SPECTROPHOTOMETRICAL INVESTIGATIONS ON ENZYME SYSTEMS IN LIVING OBJECTS

I. THE OXIDATION-REDUCTION SYSTEMS OF BAKER'S YEAST

by

H. LUNDEGÅRDH

Penningby (Sweden)

The visual observation of bands in the absorption spectrum of living specimens, or biochemical preparations, has yielded fundamental results, *e.g.* in the pioneer work of KEILIN[14]. For more detailed investigations of the steady states of the single enzymes, however, only photoelectrical methods are recommendable. I have previously shown ([21] p. 103) that bands lying closer together than 10 mμ frequently fuse to one combined band with two shoulders or peaks, which may be moved closer together than the original peaks, thus causing errors of visual observation. Exact photoelectrical recording of the spectrum can easily resolve such a combined band into its elements, provided that the true absorption spectrum of one of the components is known[21, 22, 26].

The author started work in spectrophotometry on an electronic basis before 1930[20] but satisfactory spectrograms of living objects were attained only after the introduction of photo multiplier tubes with a factor of amplification of 10^8 or more. Combined with sensitive and rapid recording millivoltmeters instruments can be constructed by means of which thick suspensions of microorganisms or thick bundles of plant roots yield excellent spectrograms. For the attainment of highest perfection, and particularily for a quantitative evaluation of the measurements, instruments of special construction are needed and the spectral properties of pure enzymes must be carefully studied.

THE RECORDING SPECTROPHOTOMETER

Two complete instruments have been built in this laboratory, one with a large quartz monochromator, the other with a Bausch & Lomb 600 mm Grating Monochromator. Only the latter instrument, which is used for the experiments with yeast, is pictured here (Fig. 1). I have put forward two leading principles for the construction: (1) the spectrum is not recorded as a continuous line, but as a sequence of distinct wavelengths; (2) both the sample and the reference beam are separately recorded for each wavelength. A number of errors, *e.g.* those caused by the inertia of the millivoltmeter and variations in the reference beam (see below), are avoided if these principles are applied. The wave-length intervals may be chosen at will, from 1 mμ (or less) to

References p. 486/487.

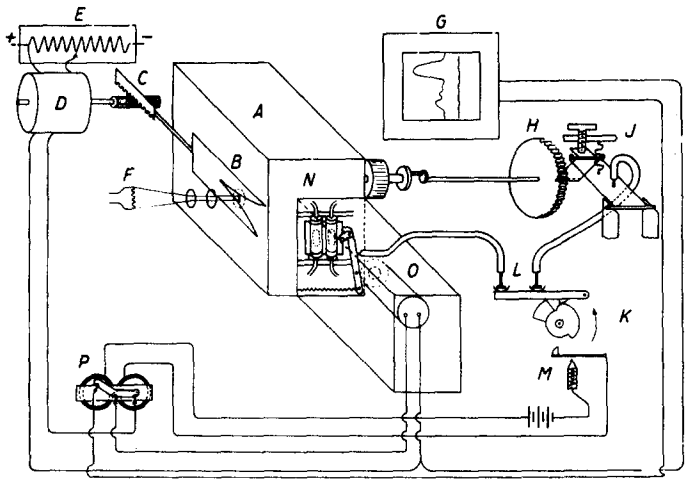

Fig. 1. Scheme of the recording automatic spectrophotometer. A Grating monochromator, B screen for adjustment of the in-going light intensity, C gear, D balancing motor, E potentiometer in balance against the photo cell, F light source, G recording millivoltmeter, H wave-length timer, I timing device for H, K cam wheel driven by synchron motor for the operation of I, L–N and M. L lever transmitting the movement of the low cam of K to I and N, N sliding object holder facing the out-going light beam, O photo multiplier tube, P electric contact device for switching over the current from O alternatingly to G or D and controlled by the high cam of K.

5 mμ (or more). I work mostly with intervals of 2 mμ. With a 6 V, 4.35 A Osram lamp, supplied from a large storage battery, and E.M.I. photo multiplier tubes No. 6260 and 6256 (with quartz window) perfect spectrograms from a 17–18 mm thick suspension of 15% yeast can be recorded at a slit width of 0.05 mm. This gives a band width of considerably less then 1 mμ (*cf.*[21]). For the ultraviolet spectrum (380 mμ downwards) a hydrogen lamp (Beckman Company) was mostly used. With 5% yeast suspension good spectrograms are here obtained at a slit width of 0.50 mm. The instrument, except of the motor assembly, is enclosed in a light-tight case provided with convenient doors and shutters. Provision is made for the use of color filters (Schott), but these are as a rule not needed.

In the Quartz instrument (*cf.*[21,22,26]) the elimination of the sensitivity curve of the photo cell is carried out by means of a mechanical device which, just before the recording of the reference beam, adjusts a movable diaphragm controlling the in-going light beam. In the Grating instrument a balancing motor steered by the photocell and a potentiometer as standard serves the same purpose (see Fig. 1). Because the intensity of the reference beam is always recorded only an approximate balancing of the sensitivity curve is needed, a fact promoting rapid work. Adjustment of the in-going light beam instead of the voltage of the dynodes eliminates errors caused by a simultaneous variation of the dark current.

With a rapid recording instrument, such as Speedomax G, all manipulations, *viz.* setting of the wave-length, adjustment of the diaphragm, recording of reference beam and sample, are accomplished in 4 seconds. For extremely rapid work the automatic adjustment of the light intensity can be omitted, or the instrument can draw a continuous curve. Several years ago I constructed an instrument with oscillographic recording of the spectrum. Extremely rapid records can of course be made by means

References p. 486/487.

of such an instrument, but the reference curve has to be separately recorded. What is gained in speed is, unfortunately, almost invariably lost in accuracy. A recording instrument, responding in 1 second, satisfies almost any wish to control the displacement of the steady states of "living enzymes", because permeability, diffusion *etc.* are delaying the response to each change of the conditions. Other investigators[30,5] are deliberately applying the principle of alternating wave-lengths for the measurement of rapid variations of the height of a special absorption band. Because of the co-variation of a number of enzymes, the bands of which are frequently over-lapping, this method must, however, be handled with great care.

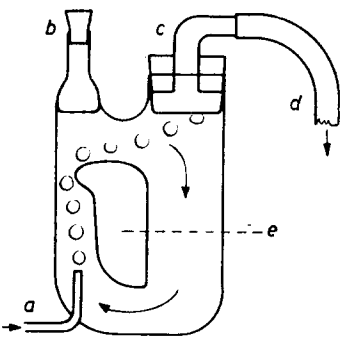

Fig. 2. Circulation vessel of quartz for combined aeration and stirring of the yeast suspension. *a* inlet of air, or N_2 *etc.*, *b* reserve outlet of gas and/or inlet of inhibitors, *c* main outlet of gas. Disturbing foam escapes through *d*.

Recipients for the sample are 17 mm quartz tubes (internal diameter), provided with inlet and outlet for the medium. For yeast, algae, bacteria, *etc.* small circulation vessels of quartz (internal diameter 17 mm) satisfy most demands as to stirring of the suspension, aeration, and rapid supply of inhibitors (Fig. 2). The aeration with air, N_2, CO, *etc.* simultaneously counteracts sedimentation. The recipients, one for the sample, and the other for the reference (see below), are clamped on the object holder (Fig. 1) which alternatingly exposes them to the out-going beam from the monochromator.

Either *direct spectra* or *difference spectra* are recorded. In the direct spectrum the reference beam is naked, corresponding to the solute in common photometry. Because no living objects devoid of all coloured substances can be provided, and the opacity of the object must be eliminated in some way, I introduced (1950) an opaque layer of filter paper or cotton wool of approximately the same transparency as the object. 4–6 layers of Munktell No. 3 filter paper are comparable to a 17 mm thick layer of a 15% yeast suspension.

To obtain a difference spectrum one vessel is filled with well-aerated starved yeast and the duplicate vessel filled with the same suspension plus inhibitor, or one vessel is aerated with air, the other with N_2, etc. The instrument then directly records the difference spectrum. The same result can of course be attained by combination of the measurements of two consecutive direct spectrograms, one with starved yeast, the second with yeast plus inhibitor, etc. I am unable to understand the statement by CHANCE ([5] p. 2) that "the selectivity that can be obtained is diminished" by this method. Recording the direct spectrum is a very important stage in each investigation of living tissues, because it reveals the features of *e.g.* the oxidized cytochromes. Incomplete knowledge of these spectra may cause considerable lack of accuracy (*cf.* Fig. 4).

In my continuous work with the new spectrophotometers during five years I have gained a complete knowledge of the degree of exactitude attained. Duplicate records have repeatedly been exposed to statistical analysis. The mean error of one single reading (sample contra reference) corresponds to an extinction value (log I_0/I) of 0.002 to 0.003. This is approximately the same value as that claimed by the manufacturer for the recording instrument. For comparison it may be mentioned that a 15%

References p. 486/487.

yeast suspension gives an extinction of 0.100–0.150 for the α-band of cytochrome *c*. The height of the main band of DPNH (reduced diphosphopyridine nucleotide) at 340 mμ is 0.300 to 0.400. The γ-bands of the cytochromes in wheat roots under similar circumstances attain a height of $\varepsilon = 0.500$–0.600.

THE OPTICAL EFFECTS OF OPACITY OR TURBIDITY

According to RABINOWITCH ([32] p. 709) a number of investigators of chlorophyll spectra in living tissues or cells have noticed that the bands in blue and violet are lowered in comparison to spectra of clear solutions. RABINOWITCH points out that the "sieve effect", *viz.* the rays escaping between the single cells, is statistically negligible in sufficiently thick suspensions of microorganisms.

The *scattering of the light* increases with decreasing wave-length, but great difficulties are met with in calculating the effect exactly. In my experience, the only practicable method in this case is to take direct measurements on each particular object.

Experiments with cotton or filter paper as scattering media give negative results: if a quartz tube is filled with loosely packed cotton wool the absorption spectrum of added reduced cytochrome *c* is identical with that from a clear solution.

The scattering in a 17 mm thick bundle of wheat roots is negligible in red, yellow, and green, but on an average lowers the Soret bands of cytochromes from calculated 100 to 75%. The effect is not constant; several experiments show no scattering effect at all.

Suspensions of yeast show a negligible scattering effect in green (α-bands of cytochromes) but a decrease of the bands in blue-violet, as may be calculated from the known relation between the γ- and the α-bands. Also in this case the results vary somewhat with the physiological state of the cells. On an average the Soret bands of yeast cytochromes are lowered from calculated 100 to 66%. This effect is only slightly dependent on the concentration (5% or 15% suspension) and the possibility cannot be excluded that it mainly depends on the molecular fixation of the enzymes in the living structure. A similar effect, however, may be observed on cytochrome added to the suspension.

The band of riboflavin at 450 mμ also shows a depression from calculated 100 to 66%. These observations are important for the calculation of the true concentration of flavoprotein in yeast. A correction factor of 1.5 has to be applied for 15% yeast. The concentration of the cytochromes should be calculated from the α-bands.

In ultraviolet, 300–380 mμ, no change of the main band of DPNH (= reduced pyridine nucleotide) was observed, if a solution of it was mixed with a suspension of 1% yeast. With 5% yeast the band was lowered from calculated 100 to 85%. Provided that the intracellular DPNH behaves similarily results from 5% yeast ought thus to be corrected by a factor of 1.18. The depression grows rapidly with rising concentration of the yeast suspension. Values from a 15% suspension must be corrected by a factor of 2.3.

It is claimed by RABINOWITCH[32] and others that scattering may distort the absorption bands. This is, however, not the case under the conditions of experiment quoted above. Quantitative measurements are as a rule confined to narrow intervals (see below), a fact which eliminates almost completely any effect of distortion.

References p. 486/487.

Incomplete knowledge of different objects sometimes arouses exaggerated ideas about distortion of absorption spectra by scattering (*cf. e.g.* a recent paper of BARER[2]). The adjustment of the diffraction by means of added protein as recommended by BARER[2] is objected to on physiological grounds if living enzyme systems are under observation. The systematical statement of the actual scattering along the lines mentioned above is the only practicable way out of the dilemma.

CALCULATION OF THE MOLAR CONCENTRATIONS OF THE ENZYMES

For accurate comparison of the spectrograms from living objects with purified enzymes it is important to compute spectral curves of the latter recorded with the same spectrophotometer (see Figs. 3–5). Pure preparations of cytochromes *c* and *f* were kindly given by Dr. R. HILL in Cambridge. I am indebted to Prof. H. KIESE in Marburg for a preparation of cytochrome oxidase from bovine heart muscle. A preparation of cytochrome *b* from the same object was made here according to the procedure recommended by KIESE[19]. For riboflavin and DPNH commercially available preparations were used (Hoffmann-La Roche and Sigma). No preparations of the cytochromes b_2 (lactodehydrogenase, 1, 3) and c_1 (or *e*, see KEILIN AND HARTREE[18]) were yet available and the data given in the literature are insufficient for calculations of the molar extinction (β). It was preliminary assumed that β is approximately the same as for cytochrome *b* and

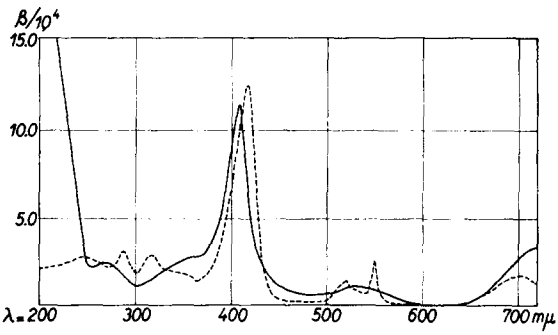

Fig. 3. The absorption spectrum of pure cytochrome *c*, recorded with the automatic spectrophotometer (Fig. 1) in wave-length intervals of 1 mμ. ——— oxidized, ------- reduced.

TABLE I

MOLAR EXTINCTION COEFFICIENTS (IN $mol^{-1}\ l^{-1}\ cm^{-1}$) OF THE PRINCIPAL OXIDATION REDUCTION ENZYMES OF BAKER'S YEAST

Enzyme	*λ mμ*	*From zero line of direct spectrum*	*From zero line of difference spectrum*	*Values of suitable intervals (diff. spectr.)*
Cytochrome *a*	604	$1.7 \cdot 10^4$	$1.6 \cdot 10^4$	604–620 ($1.0 \cdot 10^4$)
Cytochrome *b*	564	$2.0 \cdot 10^4$	$1.5 \cdot 10^4$	564–570 ($0.9 \cdot 10^4$)
Cytochrome *c*	550	$2.5 \cdot 10^4$	$2.2 \cdot 10^4$	550–542 ($1.6 \cdot 10^4$)
Riboflavin	450	$1.16 \cdot 10^4$	$1.16 \cdot 10^4$	450–500 ($1.08 \cdot 10^4$)
Dihydrocozymase	340	$5.5 \cdot 10^3$	$5.5 \cdot 10^3$	340–380 ($5.5 \cdot 10^3$)

References p. 486/487.

the figures for the concentration of the enzymes cytochromes c_1 and b_2 may accordingly need future correction. The same reservation is made for β-values of the DPN complexes quoted in the literature. The molar extinction of peroxidase and its complexes with CN and F were given in a previous communication[24]. Table I gives the molar extinction values used in the present paper, together with the corresponding values for wave-length intervals which are suitable for quantitative determination.

Many inhibitors show disturbing absorption in ultraviolet. Dithionite absorbs violently below 400 mμ, ascorbic acid gives a value of $\beta = 1.35 \cdot 10^4$ in the interval 260–310 mμ. A preparation of acetaldehyde from Eastman Co. showed a sharp peak at 290 mμ with $\beta = 4.1 \cdot 10^4$. Hydrogen peroxide absorbs only in the region below 260 mμ. Antimycin A shows no disturbing spectrum in the extremely low concentrations in which it blocks cytochrome *b*. Dinitrophenol is a very unpleasant substance for spectrophotometric measurements, because it is accumulated in the cells. The common organic acids in the tricarboxylic cycle are not disturbing, except of those with a double bond: fumaric acid shows an absorption, rapidly increasing from 300 downwards, at 240 mμ amounting to $0.5 \cdot 10^4$. Disturbing absorption in the visible region may be caused by flavons and flavanons[21,22], but these are not present in yeast, nor are carotenes. Small quantities of hemins other than cytochromes and peroxidase may be present (*cf.*[17]), but they do not disturb the measurements.

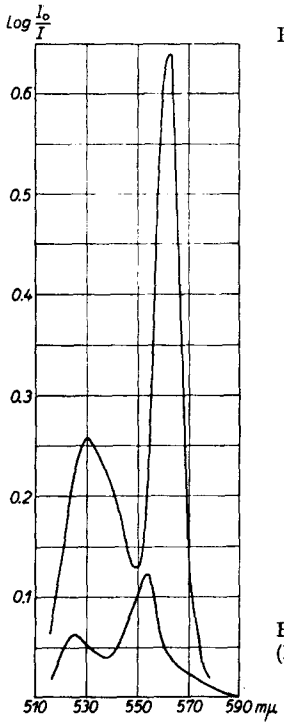

Fig. 4. Absorption spectrum of a preparation of cytochrome *b* from cow heart muscle. High curve = reduced. Low curve = oxidized.

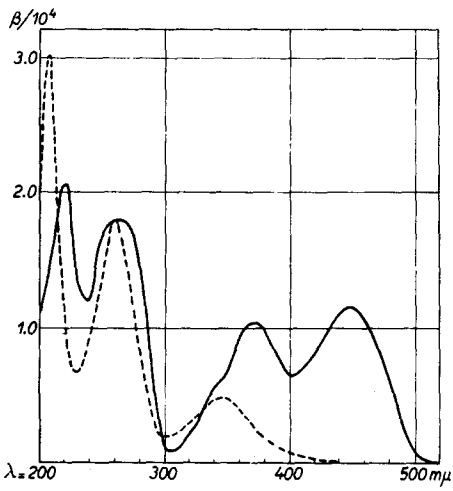

Fig. 5. Absorption spectra of commercial preparations of riboflavin (Hoffmann La Roche) ——; and reduced diphosphopyridine nucleotide (Sigma) -------.

DIPHOSPHOPYRIDINE NUCLEOTIDE

It is a well known fact that this coenzyme quantitatively strongly dominates in yeast. Its molar concentration amounts to about forty times of cytochrome *c*, *b* or cyto-

References p. 486/487.

chrome oxidase. CHANCE[6,7] has recently studied the behaviour of the coenzyme in living yeast. His spectrophotometric technique, however, does not permit the recording of finer details and measurements were only made of few wave-length intervals.

My new spectrophotometer reveals at least three or four separate bands in the region 300–380 mμ, which are related to DPN or its enzyme complexes. Because these bands are considerably broader than the bands of cytochromes they largely fuse, but may be separated after subtraction of the calculated band of free DPNH.

A band of the complex ADH (alcohol dehydrogenase)—DPNH is reported at 325 mμ[34], with a molar extinction coefficient of $5.8 \cdot 10^3$. A similar band has been reported for LDH (lactodehydrogenase)—DPNH of animal origin ([8]; peak at 330 mμ). The GDH (triosephosphate dehydrogenase)—DPN compound gives a flat band with its peak at 360 mμ and a β-value of about $3 \cdot 10^3$ [33]. Of these complexes ADH–DPNH and GDH–DPN are clearly defined in the yeast spectrum.

In starved yeast, which has been thoroughly washed in distilled water and aerated for more than one hour, the diphosphopyridine nucleotide is practically completely oxidized by the cytochromes. Subsequent treatment with acetaldehyde only adds a few percent more oxidation. The oxidation is accelerated in a salt solution, *e.g.* 0.02*M* phosphate buffer (pH 5.6) + 0.04% $MgSO_4$ + 0.01*M* KCl.

1–2 minutes after the exchange of the air for streaming N_2 about 50% of the total DPN is reduced. This process, too, is accelerated by salts. It is known that salt anions act as coenzymes in the operation of the cytochrome system[29], or, as demanded by the theory of anion respiration[28], generally in the transference of electrons between reacting oxidation reduction enzymes. The coenzymic activity of salts may thus be applied to the steady states of diphosphopyridine nucleotide also. In one experiment only a small amount of DPN was reduced in 15 minutes when starved yeast was aerated with N_2 in distilled water. After addition of phosphate buffer + 0.05*M* KCl the reduction rose to 50% of the total quantity in 10 minutes. As shown below, the steady state of DPN ⇌ DPNH in starved yeast is regulated by the combination of flavoprotein and cytochrome *b* acting as the electron donor of cytochrome *c*. This is shown in the specific response to antimycin A which blocks *b* as an electron donor but not the acceptance of electrons from DPNH and FP (= flavoprotein).

The absorption band of free DPNH at 340 mμ appearing aerobically after the addition of 2–5 μg antimycin A to 300 mg yeast is of about the same height as the band appearing after treatment with N_2 in the absence of organic nutrients but the former more completely coincides with the curve of pure DPNH (Fig. 6). Any changes in the combinations with ADH and GDH are practically absent as are also unknown bands pointing to the formation of other DPN enzymes. The observations support the idea that DPN

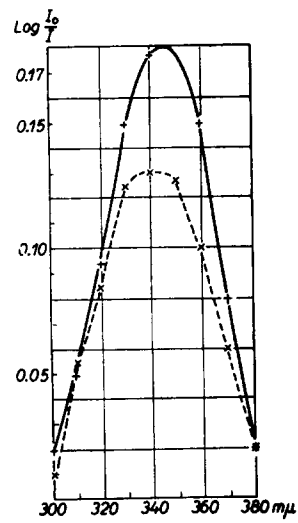

Fig. 6. Reduction of DPN in starved yeast induced by aeration with N_2 (+———+) or aerobically after addition of 5 μg antimycin A to 300 mg yeast (×----×). Difference spectra (reference oxidized yeast).

References p. 486/487.

is directly reacting with the FP-cytochrome *b* system. The simultaneously proceeding reduction of DPN and FP may be followed by observation of the FP band at 370 mμ (Fig. 7).

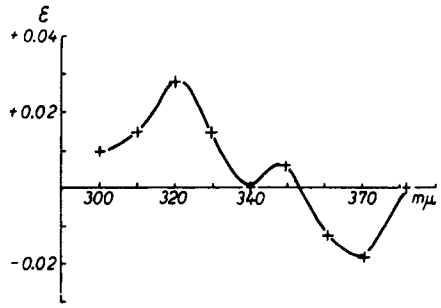

Fig. 7. Difference spectrum between yeast in 5% ethanol + N_2 and yeast in 5% aerated ethanol. The reduced band of flavoprotein is shown at 370 mμ and a slight increase of probably ADH–DPNH at 320–325 mμ.

A strong reduction of DPN starts immediately after the addition of 5% ethanol to starved yeast, but the complete spectrogram from the region 300–380 mμ is not identical with the previously mentioned one. At concentrations of ethanol below 5% the peak of the band moves to 348–350 mμ during the first 1–6 minutes and then slowly drifts back to 340 mμ. Subtraction of the calculated curve of pure DPNH from the observed spectrogram gives a curve with a peak at about 360 mμ which tentatively may be identified with that of the GDH–DPN complex. This complex partly disappears in fifteen minutes and is then followed by an elevation at 310–320 mμ. If the curve of pure DPNH is again subtracted the features of an ADH-DPNH band with its peak at 325 mμ then appear.

At higher concentrations of ethanol (10%) the ADH–DPNH complex dominates from the beginning (Fig. 8) and GDH–DPN rapidly disappears but appears again later on. The presence of ethanol thus calls the main enzymes of the glucose-alcohol balance into action. A multilateral balance obviously exists between free DPN and the enzyme combinations, the former serving as a buffering reservoir. The balance may be shifted to one side or another according to the relative concentration of substrates and products. The spectrophotometric observations support the idea that *the presence of the substrate accelerates the formation of the enzyme combination*. In the presence of ethanol accordingly the combination ADH–DPN is more favoured than GDH–DPN. A certain amount of this enzyme is obviously preformed in starved yeast as may be seen from the start period in ethanol, in which a negative band of GDH–DPN appears (Fig. 8). It may be calculated that *about* 30% *of the total DPN in starved aerated yeast is present as GDH–DPN*.

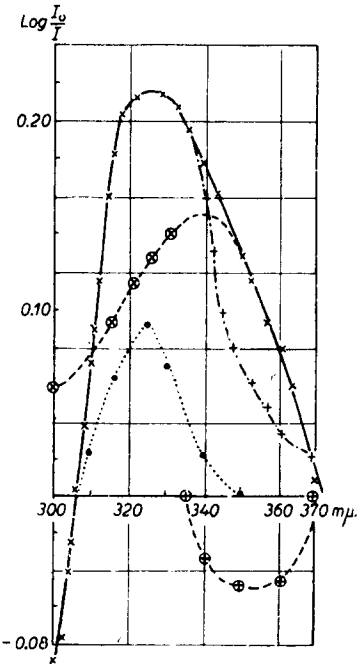

Fig. 8. Reduction of DPN and its enzyme compounds in 10% ethanol (aerated). Difference spectra (reference yeast in aerated distilled water). + — + — + 1½ minutes after the addition of ethanol; strong negative band (optically isolated below the base line) of GDH–DPN combined with a high peak at 325 mμ of ADH–DPNH. × —— × after 15 minutes; combined curve of DPNH (peak at 340 mμ ⊗ ---- ⊗) and ADH–DPNH (peak at 325 mμ ● ···· ●). 5% yeast suspension in circulation vessels.

The addition of glucose (+ phosphate and Mg) to starved yeast conveys a *sudden increase of the GDH–DPN compound* (Fig. 9). Contrary to the rapid dis-

References p. 486/487.

appearance of this compound in ethanol it is now raised above its level in starved yeast (Table II). The term total DPN in Table II means the quantity involved in the steady state balance. This quantity is, however, about 30% higher in ethanol than in glucose during the first 15 minutes, a fact indicating *a more complete total reduction in ethanol.* Shifting the aeration from air to N_2 thus has an increasing influence only with glucose. It is of interest to note, however, that with ethanol the formation of ADH–DPNH is promoted by anaerobiosis (streaming N_2, Fig. 7). The peak of the joined absorption band is here from the beginning moved to 325 mμ. The predominance of GDH–DPN in starved yeast reflects its lower dissociation constant ([11], p. 460) as compared with ADH–DPNH.

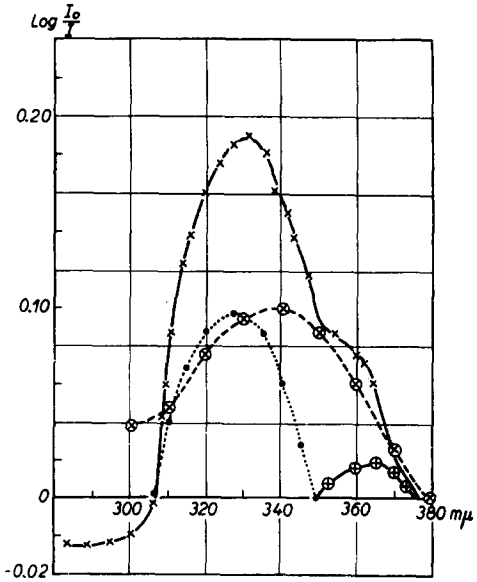

Fig. 9. Response of DPN and its enzyme compounds to an addition of 2.5% glucose (aerated). Difference spectra (reference yeast in aerated distilled water). ×——× The situation 1½ minutes after the addition of glucose. The curve can be resolved in the following components: GDH–DPN (⊕----⊕), ADH–DPN (●···●), and DPNH (⊗----⊗). 5% yeast suspension in circulation vessels.

TABLE II

THE VARIATION OF THE DPN COMPOUNDS WITH THE SUBSTRATE
Relative concentrations (total DPN = 100).

Time	*Substrate*	*ADH–DPNH*	*DPNH*	*GDH–DPN*
1–3 min	Ethanol	63	37	trace
1–3 min	Glucose	37	38	25
15 min	Ethanol	35	58	7
15 min	Glucose	32	38	30

THE CYTOCHROME SYSTEM

Baker's yeast from Svenska Jästfabriks AB (Swedish Yeast Manufacturing Co.) contains approximately equimolar quantities of the cytochromes *a* (cytochrome oxidase), *b*, and *c*, amounting to $2.0 \cdot 10^{-5} M/l$ moist yeast or $8.0 \cdot 10^{-5} M/kg^{-1}$ dry yeast. The position of the α-bands (see Table I) is the same as in Keilin-Hartree preparation of heart muscle, but according to the combination of four cytochromes (see below) in the region 550–564 mμ the combined band mostly shows the apparent peak of cytochrome *c* moved to 552 mμ and the apparent peak of cytochrome *b* moved to 562 mμ (Fig. 10). Only the accurate spectrophotometric recording of the total spectrum, made possible with my new technique, in combination with careful calculation of the single bands affords a successful disentangling of the components. It may be added here that the given figures for the wave-lengths are approximated

References p. 486/487.

to 1 mμ as a practical unit. As a matter of fact the α-band of cytochrome *c* is about three Angström lower than 550 mμ, and the band of cytochrome *b* is situated somewhere between 563 and 564 mμ.

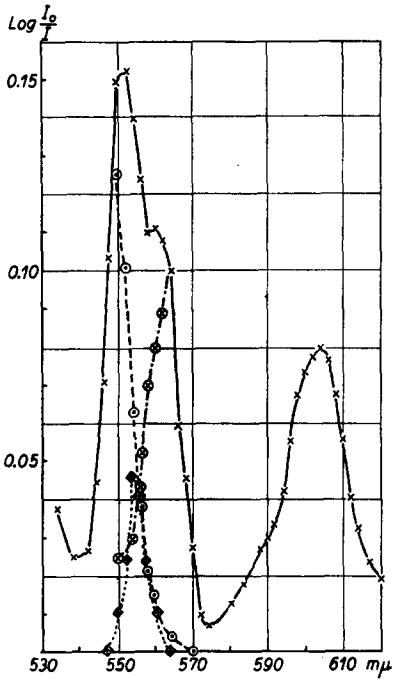

Fig. 10. α-bands of the cytochrome system of aerated yeast without washing (presence of reducing nutrients). Direct spectrum with filter paper as reference. Apparent peaks of the combined band of *c*, c_1, and *b* at 552 and 560 mμ. The band resolved in its components according to the procedure described in the text. ×——× observed curve, ◉------◉ cytochrome *c*, ⊗----⊗ cytochrome *b*, ⊕·····⊕ cytochrome c_1. 15% yeast suspension in 17 mm tubes.

Cytochrome b

Accurate measurements show a complete identity of cytochrome *c* of yeast with the pure enzyme. The identity of cytochrome *b* of yeast with that of the heart muscle appears from the position of the α-, β-, and γ-bands and from the response to antimycin A. As mentioned above, 2 μg antimycin on 300 mg yeast completely reduces cytochrome *b* and completely oxidizes cytochrome *c*.

DPN is the main donor of electrons to the cytochrome system, the steady state of which is matched against 30–50% of the total dehydrogenase. Experiments with malonate and succinate give varying results and point to a minor importance of succinic dehydrogenase in yeast. All influences blocking the *c*—*a* oxidase system or disconnecting the linkage between FP-cytochrome *b* and cytochrome *c* are simultaneously raising the level of DPNH.

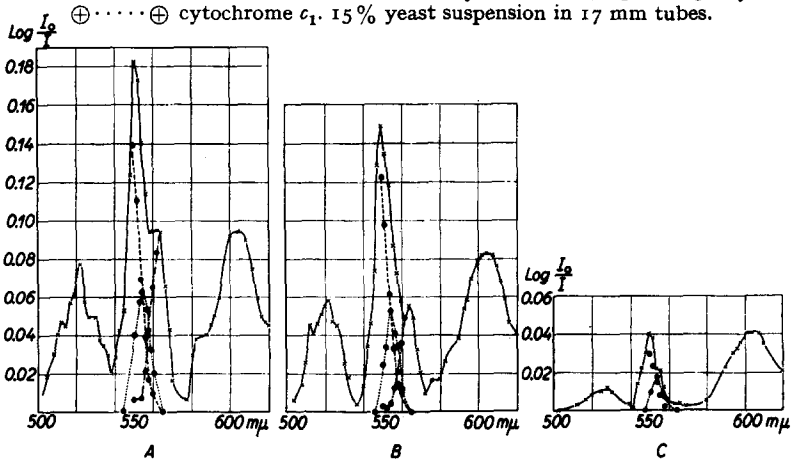

Fig. 11. Response of the cytochrome system of 15% washed yeast (in phosphate buffer + Mg) to aeration, after a period of 30 minutes in N_2. Difference spectra (reference aerated starved yeast). A complete reduction in N_2, B after aeration during 7 minutes, C after aeration during 20 minutes. ×——× observed curves, ⊗------⊗ cytochrome *c*, ⊕------⊕ cytochrome *b*, ●·····● cytochrome c_1.

References p. 486/487.

Cytochromes c *and* c_1

For an analysis of the combined band between 540 and 570 mμ the following procedure was applied: (1) calculation of the α-band of cytochrome *b* from the difference 564–570 mμ, (2) calculation of the α-band of cytochrome *c* from the value at 550 mμ obtained after subtraction of the α-band of *b* and "additional bands" (see below), (3) subtraction of the calculated curves of cytochromes *c* and *b* from the observed spectrogram; if the calculation is applied to the spectrum of starved yeast in N_2 the resulting curve now represents cytochrome c_1, the α-band of which has its peak at 554 mμ.

The influence of the band of c_1 on the height of the α-band of *c* is what was just mentioned under the heading "additional bands". Because the size of the c_1 α-band is not known beforehand an approximation must be made. As a rule a reduction of the

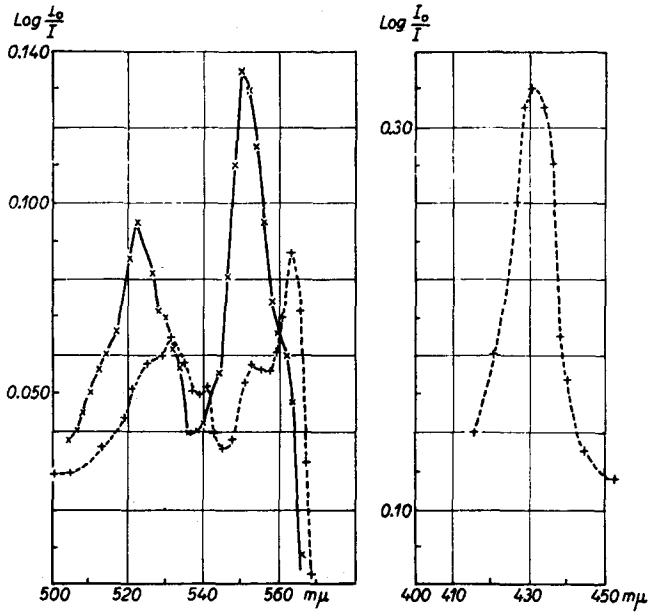

Fig. 12. Response of the cytochrome system to antimycin A (2 μg on 300 mg yeast). Direct spectrum (filter paper as reference). ×——× reduced in glucose + phosphate buffer + Mg. +-----+ 10 minutes after the addition of antimycin A. Band peaks at 430–431, 530, and 563 mμ, Partial reduction of cytochrome c_1 somewhat more pronounced than that of *c*.

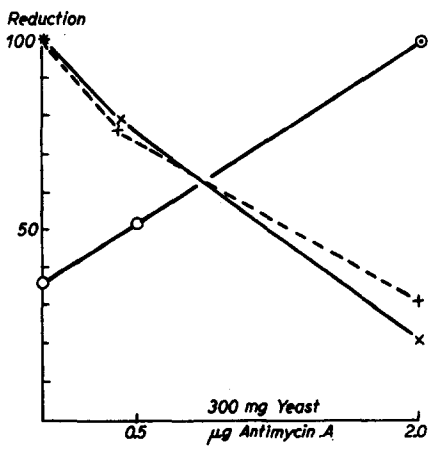

Fig. 13. The response on increased concentrations of antimycin A of the state of reduction of cytochrome *b* (◉——◉), cytochrome *c* (×——×), and cytochrome c_1 (+----+).

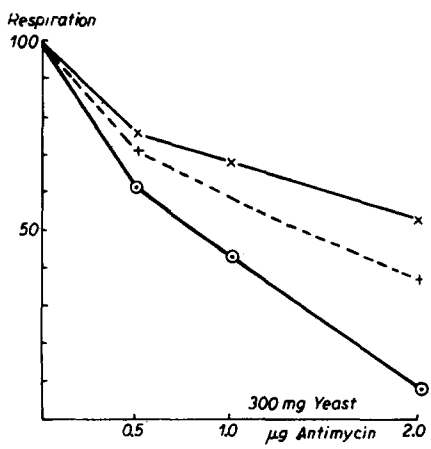

Fig. 14. The influence of increased concentrations of antimycin A on the yeast respiration, measured in the Warburg respirometer. ×——× 13 min, +---+ 25 min, and ◉——◉ 1 hour after the addition of antimycin. Compare with Figs. 12 and 13.

References p. 486/487.

band of cytochrome *c* by 15% approximately satisfies the equation. It may be added, however, that the presence of the α-band of cytochrome c_1 is clearly shown even if no preliminary reduction is made. The band then appears somewhat asymmetrical. The whole calculation is most conveniently made from the difference spectrum (see above), because both cytochromes *c* and *b* show an isosbestic point close to 580 mμ, which coincides with the base line of the spectrum. Direct spectrograms of yeast with filter paper as the reference give a spectrum which frequently is gently sloping from the Soret region towards red. The optical analysis, however, renders similar results from both kinds of spectrograms.

The residual band of c_1 resulting from the calculations is apparently the same as that originally found by OKONUKI AND YAKUSHIJI[31,38] and later on by KEILIN AND HARTREE described as cytochrome *e*[15,16]. These authors observed the new cytochrome in preparations from heart muscle, yeast, bacteria, and red algae. As will be shown in a separate publication it is present in higher plants also. In a recent publication KEILIN AND HARTREE[18] correct their previous figure 552 mμ for the α-band to 553–554 mμ, a value which fits in better with the present results. WIDMER *et al.*[37] also give the value 554 mμ. A calculation from 10 curves of yeast cytochrome, recorded with my spectrophotometer at intervals of 1 mμ, resulted in the mean value 553.7 mμ.

Somewhat varying figures are also given for the other bands of cytochrome c_1, *viz.* β = 522–524, and γ = 416–418 mμ. My records favour the figure 522 mμ for the β-band. The joined band at about 520–522 mμ has a broad peak, on which sometimes 522 protrudes as a separate peak. The latter disappears in lactate, simultaneously with the disappearance of the α-band at 554 mμ (see below), leaving the peak of cytochrome *c* at 520. The difference spectrum between reduced and oxidized yeast (N_2 and air respectively) invariably shows a shoulder or peak at 420 mμ. The difference spectrum of pure cytochrome *c* has a γ-peak at 418 mμ, 2 mμ above that of the direct reduced spectrum (*cf.*[21]). The observed peak at 420 mμ points to a position of the γ-band of c_1 at 418 mμ, or a little higher.

The α-band of cytochrome c_1 lies close to the corresponding band of cytochrome *f*, first described by HILL AND DAVENPORT[13], but the two cytochromes are obviously not identical. The spectrum of cytochrome *f*, recorded from a preparation passed on from Dr. R. HILL, shows an α-peak at exactly 554 mμ, but observations on living green objects[23] show a somewhat higher value, about 555 mμ. The γ-band of reduced pure cytochrome *f* has its peak at 422 mμ, thus at least 3 mμ higher than that of cytochrome c_1.

A mixture of pure preparations of the cytochromes *c* and *f* offers a convenient occasion for testing the abilities of photoelectric spectrophotometry to resolve fused bands. A mixture of equimolar quantities of *c* and *f* gives a uniform band with its peak at 418 mμ, composed by *c* = 416 and *f* = 422 mμ. The *c*-band at 416 mμ can now easily be optically "extracted" from the joined band after subtraction of the calculated band of *f*. This is a good illustration of the appropriateness of the technique and a similar result is obtained by resolution of the joined α-band. KEILIN AND HARTREE[18] are too pessimistic when they claim that two absorption bands "cannot be resolved spectrophotometrically if their maxima lie 4 mμ or less apart".

References p. 486/487.

Cytochrome b_2

APPLEBY AND MORTON[1] and BOERI *et al.*[3] succeeded in the biochemical isolation of cytochrome b_2. Together with an equimolar portion of flavoprotein this enzyme acts as the L-lactodehydrogenase of yeast and catalyses the reaction

Pyruvate + reduced enzyme ⇌ Lactate + oxidized enzyme

According to this equilibrium the addition of lactate to starved yeast may be expected to promote the reduction of the enzyme, even if the balance is "normally" moved to the right.

The difference spectrum between starved yeast and yeast plus 0.3 to 0.5 *M* lactate (pH 4.2), both aerated, shows nearly all cytochromes largely reduced. The combined band of *c* and *b* (see above) is somewhat widened in the direction of 560 mμ, however, (Fig. 15).

If this band is now resolved according to the procedure recommended above, the calculation presents, in addition to the bands of *c* and *b*, not the cytochrome c_1 but another cytochrome with its peak at 556.5 mμ. A corresponding analysis of the region 520–550 mμ (Fig. 15) results in the appearance of a β-band at 529–530 mμ. In the Soret region lactate gives a shoulder or peak at 424 mμ. All these values lie very close to those of prepared cytochrome b_2 and leave no doubt that the existence of this enzyme in the living yeast has now been demonstrated.

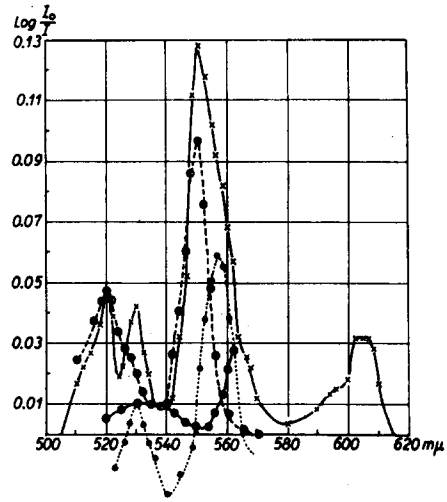

Fig. 15. Response of the cytochrome system to aerated 0.5 *M* lactate (pH 4.2) + phosphate buffer + Mg. Difference spectra (aerated phosphate buffer + Mg as reference). 15% washed yeast. ×——× observed curve. ⊗--⊗--⊗ cytochrome *c*, ⊕–⊕–⊕ cytochrome *b*, ●·····● cytochrome b_2. No reduced cytochrome c_1.

It was just mentioned that aerated lactate reduces nearly all cytochromes. The exception is namely cytochrome c_1, the reduced bands of which completely disappear in more concentrated solutions of lactate. In weaker solutions (0.2 *M*) a joined band between c_1 and b_2 is developed (Fig. 16). The reduction in 0.5 *M* lactate attains the following values, if the reduction in N_2 is set at 100:

Cytochrome	*c*	c_1	*b*	*a*
Rel. reduction	80	0	25–75	50

DPN is not reduced by lactate, an observation which is in accord with the recent findings of BOERI *et al.*[3] that the lactodehydrogenase of yeast does not dehydrogenate DPNH. Observations of the u.v. spectrum after the addition of lactate show no effect in the first 5–10 minutes except the appearance of a weak band at about 365 mμ. This is consequently a case in which the cytochromes are reduced side-ways. However, a strong band at about 330 mμ slowly develops. The simplest explanation of this development is a slow activation of the glucose-alcohol balance by products formed during the oxidation of lactate by cytochrome *c*.

The observed strong reduction of cytochrome *c* by lactate corroborates the

References p. 486/487.

observation of BOERI *et al.*[3] of a similar activity of the purified enzyme. The reduction of *c* in its turn blocks the oxidation of *b* and reduces the oxidation of the cytochrome oxidase to a medium level. The intensity of respiration, which is not changed in lactate, is still conducted by the cooperation of cytochromes and DPN.

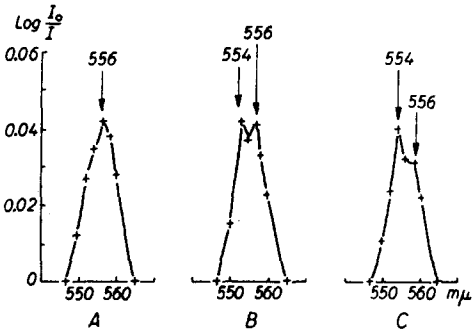

Fig. 16. The competitional behaviour of the cytochromes c_1 and b_2. A aerated 0.5 *M* lactate during 5 minutes (complete dominance of reduced b_2 at oxidized c_1). B aerated 0.2 *M* lactate during 25 minutes (reduction of both c_1 and b_2). C lactate + N_2 (both c_1 and b_2 reduced, but c_1 more).

The deviating response of cytochrome c_1 points to a comparatively independant position of this enzyme in relation to oxygen and to a competition between c_1 and b_2 in respect of their relation to cytochrome *c*. The mutual exclusion of *c* and b_2 as acceptors of electrons is shown by the fact that a joined band between both is immediately formed, and in stronger lactate solutions (0.5 *M*) this is followed by the appearance of a single band b_2 and the disappearance of the reduced band of c_1. In the absence of lactate the equilibrium (see above) is strongly in favour of oxidized b_2, and consequently its reduced band does not normally appear if starved yeast is aerated with N_2. If lactate is added to yeast under anaerobical conditions the reduction is about equally shared between b_2 and c_1 (Fig. 16).

Flavoprotein

According to the investigations of APPLEBY AND MORTON[1] and BOERI *et al.*[3] one molecule of cytochrome b_2 is firmly attached to one molecule of flavoprotein. These authors were, however, unable to correlate these results with observations on living systems.

In a previous communication[22] I showed that flavoprotein serves as a link in the ground respiration (= a fraction of the total aerobic respiration which is not inhibited by cyanide; see[23]) of roots of cereals. It was furthermore shown[25] that cytochrome *b*, which is also resistent to cyanide, probably acts as an acceptor of electrons emitted from reduced flavoprotein and in some way transfers them to oxygen without the aid of cytochrome oxidase, if the latter is blocked by cyanide.

Experiments with baker's yeast now show that a partial oxidation of *c* and *b* similar to that observed in roots occurs in an aerated solution of cyanide. About 30% of the normal respiration is attained. It may thus be expected that flavoprotein and cytochrome form a similar junction as postulated for roots.

The spectrophotometric identification and measurement of flavoprotein in living specimens is a far more intricate problem than the identification of DPNH and cytochromes. The blue band of pure riboflavin has its peak at 445 mμ. The corresponding band of flavoproteins is mostly set at 450 mμ[35] but in the "old yellow enzyme"[36] is as high as 465 mμ. The values of the molar extinction coefficient are commonly given about as high as the value I calculated from pure riboflavin (Table I). In the region between 450 and 465 mμ the difference spectrum of the cytochromes show a considerable depression below the isosbestic level, at 460 mμ amounting to

References p. 486/487.

25% for *c*, and 43% for *b*, calculated from the height of the α-band. The depressions due to the cytochromes c_1 and b_2 are not known but may be approximately estimated to be 30%. Before any reliable calculation of the concentration of FP can be made, corrections for these depressions must be applied. From this it follows that the complete spectrum covering the region 460 to 580 mμ must be recorded and calculated.

For quantitative measurements the intervals 460–500 mμ, or alternatively 460–470, and 460–480 mμ were selected. These intervals correspond to 70, 12, or 30% respectively of the full molar extinction of the blue band.

The second main band of flavin (Fig. 5) has its peak at 370 mμ. This band interferes with the spectrum of DPNH and particularly with GDH–DPN (peak at 360 mμ). Because of the high concentration of DPN compounds in yeast the flavoprotein band at 370 mμ is less suitable for calculations. In the cases in which measurements were made (*cf.* Fig. 7) the values of FP were, however, comparable to those yielded from the band in blue.

Flavoprotein is reduced in yeast in the *absence of oxygen*. The quantities correspond to 1–2 molecules per molecule of cytochrome *b*. Flavoprotein is also reduced aerobically in the presence of antimycin A. Because all other cytochromes, including c_1, are completely oxidized, if the added quantities of antimycin somewhat exceed the molar quantities of cytochrome *b*, it follows that the reduction of flavoprotein is closely linked to *b*. In one experiment $3.2 \cdot 10^{-5} M$ FP per kg yeast corresponded to $3.04 \cdot 10^{-5} M$ cytochrome *b*; these values are referred to the reduction of starved yeast in N_2.

The reduction of flavoprotein *by lactate* is conveniently studied in aerated 0.3–0.5 *M* solutions (pH 4.2). The reduction of FP amounts to $0.80–1.20 \cdot 10^{-5} M$ per kg yeast. The corresponding value of b_2 cannot be exactly calculated (see above) but if it is assumed that the molar extinction of b_2 is similar to that of *b* the quantity of b_2, which is reduced simultaneously with FP, amounts to $0.80–1.60 \cdot 10^{-5} M$ per kg or approximately the same value. The equimolar combination of FP and cytochrome b_2 found in the biochemical preparations[1,3] is thus virtually existing in the living yeast cell.

That the quantities of flavoprotein combined with *b* and b_2 are not the same appears from the fact that the amount reduced in N_2 (as opposed to air) in starved yeast is lower than that reduced in N_2 + lactate. The total enzyme-bound quantity of flavoprotein is thus somewhat higher than the molar concentration of the main cytochromes.

DISCUSSION

The enzymic properties of yeast, as compared with roots, are quoted in Table III. Yeast contains 4 times as much cytochrome *c* and 8 times more cytochrome oxidase than roots. The relative intensities of respiration, conducted by cytochrome oxidase, are approximately identical, however, in spite of the tremendous dominance of DPN in yeast. The turnover numbers of cytochrome oxidase are of similar magnitude in all living organisms ([21] p. 137, 22).

It is difficult to calculate exactly how much DPN is directly involved in the aerobic balance of the cytochrome system of yeast, but judging from the rapid reduction in N_2 of starved yeast the quantity does not exceed 30–50%. This quantity

References p. 486/487.

TABLE III

RESPIRATION AND CONCENTRATIONS OF OXIDATION-REDUCTION ENZYMES IN YEAST AND ROOTS OF WHEAT

The values are calculated per kg dry weight. They may be transformed to fresh weight by division by 3.7 for yeast and 11.0 for roots.

	Cytochromes $M \times 10^{-5}$			*Diphosphopyridine nucleotide* $M \times 10^{-5}$	*Respiration* $\mu M\ O_2/10\ mg\ 1\ h$
	a	*b*	*c*		
Yeast	7.0	7.0	7.0	280	5.0–7.5
Roots	0.9	1.7	2.7	3–13*	1.0–2.0**

* Preliminary figures.
** = anion respiration, conducted by cytochrome oxidase.

is rapidly reoxidized in air. In roots considerably more of the aerobic respiration is controlled by the succino-dehydrogenase system, a fact also reflected in the low values of DPN, and in the clear response to malonate, fumarate, and succinate in these parts. In higher plants and probably also in muscle cells the normal balance between dehydrogenases and cytochromes is moved far in the direction of oxidation of the latter. In respiring wheat roots the cytochromes are 60–80% oxidized[21] even if sufficient glucose is present. If glucose is added to yeast the balance is moved far in the direction of reduction of the cytochromes (90–100% of cytochrome *c*), simultaneously with a sudden rise of the DPNH level. The respiration is then strongly accelerated above the level indicated in Table III. Ethanol has a still more intensive effect in this respect. These experiments illustrate the simple fact that the intensity of the respiration, *viz.* the velocity of the stream of electrons from the substrate to O_2[27], is directly related to the quantity of reduced cytochrome in the steady state, provided that the dehydrogenase systems are richly dimensioned. This is obviously not the case in higher organisms.

It has been claimed[4] that cytochrome *b* would not lie in the direct path of electrons from the dehydrogenase systems to oxygen. I have previously[27] shown the incorrectness of this conclusion. It is important to measure the velocity not only of the on-reaction, *viz.* Donor $\overset{e}{\rightarrow}$ *b*, but also of the off-reaction, *viz.* *b* $\overset{e}{\rightarrow}$ Acceptor. It was shown[27] that electrons were passed with similar velocity from *b* to *c* as from *c* to *a*. The on-reaction, *viz.* the passage of electrons from dehydrogenases to *b*, is, however, considerably slowed down in roots of higher plants. It was shown[26] that this retardation of the system is partly due to a linkage of the electron transference to the steady state of high energy phosphate, this system hereby acting as buffer on the reduction of cytochrome *b*. Another retarding effect is exerted by the low or medium activity of the dehydrogenase systems.

The remarkable difference between yeast and higher plants in respect of the dimension of the dehydrogenase systems is strikingly reflected in the time-course of reduction and reoxidation. The reduction of cytochrome *b* is slow in roots, very rapid in yeast (Fig. 17). Cytochrome *c* is rapidly reduced both in yeast and in roots. That the reduction of cytochrome *c* is exclusively conducted over the *b* cytochromes, not resulting from a direct reaction between *c* and DPNH, may be concluded from the behaviour in the presence of antimycin A. Cytochrome *c* is here completely oxidized simultaneously with strong reduction of DPN. My present experience does not

References p. 486/487.

necessarily support the existence of a special "Slater factor" between cytochromes *b* and *c* outside of the cytochrome assembly. Antimycin A may as well be figured as competitively blocking the place of molecular contact between *b* and *c* but leaving the contact between *b* and FP intact. The place of electronic contact between *b* and *c* is on the whole one of the most sensitive points of the oxidation-reduction system. In addition to antimycin A urethane, malonate *etc.* attack this point.

Reoxidation of the total cytochrome system of roots is an extremely rapid process[27, 22, 26]. The reoxidation of yeast is a slow process (see Fig. 17) in which the buffering properties of the DPNH system creates an intermediate interval (between 2 and 7 minutes) of approximately constant steady state between an 80–90% reduced cytochrome system and the powerful pool of DPNH present after the previous period of reduction. Only after considerable decrease of the DPNH reserves does the final oxidation of the cytochrome system set in.

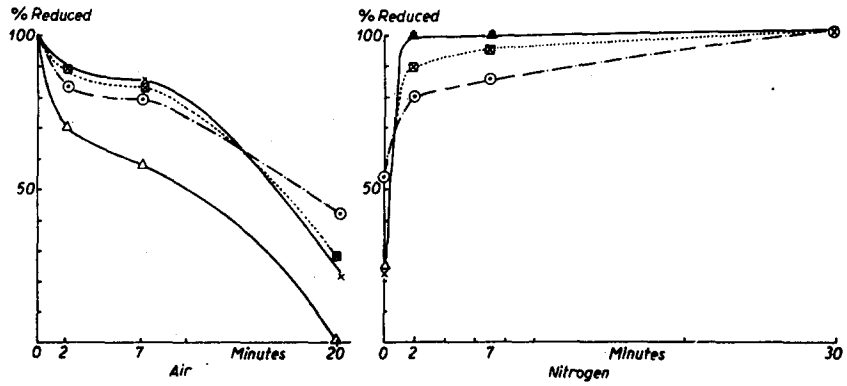

Fig. 17. The time-course of reoxidation and reduction in N_2 of starved yeast in phosphate buffer. Difference spectra. ×——× cytochrome *c*, ◉----◉ cytochrome oxidase, △——△ cytochrome *b*, ⊠·····⊠ cytochrome c_1.

The close cooperation between flavoprotein and cytochromes of the *b*-type, exemplified in FP-b_2[1, 3] and the combination FP-*b* studied in this laboratory, obviously serves as a tool for simultaneous transportation of protons and electrons according to the following tentative scheme:

$$\text{DPNH} + \text{FP} \longrightarrow \text{DPN} + \text{FPH} \qquad (1)$$

$$\text{FPH} + b(\text{Fe}^{3+}) \longrightarrow \text{FP} + b(\text{Fe}^{2+}) + \text{H}^+ \qquad (2)$$

The equimolar concentrations of FP and the cytochromes *b*, *c*, and *a* in the yeast cell point to the existence of a compact structural unit, in which, however, at least some of the participants maintain an independent mobility, as shown by the competitive phenomenons mentioned above. Regarding substrates, the combination FP-cytochrome *b* of yeast is obviously specific to DPN, whereas FP-b_2 is specific to L-lactate. It has recently been shown that flavoprotein also is a component of succinodehydrogenase[12]. This would mean a third group of specific FP. As to a hemin component of this enzyme, however, opinions are still conflicting[12, 10]. It may be tentatively assumed that the substrate specifity is primarily a property of the flavoprotein moiety, whereas the hemin moiety is specific to its electron acceptor. The early results of WARBURG AND CHRISTIAN[36] with the "old yellow enzyme" from yeast

References p. 486/487.

may probably be explained as a splitting up of the FP-cytochrome *b* combination, the isolated FP hereby still retaining some of its enzymic properties.

The position of c_1 in the "electron ladder" must still await further elucidation. According to YAKUSHIJI AND OKUNUKI[31,38] its position would be between *b* and *c*. Its oxidation in aerated lactate, at simultaneous reduction of *b* and *c*, however, points to a more independent position. The competitive behaviour of b_2 and c_1 suggests adjacent points of contact with the molecule of cytochrome *c*. When the contact between *b* and *c* is abolished by antimycin A, c_1 is more slowly oxidized than *c*. This points to the possibility that cytochrome c_1 serves as an auxiliary donor of electrons to *c* and *a*. The possibility cannot be excluded that cytochrome c_1 is identical with the "Slater factor" (*cf.*[37]).

KEILIN AND HARTREE[18] deny the possibility of an independent autoxidation of c_1, but its behaviour in this respect in living cells has to be investigated. It is quite obvious, however, that cytochrome c_1 is not identical with an autoxidizable cytochrome b_5 recently described by CHANCE AND PAPPENHEIMER[9] from homogenates of *Cecropia* midgut.

The cooperation of FP in the respiratory chain of yeast and roots actualizes the possible formation of H_2O_2 and its utilization by peroxidase. Roots contain large quantities of peroxidase[22,24]—more than six times as much as yeast, in which the quantity of peroxidase amounts only to about 30% of that of cytochrome *c* (in roots the relation is 200–400%). Measurements on roots showed[22] that peroxidase certainly cooperates in the total aerobic respiration but to a much smaller degree than the cytochromes. No trace of an peroxidase-H_2O_2 complex has been found in baker's yeast and the spectrophotometric picture of yeast respiration under varying conditions do not support the idea advanced by CHANCE[6,7] that a combination of cytochrome *b* and the peroxidase complex II would appreciably contribute to the aerobic respiration. This problem must be considered quite apart from the fact that, according to CHANCE[6], the H_2O_2 complex of peroxidase is extremely reactive.

SUMMARY

Spectrophotometric records were made from suspensions of living yeast by means of a new automatic recording instrument of special construction. The technique enables a detailed investigation to be made of the steady states of the following enzymes and coenzymes: diphosphopyridine nucleotide and its combination with alcohol dehydrogenase and triosephosphate dehydrogenase, flavoprotein and its combination with cytochromes of *b*-type, cytochromes *c*, *b*, and *a* (cytochrome oxidase), cytochromes c_1 and b_2, and peroxidase. The author comments on the presence and conditions of oxidation and reduction of the cytochromes c_1 and b_2 in living yeast and the steady states of the DPN compounds. The results are compared with previous investigations on roots of higher plants.

The investigation has been aided by a grant from the Swedish Research Council of Science.

REFERENCES

[1] C. A. APPLEBY AND R. K. MORTON, *Nature*, 173 (1954) 749.
[2] R. BARER, *Science*, 121 (1955) 709.
[3] E. BOERI *et al.*, *Arch. Biochem. Biophys.*, 56 (1955) 487.
[4] B. CHANCE, *Nature*, 169 (1952) 215.
[5] B. CHANCE, *Science*, 120 (1954) 767.
[6] B. CHANCE, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 399.
[7] B. CHANCE, *Harvey Lectures*, Ser. XIL (1953–1954) 145.

[8] B. CHANCE AND J. B. NEILANDS, *J. Biol. Chem.*, 199 (1952) 383.
[9] B. CHANCE AND A. M. PAPPENHEIMER, *J. Biol. Chem.*, 209 (1954) 931.
[10] H. W. CLARK, H. A. NEUFELD, C. WIDMER AND E. STOLZ, *J. Biol. Chem.*, 210 (1954) 851.
[11] *The Mechanism of Enzyme Action*, edited by W. D. MCELROY AND B. GLASS, 1954.
[12] D. E. GREEN, P. M. KOHOUT AND S. MII, *Biochim. Biophys. Acta*, 14 (1954) 295.
[13] R. HILL AND H. E. DAVENPORT, *Proc. Roy. Soc. (London), B*, 139 (1952) 327.
[14] D. KEILIN, *Proc. Roy. Soc. London, B*, 98 (1925) 312.
[15] D. KEILIN AND E. F. HARTREE, *Nature*, 164 (1949) 254.
[16] D. KEILIN AND E. F. HARTREE, *Nature*, 165 (1950) 504.
[17] D. KEILIN, *Nature*, 172 (1953) 390.
[18] D. KEILIN AND E. F. HARTREE, *Nature*, 176 (1955) 200.
[19] G. HÜSCHER, M. KIESE AND R. NICOLAS, *Biochem. Z.*, 325 (1954) 223.
[20] H. LUNDEGÅRDH, *Z. Physik*, 66 (1930) 114; *Die quantitative Spektralanalyse der Elemente*, G. Fischer, Jena, 1929–1934, Vol. 1 and 2.
[21] H. LUNDEGÅRDH, *Arkiv Kemi*, 5 (1952) 97.
[22] H. LUNDEGÅRDH, *Arkiv Kemi*, 7 (1954) 451.
[23] H. LUNDEGÅRDH, *Physiol. Plantarum*, 7 (1954) 375.
[24] H. LUNDEGÅRDH, *Physiol. Plantarum*, 8 (1955) 84.
[25] H. LUNDEGÅRDH, *Physiol. Plantarum*, 8 (1955) 95.
[26] H. LUNDEGÅRDH, *Physiol. Plantarum*, 8 (1955) 142.
[27] H. LUNDEGÅRDH, *Nature*, 171 (1953) 521.
[28] H. LUNDEGÅRDH, *Ann. Rev. Plant Physiol.*, 6 (1955) 1.
[29] H. LUNDEGÅRDH, *Nature*, 171 (1953) 477.
[30] G. A. MILLIKAN, *Proc. Roy. Soc. London, B*, 123 (1937) 218.
[31] K. OKUNUKI AND E. YAKUSHIJI, *Proc. Imp. Acad. (Tokyo)*, 17 (1941) 263.
[32] E. RABINOWITCH, *Photosynthesis and Related Processes*, Vol. II, Pt. 1, Interscience Pub., New York, 1951.
[33] E. RACKER AND I. KRIMSKY, *J. Biol. Chem.*, 198 (1952) 731.
[34] H. THEORELL AND R. BONNICHSEN, *J. Biol. Chem.*, 5 (1951) 1105.
[35] H. THEORELL, *The Enzymes*, edited by J. B. SUMNER AND K. MYRBÄCK, Academic Press, New York, 1951, Vol. II, Pt. 1, p. 355.
[36] O. WARBURG AND W. CHRISTIAN, *Naturwiss.*, 20 (1932) 688, 980.
[37] C. WIDMER, H. CLARK, H. A. NEUFELD AND E. STOTZ, *J. Biol. Chem.*, 210 (1954) 861.
[38] E. YAKUSHIJI AND K. OKUNUKI, *Proc. Imp. Acad. (Tokyo)*, 16 (1940) 299.

Received September 3rd, 1955