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REFERENCES

1 G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 32 (1959) 362.
2 J. M. MUELLER, J. G. PIERCE, H. DAVOLL AND V. DUVIGNEAUD, *J. Biol. Chem.*, 191 (1951) 309.
3 J. BRUEGGEMANN AND K. DREPPER, *Naturwissenschaften*, 39 (1952) 301.
4 E. KAWERAU AND T. WIELAND, *Nature*, 168 (1951) 77.

SPECTROPHOTOMETRIC INVESTIGATIONS ON ENZYME SYSTEMS IN LIVING OBJECTS

IV. KINETICS OF THE STEADY STATES

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SUMMARY

A spectrophotometric technique was developed by means of which changes in the steady states of the respiratory enzymes after removal or addition of oxygen, could be accurately recorded. The results point to the existence of a spatial organization of the respiratory system, in which the individual enzymes form multimolecular units.

INTRODUCTION

According to current opinions the main cytochromes act in an "electron ladder", *e.g.*

$$\rightharpoonup b \rightharpoonup c \rightharpoonup a_3 \rightharpoonup O_2,$$

the letters representing single molecules. Recent observations point to more complicated couplings[1-4] and an organization in multimolecular entities or groups, which interact only at special points or surfaces[5,6]. The fact that a specific time order of reduction of the steady states is observed in two very different materials, wheat roots and baker's yeast[6], indicates not only the existence of a common scheme of spatial organization in the respiratory system but also a uniform access to oxygen in the state of normal aeration.

Observations on baker's yeast[5] have shown that the steady states of the cytochromes a_3 and c vary approximately synchronously and the same is true for cytochrome b and flavoprotein (FP). The latter begin to be reduced only after the cyto-

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chromes a_3 and c are more than 50 % reduced. These observations support the idea that cytochrome c is probably spatially co-ordinated with a_3 (cytochrome c oxidase). Observations on homogenates of wheat roots showed that the molar ratio a_3/b remains approximately constant for particles of different size[7]. The fact that cytochrome c is easily detached from the group a_3-(a-)-c and flavoprotein detached from the group b-FP may hypothetically be interpreted by supposing that cytochromes a_3 (a) and b form the structural skeleton, and cytochrome c and FP are in some way interposed.

GREEN and co-workers[1,8] found that further fragmentation of the "electron transport particles" (ETP), which carry out oxidation of metabolites but have lost the power of phosphorylation, yields 3 still smaller particles. One of these is green and contains all the cytochrome oxidase and much of the system for oxidation of DPNH. Another small particle obtained by enzymic digestion of ETP contains purified cytochrome oxidase. It is difficult to imagine how cytochrome oxidase could be isolated as a multimolecular particle, if this enzyme did not exist as multimolecular groups in the living structure.

EXPERIMENTAL

Drainage and aeration of root recipients

A suitable device for investigating the absorption spectrum of wheat roots has been delineated in previous communications[9,10].

The recipient was drained by aerated solutions flowing at a rate of 2 drops/sec, thus maintaining an O_2-concn. about 4–5 times above *the minimum at which visible reduction begins*. At 20° the oxygen in the recipient is consumed in about 2 min if the flow of aerated solution is stopped. The start of reduction of cytochromes a_3 and c is then retarded for about 30 sec. The O_2 present in the root tissue, viz. inside the cells and in the intercellular space, is consumed in less than 1 min, thus reducing the previous retardation to 5–10 sec, if the aerated medium is rapidly exchanged for an O_2-free solution.

Aeration of yeast suspensions

Baker's yeast (from Svenska Jästfabriks AB) was washed twice in distilled water and vigorously aerated in order to remove residual glucose. Because the reduced phosphopyridine nucleotides slowly disappear during aeration (see Table I), the

TABLE I

INFLUENCE OF VARIOUS AGENTS ON THE BAND AT 562 mμ OF AERATED YEAST

	*562–566 mμ**	*340–360 mμ**
Water aerated 2 h	100	100
Water aerated 12 h	95	100
Water aerated 24 h	90	31
Water aerated 30 h	82	25
Water aerated 30 h + 0.05 *M* KCl	70	
Pyrophosphate (0.1 *M*)	73	
Malonate (0.1 *M*)	63	
Fumarate (0.1 *M*)	57	

* Relative extinctions of band portion.

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velocities of reduction decrease and the velocities of reoxidation increase on prolonged aeration[9,11].

The yeast suspension (mostly 15 % for studying α-bands of cytochromes and 5 % for γ-bands, diphosphopyridine nucleotide [DPN] and flavoprotein) was transferred to a "circulation vessel" (see Fig. 2 of ref. [11]) and vigorously aerated (air, O_2, or 99.99 % N_2 from AB Gas accumulator). The start times of reduction after switching on N_2 instead of air (or O_2) of course depend on the amount of O_2 dissolved in the medium, *i.e.* on the length of the periods of aeration (Table I of ref. [5]). The minimum concn. at which visible reduction of the respiratory enzymes start, lies at about 0.1 of full saturation with air. Yeast fed with 1 % glucose, however, responds positively to aeration with pure O_2[12].

Spectrophotometers and measuring of bands

Two of the recording spectrophotometers, which were constructed and built in this laboratory, have been described in previous publications[5,9,10]. They are built along principles which enable accurate calculation of absorption bands. Precautions in order to avoid the effects of scattering in turbid media have been discussed in previous communications[4,11].

These spectrophotometers may be used for accurate recording of comparatively slowly proceeding changes in the steady states. A duplicate recording (reference + sample) at one wavelength takes 2 sec and the region 530–570 mμ, covering the α-bands of cytochromes *c*, c_1, b_2, b_3 and *b*, is recorded in 40 sec, if the wavelength intervals are set at 2 mμ. This moderate speed is often sufficient for tracing shifts in the steady states of the respiratory enzymes in wheat roots and other plant tissues[4,9,10].

For the more rapidly changing steady states in yeast[5] or in chloroplasts[4] continuous recording of the band tops enables more rapid work. By special adjustment of a recording Speedomax G millivoltmeter the sensitivity attained (full scale deflection in about 0.5 sec) was sufficiently high to enable recording of the change in steady states of the respiratory pigments[5,12]. Synchronization of the readings of the whole set of pigments (cytochromes a_3, *a*, *c*, c_1, b_2, *b*, flavoprotein, pyridine nucleotide) was achieved by means of a special device switching over from air to N_2 at constant intervals[5].

The specially trimmed Speedomax G may also be used for the recording of scanning spectrograms, viz. automatic repetition of a portion of the spectrum. Fairly good records have been obtained by intermittent recording of an 80-mμ long spectrum in 6 sec. The scanning is performed by a device which moves the wavelength screw of a Bausch and Lomb grating monochromator (60 cm 1200 lines) to and fro. The changes of the steady states of baker's yeast may be followed quite well by means of this simple device, if the reactivity is slowed down by cooling the sample to a few degrees above 0°.

For tracing the finer details of the spectrum a special *scanning spectrophotometer* was built. This instrument enables records of sufficiently large portions of the spectrum in fractions of a second. The recording millivoltmeter is a sensitive oscilloscope combined with a film camera. The automatic oscillation of the wavelength screw works satisfactorily down to about 1/3 sec/single record. For still more rapid work a rotating mirror was introduced, which flashes the spectrum on the sample. The only practical

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speed limit set here is the inevitable hum, notably if this is caused by frequencies near the time value of the intervals. It is fortunate that the shifts in the steady states of microorganisms seldom necessitate shorter time intervals than 1/3 sec. For a study of the kinetics of the steady states in yeast at 20° intervals of 0.5 sec proved useful (see Fig. 1).

Oscillographic recording has the advantage of convenient marking of wavelengths on the record. Most oscilloscopes are provided with a device for suppressing the light spot. In our instrument a circular disc on the axis of the wavelength screw carries "riders", the position of which may be fixed at any wavelength. Turning the axis brings the riders in touch with a contact, which for a very short moment (corresponding to about 2 Å) suppresses the image.

Recording of complete spectra is to be preferred to recording only the difference between 2 wavelengths[17], because it permits the tracing of accessory pigments, *e.g.* cytochromes c_1 and b_3, and the controlling of shifts in the background of the bands caused by positive or negative coincidences (above or below the isosbestic level). The α-band of cytochrome *b* is lowered by coincidence with the negative portion of the cytochrome *c* band at 550 mμ. For accurate measurements of the b band a small portion is therefore selected, *e.g.* the difference 562–566 mμ (*cf.* Table I), where the depression of the c band is approx. uniform.

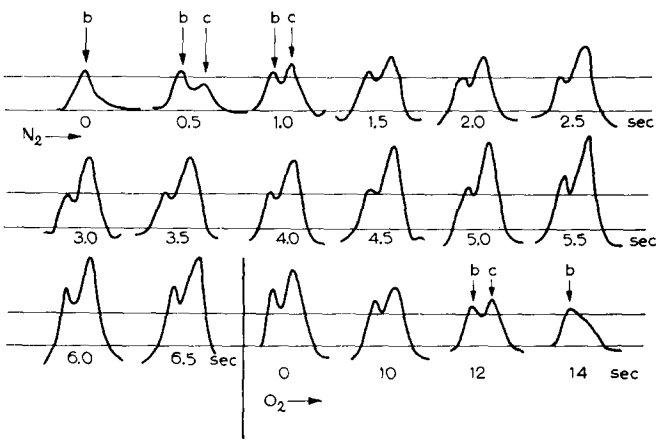

Fig. 1. Increasing reduction (in N_2) and increasing reoxidation (in air) of a 15% suspension of baker's yeast. Direct copies from the oscillograms of the scanning spectrophotometer. Note the starting lag between cytochromes *c* and *b* and the initial decrease of the *b* band owing to the depression at 562 mμ in the difference spectrum of cytochrome *c* (*cf.* Fig. 7).

EXPERIMENTAL RESULTS

Steady states at full oxygenation

In the steady state of maximum oxidation the direct absorption spectrum of wheat roots (filter paper as reference) invariably shows a 2-banded spectrum in the green with broad elevations at about 530 and 557 mμ (the latter band is seen in Fig. 2). It was tentatively assumed[7] that this spectrum of oxygenated wheat roots is composed of fused bands of about 70% oxidized cytochrome *b* and 30% reduced cytochrome *b*, the broad band at 530 mμ of oxidized cytochrome *c* and smaller quantities of cytochromes c_1 and b_3.

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Starved baker's yeast also shows a 2-banded spectrum in the fully oxygenated state[5], viz. a broad band at 530–535 mμ and a more pointed band at 562 mμ. These bands are only slightly changed by prolonged aeration (up to 48 h) and repeated washing. The band at 562 mμ is lowered on treatment with 0.1 *M* pyrophosphate, malonate and fumarate, agents which are known as promotors of oxidation of the cytochrome system (see Table I). The average ratio (22 spectrograms) between the measured differences 562–566 mμ and 530–536 mμ was 100/23, which is not very different from the figure, 100/18, found with a pure preparation of reduced cytochrome *b*. The strong development of a broad band at 525–535 mμ in oxygenated yeast may obviously be considered as a summation of the broad bands of oxidized cytochromes *c*, c_1 and *b*.

The broad band at about 530 mμ disappears with increasing reduction, both in preparations of cytochrome *b* (courtesy of Prof. OKUNUKI) and in living yeast. As a consequence, the difference spectrum shows a comparatively lower β-band than the direct spectrum, whereas the appearance of the α-band is not measurably disturbed.

Besides the cytochromes, wheat roots contain large quantities of peroxidase[10,13]. Peroxidase is not directly reduced under anaerobiosis, but combines with a number of hydroxylated compounds[14]. The complexes are extremely autoxidizable, but a "peroxidase-dh" complex[4,13] appears in the complete absence of O_2. The spectrum of this complex is strikingly similar to that of a cytochrome. Because oxidized peroxidase has a broad band at 500 mμ, which slopes gently towards 600 mμ, the disappearance of this band simultaneously with the formation of the dh-complex changes the background of the cytochrome spectrum; this must be taken into consideration in "optical resolution" of the absorption spectrum[4]. Measurements at small intervals, similar to those recommended in this communication, are, however, only slightly affected by peroxidase. Published spectrograms of wheat cytochromes[4,9,10] and the elucidation of the dh-complex show that wheat roots are not such an "exceptionally difficult material for spectrophotometric studies" as SMITH AND CHANCE[15] claim.

Clear knowledge of the spectral properties of the oxygenated systems is a prerequisite for studying the changes caused by varying O_2 supply. Measurements with the scanning photometer show that the extinction at 530 mμ in the first 20 sec of increasing reduction of cytochrome *c* remains apparently stationary. Because the band of oxidized cytochrome *c* at about 530 mμ has an intensity about the same as that of the reduced β-band at 520 mμ (*cf.* Fig. 3 of ref. [11]) no appreciable alteration can be expected in this region. The extinction of the combined β-bands of *c* and *b* are measurably altered only when the reduction of cytochrome *b* begins. These observations do not support the assumption of the existence of an additional respiratory pigment with a band at 530 mμ.

Summing up all observations on the state of oxidation of the cytochromes in oxygenated roots and aerated starved yeast it may be concluded that cytochrome *c* is predominantly (at least 90 %) oxidized. The steady states of cytochromes $a_3 + a$ are also moved far to the side of oxidation. With regard to c_1 the evidence is less clear. There is a possibility that this enzyme, which links *b* to *c* and/or dehydrogenases[5], is more reduced than *c*. Cytochrome *b* remains oxidized to the extent of only 50–70 % but can become somewhat more oxidized under the influence of agents which retard the transference of electrons from the dehydrogenases (Table I). The stationary partial reduction of cytochrome *b* is more pronounced in yeast than in wheat roots. This

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fact may be connected with the higher concn. of pyridine nucleotide in yeast. In both materials the amount of flavoprotein is about double that of cytochrome *b*[5,10]. The fact that the steady state of cytochrome *b* is moved considerably more towards reduction than that of cytochrome *c* is surprising, because the rapid reduction of cytochrome *c* points to a ready delivery of electrons from cytochrome *b*. It may be remembered here, however, that cytochrome *c* probably receives electrons from both *b* and c_1. Also the reversible trapping of electrons in high-energy phosphate must be taken into consideration.

Time course of reduction in wheat roots

Spectrograms of this process have been published in a previous communication (Figs. 2 and 3 of ref. [7]). It was shown that the α-bands of reduced cytochromes a_3 and *c* appear almost immediately, followed in 1–2 min by increasing reduction of cytochrome *b*. The peroxidase-dh complex is not formed during the first 10–15 min of reduction; 1–2 h elapse before its α-band at about 571 mμ is fully developed. The delayed appearance of the dh-band facilitates observation of the cytochromes, which are largely reduced before the disappearance of the free peroxidase alters the background of the spectrum.

That cytochrome c_1 participates in the respiratory system of wheat roots appears from Fig. 2. In this case the region 540–570 mμ was recorded at intervals of 1, 3, and 50 min, respectively, from the start of anaerobiosis. No alteration of the spectrum can be noticed at 563 mμ (cytochrome *b*) during the first minute, whereas a considerable rise in extinction occurs between 545 and 555 mμ, corresponding to an increased reduction of cytochromes *c* and c_1, the latter showing a distinct peak at 553 mμ.

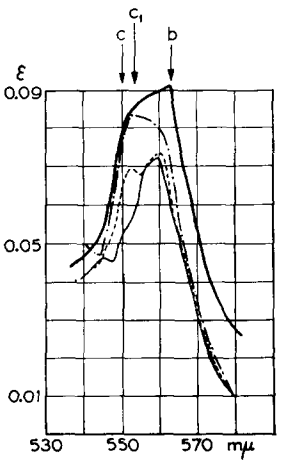

Fig. 2.

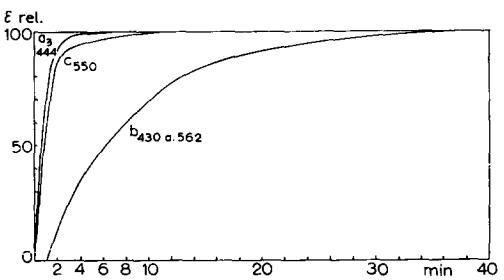

Fig. 3.

Fig. 2. Increasing reduction of the cytochromes *c*, c_1, and *b* in wheat roots. ——— aerated solution, ------- O_2-supply stopped for 1 min, —·—·— for 3 min, ——— for 50 min. No interference, with peroxidase. See the text.

Fig. 3. Increasing reduction of the cytochromes a_3, *c* and *b* in wheat roots after stopping the O_2 supply.

The relation between cytochromes *c* and c_1 is consequently similar to what is known about yeast[5].

The extinction at 563 mμ does not start for about 3 min. A distinct α-peak of reduced cytochrome *b* is observed in 50 min. The reduction of *c* and c_1 is completed in 3 min, whereas the reduction of cytochrome *b* continues and finishes considerably later. These observations, which were repeated in several expts. (Fig. 3), refute the

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"theory" that no cytochrome "should reach complete reduction greatly out of step with the others"[15].

The time course of increasing reduction in the steady states was also studied in *expts. with O_2-free dithionite* (0.02 *M*, adjusted to pH 6) *as the reducing agent.* For these expts., a special device was constructed. The solutions, 0.02 *M* dithionite and aerated distilled water, were alternately conducted through the recipient. The intervals (2 + 2 min) were controlled by electric valves and a timer. The spectrum was continuously recorded by means of the scanning spectrophotometer. The results of these expts. (Figs. 4 and 5) are very similar to those observed in the expts. in which the flow of aerated water was stopped (Fig. 3); cytochromes $a + a_3$ were first reduced, followed by *c* and then *b*. A difference was noticed, however, in that the reduction of cytochrome *c* was somewhat retarded, as compared with aqueous media, and that of cytochrome *b* was somewhat accelerated. Flavoprotein was reduced at about the same rate as cytochrome *c*. The total process was considerably shorter than in anaerobiosis, because no residual oxygen had to be consumed before reduction could start.

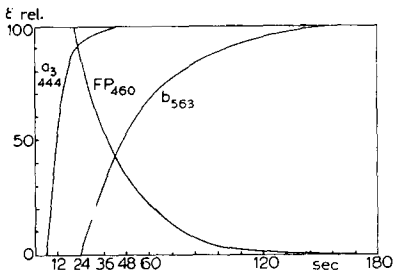

Fig. 4. Increasing reduction of the cytochromes a_3 and *b*, and of flavoprotein (FP) during treatment with O_2-free 0.02 *M* dithionite at pH 6.0.

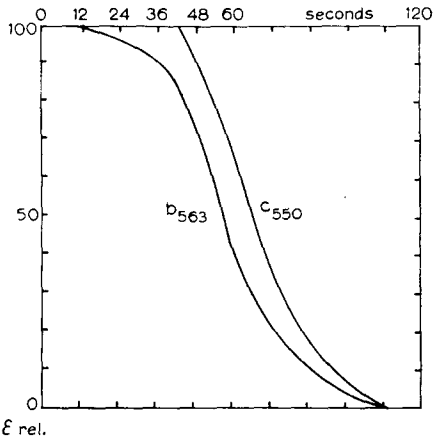

Fig. 5. Reoxidation in aerated water after treatment with dithionite.

The fact that anaerobiosis and dithionite both display a definite starting order of the respiratory pigments supports the conclusion that the rhythmical sequence of increasing reduction is an effect of spatial organization and not of a dubious "oxygen diffusion"[15]. Dithionite is known to reduce all cytochromes directly. The expts. quoted in Figs. 4 and 5 show, however, that the reduction starts with the cytochromes $a_3 + a$ and then follows the same order as in anaerobiosis. Dithionite apparently primarily attacks the cytochrome oxidase (see below), hence the observed time order. A certain direct effect on *b* may be concluded from the "apparent velocity" (see below).

As to the *velocity of the changes of the steady states* observations were made of the half-time of reduction, the inverse value of which gives the "apparent velocity" of the processes (see Table II).

It appears from Table II that cytochromes a_3 and *c*, taking account of their different concns., act with about the same molar velocity, whereas the reduction of *b* is considerably slower.

Reduction in dithionite shows figures which are somewhat different from those

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TABLE II

APPARENT VELOCITY OF REDUCTION OF COMPONENTS IN WHEAT ROOTS WHEN THE SUPPLY OF OXYGEN IS STOPPED

Average of 15 expts.

Cytochromes	a_3	*c*	*b*	*dh-complex*
1/half time (sec) of reduction	0.031	0.015	0.003	0.002
Relative concentration	1	2	4	—

in Table II. The half time of reduction of cytochromes a_3 and *c* amounts to about one half the value obtained for cytochrome *b*, compared with the relation 1:5 in anaerobiosis. This may be interpreted as a "walkover", the dithionite obviously to some extent directly reducing cytochrome *b* (see above).

Time course of reoxidation in wheat roots

Owing to the rapid movement of O_2 into cells and tissues reoxidation is a rapid process. The sequence of oxidations and the apparent velocities are more uniform than during reduction, but there is a tendency to reversal of the starting lags, viz. reoxidation in the order $b > a_3$-*c*, which appears also during reoxidation after reduction with dithionite (Fig. 5). The order is, however, liable to variations caused by varying relative quantities of the reacting enzymes. The apparent velocity of reoxidation, too, shows the relatively faster reaction of cytochrome *b* (Table III). The total process is completed in about 3 min at 20°.

Reoxidation of cytochrome a_3 closely follows a first order reaction (Fig. 6). The molar velocities of all cytochromes are about the same.

TABLE III

APPARENT VELOCITY OF REOXIDATION OF COMPONENTS IN WHEAT ROOTS IN AERATED WATER

Average of 12 expts.

Cytochromes	a_3	*c*	*b*	*dh-complex*
1/half time (sec) of oxidation	0.095	0.042	0.026	0.033
Relative concn.	1	2	4	—

Time-course of reduction and reoxidation in baker's yeast

The velocity of reduction is controlled by the activity of the pyridine nucleotides (see Table I). All expts. were performed at 20° with sufficiently aerated suspensions (mostly 15 %) of washed yeast.

The scanning spectrophotometer permits measurements of spectral changes at any wavelength in the recorded spectrum. In Figs. 7 and 8 reduction and reoxidation were measured at 522, 535, 550, 562 and 572 mμ. In this expt. cytochrome *c* (550 mμ) started reduction 1 sec after switching over to N_2. Its reduction follows the curve of a first-order reaction (Fig. 9). Synchronously with the rise of extinction at 550 mμ the depression below the isosbestic line appears at 535 mμ. The final value is here 20 % lower than that calculated from pure cytochrome *c*, obviously because of an interference by the corresponding depression of cytochrome c_1. Synchronously with

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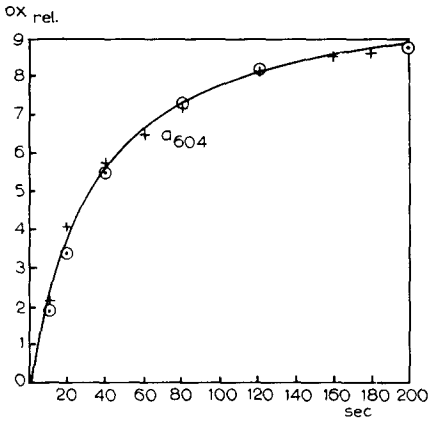

Fig. 6. + Reoxidation of cytochrome *a* in wheat roots, after complete anaerobiosis, upon addition of aerated water, ⊙ calculated curve according to the formula $A = A_0 \cdot e^{-kt}$, A_0 representing the quantity of fully reduced cytochrome and A the amount at time t.

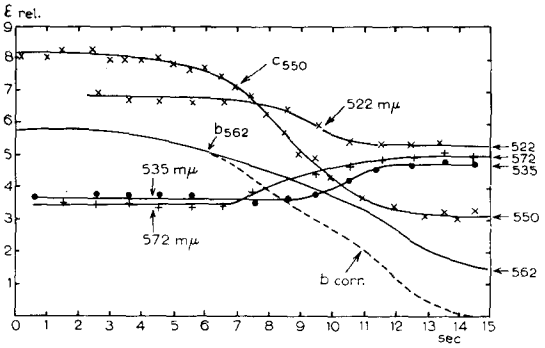

Fig. 8. As in Fig. 7. Reoxidation on switching on air. The position of the starting points on the scale is arbitrary.

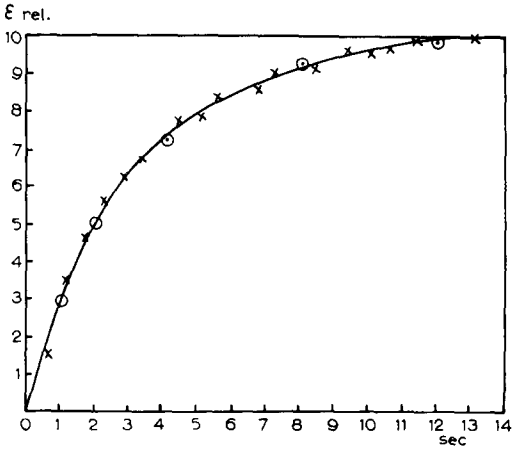

Fig. 9. Curve of increasing reduction of cytochrome *c* of yeast (rise of the α-band at 550, measured as the difference 550–554 mμ). ⊙ Values calculated for a first order reaction (*cf.* Fig. 6): $k = 0.34$.

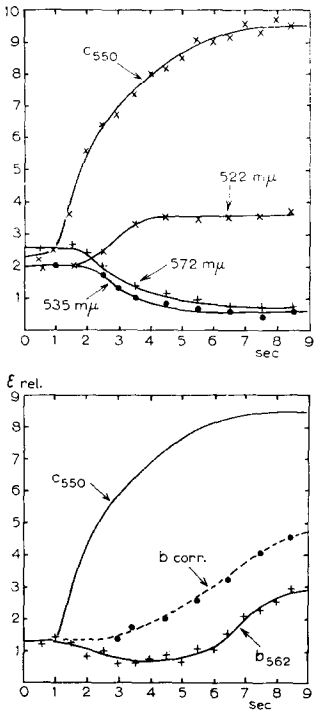

Fig. 7. Measurement of spectrograms recorded with the scanning spectrophotometer at intervals of 0.5 sec (*cf.* Fig. 1). Material: 15% washed yeast in water. Upper diagram: changes in the extinction upon switching on N_2. Lower diagram: increasing reduction of cytochromes *c* and *b*.

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the α-band at 550 mμ the combined β-bands of cytochromes c and c_1 at 522 mμ increase. Here again the final value exceeds the calculated value by 20–25 % because of the coincidence with c_1.

The extinction at 562 mμ (Fig. 7) first decreases, due to reduction of cytochromes $c + c_1$, the difference spectrum of which is lowered below the isosbestic line at this wavelength (*cf.* Fig. 1). The depression amounts to about 27 % of the corresponding height of the α-band of cytochrome c. Correction for this depression gives the true curve of an increasing α-band of cytochrome b (see Fig. 7). The increase of this band begins about 2 sec later than that of cytochrome c and the curve of b is considerably flatter than that of c.

Measurements at 572 mμ, at which wavelength the α-band of cytochrome b is not far from the isosbestic point, clearly show the depression caused by cytochromes $c + c_1$. Because the depression is about the same at 562 mμ and 570 mμ (the deviation is of the order 5 %) measurements of the difference 562–570 mμ, or 562–566 mμ (*cf.* Table I), represent the spectral changes of the α-band of cytochrome b. Cytochrome b_2 does not participate in the anaerobiotic reduction of the respiratory chain[5] and other b-cytochromes (b_3 etc.) apparently do not participate in appreciable quantities[7].

During reoxidation (Fig. 8) the bands of cytochromes c and b decrease approx. simultaneously. Frequently, however, reoxidation of b starts a little earlier than that of c.

The time courses of reduction and reoxidation of cytochrome oxidase (a_3) and flavoprotein (FP) were measured by the differences between the top of the bands (444 and 470 mμ respectively) and the isosbestic point of a_3 viz. 462 mμ (*cf.* Fig. 2 of ref. [12]). During reduction the values of a_3 increase and those of FP decrease. The curves in Fig. 10 show a starting lag of about 2 sec between a_3 and FP and a considerable difference both in the shape of the curves and in the duration of the processes, a_3 being fully reduced several seconds before FP; similar differences were observed between cytochromes c and b. These facts corroborate the conclusion drawn in previous publications[5,6] that a_3-c form a group which reacts more rapidly than the group b-FP during reduction. Cytochromes a_3 and c cannot easily be recorded in the same flash spectrum; for the difference in the sensitivities of the photo cell at 444 and 550 mμ, respectively, is too large. During reoxidation flavoprotein reacts

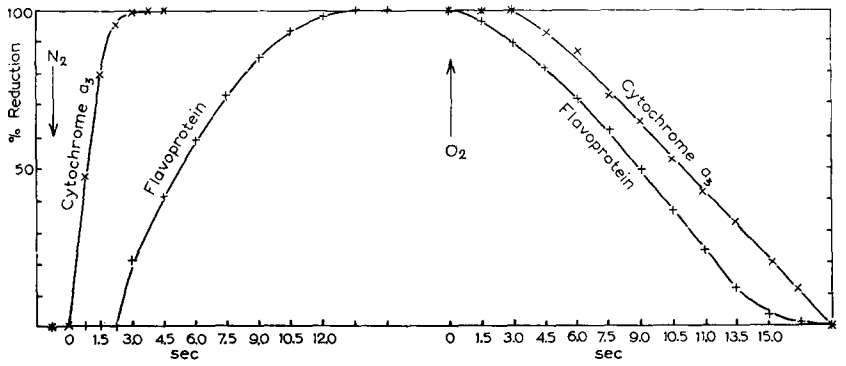

Fig. 10. Increasing reduction (N_2) and reoxidation (air) of cytochrome oxidase and flavoprotein in a 15 % yeast suspension. Scanning intervals 0.75 sec.

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more quickly than the cytochrome oxidase (Fig. 10). The time lag is thus here the same as between cytochromes *c* and *b*.

If the starting time of cytochromes a_3 and *c* is taken as zero, the mean *starting lags of cytochrome* b *and flavoprotein* amount to 2.7 sec and 2.2 sec, respectively. *The apparent velocity of reduction* is shown in Table IV.

Here again cytochromes a_3 and *c* on the one hand, and cytochrome *b* and FP on the other follow one another closely. The *apparent velocities of reoxidation* were measured only on a few spectrograms. Very similar values were obtained, viz. 1/half time of reduction = 0.20–0.24 for cytochrome a_3, cytochrome *b* and flavoprotein.

TABLE IV

APPARENT VELOCITY OF REDUCTION OF COMPONENTS IN STARVED YEAST AFTER SHIFT FROM AIR TO N_2

Average of 27 experiments.

	a_3	*c*	*b*	*Flavoprotein*
1/half time (sec) of reduction	0.45	0.45	0.24	0.24

These values for the starting lags and apparent velocities refer to "standard conditions" of starved (washed) and aerated yeast. Variations may, however, be observed under changed conditions, notably at higher levels of the stationary reduction of cytochrome *b* in the oxygenated state (see above).

DISCUSSION

The starting lag of reduction of cytochrome oxidase is obviously caused by residual oxygen. If an aerated suspension of starved 15 % yeast is switched over to N_2 the residual O_2 amounts to about 50 times the quantity of cytochrome oxidase, corresponding to 200 turnovers of each molecule. At a turnover rate of about 20/sec[4,5,9] the residual O_2 ought to be consumed in about 10 sec. Actually, somewhat larger lags are observed, because the concn. of O_2 continuously decreases. Most of the residual O_2 is consumed before the steady state starts moving towards reduction. The same was observed in wheat roots. Several arguments support the idea that the last traces of O_2 are adsorbed in the protoplasm and that reduction starts only after the disappearance of the O_2 which is dissolved in the vigorously stirred medium[4,5,10].

If the half-time of reduction of cytochrome a_3 amounts to 2 sec and the turnover number to 20/sec, it may be concluded that at the moment of reduction the molar quantity of O_2 present in the vicinity of the respiratory system exceeds that of cytochrome a_3 by a factor of 10–20. The fact that the process is of first order (see above) supports the assumption that the residual O_2 is accessible at random and that "diffusion" plays a subordinate role.

During the elimination of the residual O_2 electrons flow continuously through the respiratory chain. If we assume that cytochrome a_3 builds up the main lattice (or some kind of spatial organization) and that *c* occupies the interstices, the rapid turnover of the electrons makes it difficult to trace any differences in apparent

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velocity. If the affinity of a_3 for oxygen exceeds that of c for a_3, a somewhat lower apparent velocity of reduction of a_3 compared with that of c would be expected; this has actually been observed (Table II). A most surprising result is the large difference in apparent velocities of reduction with cytochromes a and a_3. Cytochrome a does not react with oxygen[12]. Its close spatial attachment to a_3 is apparent not only from the extreme difficulties encountered in separating the 2 enzymes but also from the fact that the reduction of both begins approx. simultaneously[5,12]. The slower reaction of a, however, points to a line of reduction which is not linked to cytochrome c.

A "b-shunt to oxygen"[4] is only weakly developed in yeast and it is doubtful if this shunt, even in wheat roots, competes appreciably with the rapid oxidation of b by cytochrome c. The starting lag between c and b at increasing reduction, in which reduction of cytochrome b begins after about 70 % reduction of cytochrome c, cannot be attributed to "diffusion of oxygen"[14] but only to the interaction of c and b. This is also evident from the expts. with dithionite. If we symbolize the interaction of b as follows: $\xrightarrow[v_{red}]{on} b \xrightarrow[v_{ox}]{off}$ c, where v_{red} and v_{ox} symbolize the velocities of electron transference, a steady state implies $\frac{v_{red}}{v_{ox}} = 1$. If the path of electrons from c is blocked, v_{ox} decreases. At undiminished v_{red} this leads to rising reduction of b. The fact that a decrease from about 100 % oxidized c to about 30 % does not affect the steady state of cytochrome b cannot easily be explained except as a curtailment of the interaction between b and c owing to a spatially regulated path of electron transference. A hypothetical scheme of canalized electron transference is pictured in Fig. 11 (*cf.* also Fig. 1, ref. [6]).

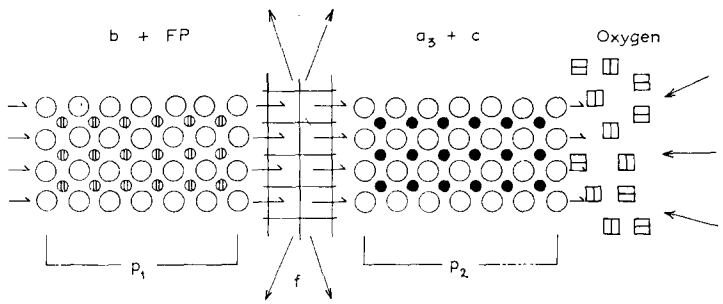

Fig. 11. Hypothetical scheme of the spatial organization of the cytochromes.

The electrons in Fig. 11 move along the lattices of the multimolecular enzymes. If c is completely oxidized at the start, blocking of the electron transference at the oxygen side would affect the electron transference at the place of interaction between b + FP and $a_3 + c$ only after p_2/TN sec (TN = turnover number). A layer of 40 molecules of cytochromes $a_3 + c$ would thus delay the start of visible reduction of cytochrome b by about 2 sec if TN = 20/sec. During this interval the reduction of c would proceed, starting at the interphase between $a_3 + c$ and O_2 and moving to the left.

The presence of a "factor" (f) between the 2 groups of cytochromes, as pictured in Fig. 11, would also delay the response to a change in the conditions. The arrows spreading from the factor symbolize co-ordinated processes which may trap, or

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additionally supply, electrons. An objection to this very schematical visualization of the path of electrons may perhaps be raised, namely that a series of 20–40 molecules would result in a fairly thick membrane. There is, however, the possibility that the molecules are arranged helically, and, in fact, this structure would suit the hypothetis. It should be mentioned that BUVAT AND LANCE[18] recently observed coiled threads in plant mitochondria.

It is difficult to imagine any retardation except by an elongation of the path of electrons through each enzyme, because the exptl. results point to an undiminished speed (turnover) of the electrons during the observed changes in the steady states. The idea of spatial organization of the respiratory system, including cytochromes, flavoprotein and "factors" is supported by the fact that these enzymes frequently show fairly simple quantitative relations[4,5,7].

The guided electron transference pictured in Fig. 11 cannot be expected to result in reactions of first or higher order, because the flux of electrons is probably similar to that in a metallic conductor. This would imply a tendency to reactions of zero order. The frequently flattened curve which illustrates the increasing reduction of cytochrome *b*, as compared with that of cytochromes *c*-a_3, supports the idea of a straight flux of electrons. Similar conclusions may be drawn from calculations of the relation between quantity and apparent reaction velocity in the case of the enzymes cytochrome *c* and FP[16].

In contrast to the *guided electron transference through the organized respiratory system* the transference of electrons to oxygen obviously follows the conventional scheme of reactions in a Newtonian medium. The O_2 molecules are freely movable, even if they probably are transiently adsorbed in the protoplasm. Curves of first order were observed both during variation of the O_2-pressure in the medium[12] and during the elimination of the residual O_2 (see above).

The fact that visible reoxidation frequently starts at cytochrome *b*, but not at a_3 or *c*, was emphasized in a preliminary communication[6]. Here the contact surfaces between the interacting enzymes and the specific velocity of electron transference regulate the process. A leading role is played by the transition $b \rightarrow c$ (*cf.* above; see ref. [16] for "reaction ratios").

In wheat roots the interval between starting reduction of cytochrome *b* and starting reduction of cytochrome *c* is considerably larger than in yeast. Observations on homogenates show, however, that cytochrome *b* and the group *c*-a_3 are spatially linked together. There is a possibility that cytochromes *b* and *c* are separated by a substantial factor, through which the electrons are sluiced, but the assumption that *b* interacts in some way with the peroxidase·dh-complex is more likely. If a factor (*cf.* Fig. 11) divides the stream of electrons from cytochrome *b* in 2 pathways, one through *c* and a_3 and the other through the dh-complex of peroxidase, this would delay the reduction of *b*. Observations show that the time course of reduction of *b* and dh is co-ordinated, but that the development of reduced dh proceeds somewhat more slowly than the reduction of *b*. The reduced peroxidase·dh complex is autoxidizable and it was calculated that it takes over about 20 % of the oxygen consumption of wheat roots[3]. Because the "b-shunt to oxygen"[6] operates in the presence of cyanide, which blocks both a_3 and peroxidase, it must be concluded that, besides dh, cytochrome *b* may directly, or *via* some unknown oxidase, deliver electrons to oxygen. These questions await further exptl. elucidation.

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The rhythmic proceeding of the reduction of the respiratory chain reported in this communication has not been observed by CHANCE and co-workers. CHANCE (see ref. [4,15]) recommends a "rapid flow" apparatus, but this apparatus is useful only in studying the process of reoxidation, which is less elucidatory than that of increasing reduction. The only reliable method for observation of increasing reduction is removal of the last traces of oxygen by the activity of the living cells. SMITH AND CHANCE[15] controvert our observations of the rhythmic time-course of reduction on the basis of hypothetical suppositions as to "mixing" and "diffusion of oxygen", the inadequateness of which was shown above. CHANCE apparently disregards the possibility of "kinetic deviations" caused by spatial organization. Most astonishing is CHANCE's declaration (see ref. [15]) that his values for the oxidation of cytochrome a_3 are 10^6 fold greater than ours. We have shown that the turnover number of the cytochrome oxidase in wheat roots and in starved yeast amounts to 20–30/sec, an order of magnitude also appearing in CHANCE's calculations. Chance has probably overlooked the fact that in our calculations of the relative or "apparent" velocity of reoxidation[5] of the cytochrome oxidase the concn. of the enzymes was given in μmoles/l.

The circumstance that *on reoxidation* the spatial organization of the respiratory chain yields less clear "kinetic deviations" depends on the fact that all enzymes are initially fully charged with electrons. A sudden access to oxygen—which is satisfactorily accomplished with the technique recommended here—as a rule develops a passing "vacuum" behind cytochrome *b*, obviously because the fully reduced a_3 $(+ c)$ emits electrons more rapidly than the dehydrogenases can deliver them. The observed early start of oxidation of cytochrome *b* illustrates the unrestricted passage of electrons between *b* and *c*. The oxidation of the group c-a_3 which immediately follows slows down the flux of electrons to a value which is in accord with the capacity of the dehydrogenases. This is the steady state of normal oxygenation.

REFERENCES

[1] *Chem. Eng. News*, (1958), Sept. 8, p. 48.
[2] O. GAEBLER, *Internatl. Sympos. on Enzymes, Units of biological structure and function*, Academic Press, New York (1956).
[3] F. LEUTHÅRDT, *Chimia*, 11 (1957) 1.
[4] H. LUNDEGÅRDH, *The cytochrome-cytochrome oxidase system*. Encyclopedia of Plant Physiology (Ruhland), 12 (1959), in the press.
[5] H. LUNDEGÅRDH, *Acta Chem. Scand.*, 10 (1956) 1083.
[6] H. LUNDEGÅRDH, *Biochim. Biophys. Acta*, 27 (1958) 653.
[7] H. LUNDEGÅRDH, *Biochim. Biophys. Acta*, 27 (1958) 355.
[8] F. L. CRANE, *Soil Science*, 85 (1958).
[9] H. LUNDEGÅRDH, *Arkiv Kemi*, 5 (1952) 97.
[10] H. LUNDEGÅRDH, *Arkiv Kemi*, 7 (1954) 451.
[11] H. LUNDEGÅRDH, *Biochim. Biophys. Acta*, 20 (1956) 469.
[12] H. LUNDEGÅRDH, *Biochim. Biophys. Acta*, 25 (1957) 1.
[13] H. LUNDEGÅRDH, *Nature*, 181 (1958) 28.
[14] H. S. MASON, *Internatl. Symposium on Enzyme Chemistry, Tokyo a. Kyoto, Proceed.*, (1957) 220.
[15] L. SMITH AND B. CHANCE, *Ann. Rev. Plant Physiol.*, 9 (1958) 449.
[16] H. LUNDEGÅRDH, *Internatl. Symposium on Enzyme Chemistry, Tokyo a. Kyoto, Proceed.*, (1957) 272.
[17] B. CHANCE, *Nature*, 169 (1952) 215.
[18] R. BUVAT AND L. LANCE, *Compt. rend.*, 247 (1958) 1130.