ$ — $\circ$ — $\circ$ spectrum of cytochrome c_1 calculated as the observed curve minus c minus b.

the Soret bands are depressed about 33 % (factor 1.33). The same is true with regard to the band of FP at 450—460 $m\mu$, whereas the values of the DPNH band at 340 $m\mu$ have to be corrected with the factor 2.3. Dilution to 5 % here lowers the correction to only 1.25.

Technique for studying rapid changes in the steady states. As shown in Fig. 3 a suspension of washed yeast in aqua dest. or 0.02 M phosphate (pH 6—7) is predominantly oxidized if aerated for some 30 minutes. According to frequent variations in the concentration of DPN the balance may be attained in less than one minute but may occasionally take several minutes. Because there is always a residual reduction of several enzymes, *e. g.* cytochrome b (Fig. 3), in aerated yeast, a fact reflecting the intensive aerobic respiration, observations of the kinetics of reduction have to start from this state as the bottom level. Addition of glucose or still more of ethanol of course moves the steady state far more to the reduced side⁴.

Continuous recording of the involved enzymes now shows that reduction does not start immediately after the shifting over from air to nitrogen. As shown in Table 1 the time lag is directly influenced by the intensity of the previous aeration, *viz.* obviously by the quantity dissolved oxygen in the

Fig. 6. Optical analysis of the spectrum of DPNH. 3 ml of the sample contained 0.1 mg DPN, 3 mg ADH (from a commercial preparation) and 0.05 ml alcohol. Depth of the cuvette 1 cm. ●—● recorded spectrogram. ○---○ calculated spectrum of DPNH. ▲—▲ the difference between both, corresponding to the spectrum of ADH · DPNH.

medium surrounding the cells. All experiments were performed in "circulation vessels", made of molten quartz and holding 18 ml (Fig. 7). According to the shape of the vessel the suspension is vigorously moved by the stream of air or nitrogen blown from the narrow tube near the bottom. No yeast is accordingly sedimented, a matter of importance for the maintaining of constant light absorption, and the gas exchange is exuberant.

As may be seen from the direct records in Fig. 2 the reduction of the enzymes, primarily those of highest oxidation potential, $a + a_3$ and c , starts abruptly and proceeds practically linearly, at least up to more than 50 % reduction. This sudden reduction after a comparatively long lag period supports the idea that oxygen forms an adsorption layer in close contact with

Table 1. Relation between the duration of the aeration and the start lag of reduction in N_2 . Single values. Times in seconds.

Enzyme	DPN	FP	cytochrome b	cytochrome c	cytochrome a
Band $m\mu$	340	460	562	550	604
5 min. aeration	82	81	70	52	76
2 » »	76	77	69	46	70
1 » »	54	60	60	39	59
30 sec. »	—	43	40	24	—
15 » »	25	25	29	14	20
5 » »	5	5	5	—	5

Fig. 7. Scheme of a device for automatic alternating of streams of air and N_2 through the yeast suspension. A Cam wheel rotating with a speed of one revolution in 110 seconds. The cam closes an electrical contact which changes the position of the relay B. A V-shaped mercury contact tube alternately closes the current through the electrical valves V_1 and V_2 , one for air the other for nitrogen. On its passage from one position to the other the anchor of the relay touches the contact D which by means of a special device draws a short line on the record (see Fig. 2). C is the "circulation vessel" made by molten quartz, containing 18 ml yeast suspension, and with a diameter of the main tube of 17 mm. The hatched circle marks the centre of the optical field. The suspension is held in continuous movement by the gas current which escapes through a tube on the top of the vessel. The addition of inhibitors etc. may be made through the smaller tube to the right.

cytochrome oxidase, a layer operating as a buffer between the dissolved oxygen and the process of electron transfer. It may be tentatively assumed that the absorbed gas layer is carried by a hemoglobin-like pigment (*cf.* Ref.⁸), but it may otherwise be supposed that the apoenzyme of the oxidase adsorbs oxygen.

Preliminary experiments now showed that the lag phase of reduction remains constant within one second or less, if the two gases, air and nitrogen, are alternating in fixed intervals, provided that the gas stream is maintained at a constant value, and that the system has come to a balance. This observation together with the fact that the length of the lag phase is not identically the same for all enzymes served as the basis of a method for determination of the apparent kinetics of a complex system. The experimental outfit is shown in Fig. 7. Air and nitrogen (99.9 %) are fed to the circulation vessel *via* two electrical valves which are operated by means of a relay and a clockwork. Both gas streams are adjusted to the same speed, amounting to about 250 ml in one minute, which gives an intense bubbling in the right side of the vessel. The gas escapes through a tube leading to a flask with a layer of water above the gas outflow. The clockwork was mostly set for periods amounting to 35 seconds for the air and 75 seconds for the nitrogen. In order to obtain a precise record of the start moments the relay is provided with a guide-contact marking a line on the record each time the lever of the relay shifts position.

Apparent velocity constants. Thermodynamical velocity constants cannot be calculated for living enzyme systems, mainly for two reasons: 1) the enzymes are not in true solution but with few exceptions (DPN and possibly cytochrome c) attached to structural moieties ("mitochondria"); each enzyme

is structurally linked to at least one, frequently two or more other enzymes, a situation creating difficulties in clearly distinguishing between individual kinetics and group kinetics. An approximate estimation of the reaction velocity may be calculated from the half-time ht (cf. Ref.⁹), viz. the time elapsing from the start to the point of 50 % increase or decrease of the reduction. The value ht is inversely related to the reaction velocity. The apparent velocity constant is calculated as $K_{app} = c/ht$, where c = the apparent molar concentration, viz. that fraction of the actually present enzyme which partakes in the reaction.

EXPERIMENTAL RESULTS

Start lag and reaction velocity of the respiratorical enzymes.

The experiments were conducted at 20° C with 15 % baker's yeast (from Svenska Jästfabriksaktiebolaget), washed twice and resuspended in double distilled water. Aeration during 1—2 hours before start.

Table 2. *Start lags and apparent reaction velocity at alternating reduction and reoxidation (35 sec. air, 75 sec. N₂). $c = \mu\text{mole} \times \text{lit.}^{-1}$ moist yeast. Time unit seconds.*

Enzyme or coenzyme	a ₃	a	c	c ₁	b	FP	DPN
Measured band mu	444	604	550	554	562	460	340
Start lag of reduction	32.1	31.4	32.9	34.5	39.6	38.8	56.2
» » » oxidation	—	2.7	—	—	1.6	2.6	2.5
ht_{red}	1.26	5.42	1.75	—	3.10	4.51	10.0
ht_{ox}	4.36	3.78	5.60	6.7	2.54	4.87	1.3
$K_{app \text{ red}}$	7.1	1.6	11.6	—	5.8	8.6	45.0
$K_{app \text{ ox}}$	2.1	2.3	3.7	—	7.4	8.2	347.0

The start lags of reduction of the group a₃ + a and c show that these enzymes are the first to be "saturated by electrons" when the supply of oxygen is cut off. The slightly schematical picture of the start field in Fig. 8 clearly shows the close connection between a₃ and c, whereas a while starting as rapidly as a₃ takes a quite deviating course of reduction. In its time course it is very similar to c₁. These two enzymes are leading over to group b — FP which enzymes are starting about synchronously with the final stage of reduction of the group a₃ and c. The probably close linkage between cytochrome b and flavoprotein has been emphasized in a recent communication⁴. The existence of a lag of six to seven seconds between the first response of the groups a — c and b — FP points to the presence of a "buffer system" between these two. A member of this intermediate system is probably cytochrome c₁ and possibly also cytochrome a. The situation is complicated by side-lines which are certainly branching off from some of the enzymes, if not all of them, side-lines which imply a display of energy. Studies in the cytochrome

Fig. 8. Slightly schematical reproduction of the course of reduction of the respiratorical enzymes of yeast (cf. Table 6). The start lags are calculated from the moment when the reduction of cytochrome *a* begins. The first half-part of the reduction approximately follows a straight line, the course of the reduction above the 50 % line is somewhat more varying, but generally slightly curved. The exact position of 100 % reduction is difficult to ascertain.

system of wheat roots strongly support the assumption that the oxidation — reduction of cytochrome *b* is conducting synthesis of high energy phosphate. Such a coupling between processes giving off and taking up energy will, if they are reversible, act as a buffer system, too¹². The scheme in Fig. 8, however, may possibly be significant of the pulsations of the stream of electrons, because it shows that the group *b* — FP does not start reduction until the group *a*₃ — *c* is nearly completely reduced, and DPN does not start reduction until the group *b* — FP is completely reduced. This "pulsation" of the total process cannot be taken as an argument for disruptions in the structural integrity of the total system. It is more likely to assume that it reflects a grouping of oxidation potential states. From this point of view the deviating course of the cytochromes *a* and *c*₁ is most interesting. — Certain minimum quantities of DPNH are of course present from the beginning, even in well aerated yeast, and act as a continuously working source of electrons feeding the group *b* — FP and so on. Electrons are fed to DPN by, among others, the alcohol and glyceraldehyde phosphate dehydrogenases. The proceeding of the reduction of ADH can be measured on the ADH · DPNH band at 323—325 mμ (Ref.⁴, Fig. 6). Observations of the time course of the development of this band show that it starts considerably slower than the DPNH band, an observation alluding to the fact that a certain level of DPNH is needed before measurable quantities of the enzyme combination are formed.

Cytochromes a₃ and a. In their important work on the spectral characteristics of the cytochrome system Keilin and Hartree¹³ concluded that in respect of cytochrome *a* "we are dealing here with two distinct but closely allied and intimately connected compounds *a* and *a*₃". There has been much discussion in the literature as to this point. One of the main difficulties is the fact that the quantitative relation $\lambda_{444}/\lambda_{604}$ remains constant irrespective of the path of reduction¹⁰. A fixed combination of two pigments in equimolar concentrations would, however, render a similar result. The present observation of a deviating time course of reduction and reoxidation of the two bands 444 and 604 undoubtedly corroborates the original conception of Keilin and Hartree.

The results clearly show that a_3 is the oxidase, because it reacts about four times as rapidly as a at reduction. Some peculiarities of a were emphasized above. Others are appearing under the influence of antimycin A, as will be shown below.

The actually reacting quantities. As already mentioned these are not necessarily identical with the total quantities. Previous measurements⁴ pointed to equimolar total quantities of a , c and b . In the present experiments calculations of the activity of a only give *ca.* 50 % of the total quantity (see Table 3). Calculations from 444 $m\mu$ give higher values (see Table 4). Because no really pure preparation of $a + a_3$ is at hand all figures of molar concentrations are somewhat uncertain. The results, however, support the conclusion that under the conditions of the experiments the actually reacting quantities of a_3 and a are not always the same, even if the total amounts may be alike. Regarding the group b and FP the results corroborate the authors previous calculations. Experiments with N_2 , lactate, and antimycin A showed⁴ that cytochrome b is most probably connected to an equimolar quantity of flavoprotein. A similar close connection has actually been shown to exist^{5,6} regarding cytochrome b_2 . The fact that the actually reacting quantity of flavoprotein is twice as large as that of b , even if b_2 does not partake in the balance (see above), points to the existence of still another fraction of FP, which participates in the respiratorical chain but is not intimately linked to b . The following experiments with antimycin A will further elucidate this question. The high concentration of DPN in yeast is a well known fact. The experiments show that the total reacting quantity is 50—100 times as large as that of the single cytochromes and that nearly 50 % of DPNH is within 1—2 minutes bound to the alcohol dehydrogenase.

The response to antimycin A. Antimycin is claimed¹¹ to inhibit the transfer of electrons from cytochrome b to cytochrome c . If this antibioticum is added in increasing quantities to 18 ml of an 15 % yeast suspension (containing 2.7 g yeast) a visible reduction of cytochrome b starts at *ca.* 1 μg and an optimum

Table 3. Reacting quantities of the enzymes. Values in $\mu\text{mole} \times \text{lit.}^{-1}$ moist yeast.

Enzyme or coenzyme	a_{604}	c_{550}	b_{563}	FP ₄₆₀	DPNH ₃₄₀	ADH ₃₂₅
Absolute quantities	9.0	20.3	18.0	39.0	450.0	330.0
Relative, approxim.	1	2	2	4	50	40

Table 4. The effect of antimycin A on the cytochromes c , c_1 , and b in 15 % yeast (aerated) in vessels of 17 mm diameter.

Cytochromes	c	c_1	b
Observed extinction in:			
0.0 μg antimycin A	0.000	0.000	0.059
1.2 » » »	0.076	0.038	0.133
2.4 » » »	0.000	0.022	0.144

is attained at *ca.* 2.4 μg antimycin A. Larger quantities do not change the spectroscopic picture. It could, however, be shown that the initial effect of antimycin A is not exclusively confined to cytochrome *b*. Also cytochrome *c* is affected in the start period, and cytochrome *c*₁, too (Table 4). This intermediate reduction of cytochrome *c* reflects the comparatively slowly proceeding combination of antimycin A with cytochrome *b*, as can be observed by continued recording of the band 563 $m\mu$. The proportions between quantities of antimycin A needed for optimal effect on *b*, and the enzyme itself, are about equimolar. The reaction takes at least a couple of minutes. It could furthermore be shown that the reaction stops at *ca.* 80 %, the rest may then be reduced in nitrogen. The residual quantity of noninhibited cytochrome *b* is possibly responsible for the residual aerobic respiration of about 5–10 %, frequently observed after treatment with antimycin A, but this observation may be explained otherwise, too (see below). The effect of antimycin is reversible. It may be abolished by treatment with some dithionite followed by aeration.

Change of activities after treatment with antimycin A. The deepgoing effects of antimycin A are illustrated in Tables 5 and 6, computed from experiments with alternating reduction and reoxidation. If attention is first paid to the

Table 5. *Reacting quantities of cytochromes, FP, and DPN in one experiment with added antimycin A (ca. 4 μg per 2.7 g yeast). Values in $\mu\text{mole} \times \text{lit.}^{-1}$.*

Enzymes and coenzymes	a_{444}	a_{604}	c_{550}	$c_{1\ 554}$	FP	DPN
Control	5.5	8.5	20.3	—	39.0	450.0
Antimycin added	7.5	8.1	18.5	—	23.0	225.0
Relative values, control	100	100	100	100	100	100
» » + antimycin	135	95	91	52	59	50

actually reacting quantities (Table 5) these are remaining practically constant as far as *a* and *c* are concerned whereas the activity of *b* is practically completely abolished (not shown in the table) and that of cytochrome *c*₁, FP and DPN reduced to 50 %. The apparent rise of the activity of *a*₃ may be caused either by a more far going activation of the oxidized state under the influence of the lessened effect of the dehydrogenases, or simply by some interfering effect which is still obscure. The first explanation is, however, good enough. The observed 50 % reduction of the reacting quantities of FP strongly support the idea of a close molar linkage between cytochrome *b* and this pigment. The other half quantity of flavoprotein is obviously cooperating with the rest of the cytochrome system probably *via* the remaining quantities of cytochrome *c*₁. The fact that the reacting quantity of DPN is reduced to about the same percentage as FP is difficult to understand unless we assume the existence of two different enzymes performing the transference of electrons from DPNH to FP. One of these enzymes will only react with that fraction of total flavo-

Table 6. Effects of antimycin A on the kinetics of the respiratorical enzymes. Values in seconds. — control. + with antimycin A.

Enzymes or coenzymes	a ₃	a	c	c ₁	b	FP	DPN	ADH
Measured band	444	604	550	554	562	460	340	325
Start lag of reduction —	40.3	38.5	39.8	—	41.6	47.8	91.0	97.7
+	149.5	143.0	134.0	152.0	—	155.0	182.0	215.0
Start lag of oxidation —	values varying between 1.2 and 2.5							
+	» » » 1.9 » 2.5							
<i>ht</i> _{red} —	1.1	8.2	1.8	—	4.2	5.4	8.7	10.0
+	5.0	4.4	4.0	4.3	—	4.2	12.5	15.0
<i>ht</i> _{ox} —	2.9	1.7	2.5	—	1.7	3.8	1.4	—
+	1.3	1.8	0.7	1.3	—	1.2	3.3	2.5
<i>K</i> _{app red} —	5.0	1.0	11.3	—	—	7.2	52.0	—
+	1.5	1.8	4.6	—	—	5.5	18.0	—
<i>K</i> _{app ox} —	1.9	5.0	8.1	—	—	10.3	32.0	—
+	5.8	4.5	26.4	—	—	19.1	68.0	—

protein which is directly linked to cytochrome b. After blocking the b — FP moiety a corresponding quantity of DPN is put out of circulation.

The strongly reduced oxygen consumption (about 5—10 % of the normal) in yeast which has been treated with antimycin A is illustrated by the increase in the start lag of reduction from about 40 to about 150 seconds (Table 6). The retardation of the velocity of reduction (*K*_{app red}) of the cytochromes a₃ and c amounts to about 1/3 and is a consequence of the drop in DPN activity, viz. in the intensity of the stream of electrons, at preserved activity of the oxidase. Inversely a slower working mechanism of reduction raises the speed of reoxidation (from 1 to 3). It is significant that these reciprocal relations between *K*_{app} before and after the addition of antimycin A are less exaggerated with regard to flavoprotein, because the velocities are here calculated on the basis of 50 % lower activity. The residual quantity of FP must then, of course, speed up its *K*_{app ox} in relation to the oxidase. We are in Table 6 again meeting the deviating response of cytochrome a, as compared with a₃ and c. Its activity is seemingly only little affected by the sharp drop in the activity of reduction of DPN. Because it is not hampered with regard to active concentration one must conclude that it is involved in some oxidation-reduction balance outside of the scope of DPN and FP, perhaps in relation to high energy phosphate. This point needs further experimental elucidation.

Alternate pathways of electron transfer. Even if antimycin A is not inactivating cytochrome b to fully 100 % it could be calculated that under the conditions quoted in Table 6 the state of oxidation and reduction of this enzyme is not visibly altered. Reduction only causes a negative response at 562 mμ

(cf. Fig. 2) the magnitude of which is quite in accord with the calculated depression of the extinction curves of *c* and *c*₁ at this wave-length (see Fig. 4). It must consequently be concluded that the electrons largely bypass the inhibited cytochrome *b* and from the residual FP go to cytochrome *c*, probably *via* *c*₁, the behaviour of which in some way resembles that of *b*. This would be an alternate pathway of electron transference from DPN to *a*₃ and oxygen. But this way offers a poor substitute for the main line through cytochrome *b* which normally transmits 80—95 % of the electrons conducting aerobic respiration. The sharp drop in effectivity is nevertheless surprising, because a reduction of DPN and FP to half activity would only cause a retardation of $\frac{1}{2}$. The real depression, however, approximates $(\frac{1}{2})^3$ or even $(\frac{1}{2})^4$. For explanation two possibilities present themselves. It may either be assumed that the cytochrome *b*, in spite of the spell under which it is held by antimycin A, still maintains a strongly reduced activity, or it may be assumed that the alternate pathway from FP to *c*₁ and *c* is far less convenient (perhaps for structural reasons) as a track for electrons. In view of what was said above of the obvious outruling of cytochrome *b* and of the fact that its partner, *viz.* the halfpart of the total flavoprotein, is inactivated too, the odds are obviously more in favour of the second scheme of explanation.

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