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Commentary by

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on 'New methods for the study of the carbon monoxide compounds of respiratory enzymes'
by B. Chance, L. Smith, and L. Castor
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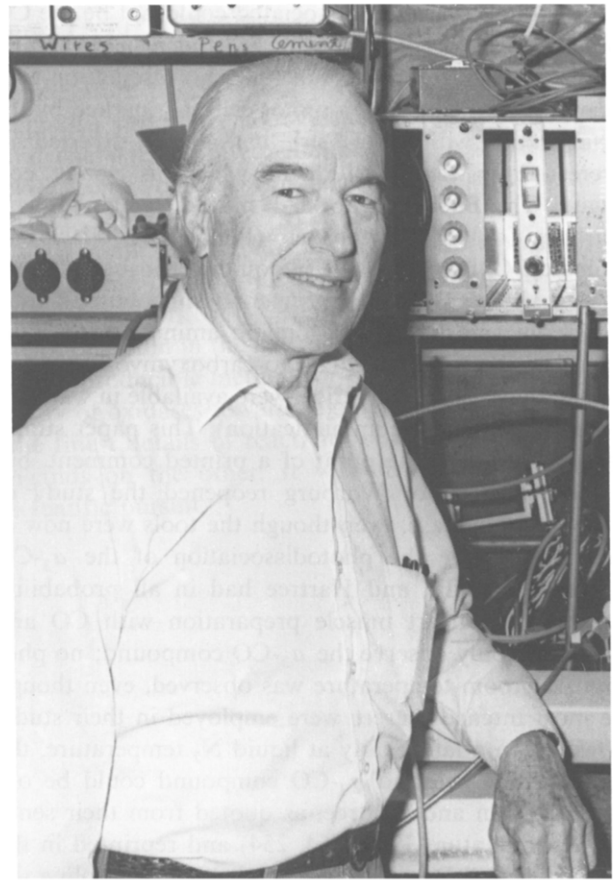
If one had been privileged to peregrinate between the laboratories of Otto Warburg and David Keilin in the '30s, one would have found a striking difference of attitude, difference of pace and difference of technological sophistication. David Keilin, the discoverer of cytochrome, was a simple man, one of rare intellect and insight on matters of physiological importance. His instrumentation was primitive but effective, using the respirometer, the microspectroscope, and together with Hartree, Mann and others, they had great preparative skills. But generally he had a fascination with life, respiration and parasitism as they existed over a wide range of biological materials.

Otto Warburg, on the other hand, descended from a 'photochemist father' and trained in the then 'high tech' of German scientists, was a master of technology. We had a special interest and talent in assembling technically skilled laboratory assistants, such as Haas and Negelein just to mention a few. The photochemistry came readily to him, and it was no surprise that the discovery of Smith and Haldane on the effect of Summer and Winter upon hemoglobin dissociability should have soon reached the mind of a man who was said often to be in Newmarket, England (but rarely, if ever, in Cambridge). He soon thereafter obtained his first absolute spectrum of the carbon monoxide compound of cytochrome oxidase in cells of *Torula utilis*. It was a remarkable achievement, in fact; an attempt to reproduce or even extend the result in the hands of Stern and Melnick got the wrong result; it was not until Chance, Smith and Castor, with the New Methods paper, that the original results could even be reproduced. They were extended to other cells with the discovery of cytochrome *o* now found to be a protoporphyrin IX analog of mammalian cytochrome oxidase. In fact, Warburg mastered the microspectroscope technique as well, and together with Negelein observed the spectra of cytochromes in the bee thorax and, by taking special pains to employ copper sulfate and appropriate filters, was able to identify the Soret band of reduced 'Atmungsferment' which appeared at 445 nm instead of at 430 nm as was found for the carbon monoxide compound of the yeast oxidase. Whatever may have been Warburg's reaction to this result, he failed to pursue it and perform the crucial experiment and put



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the bee in an atmosphere of carbon monoxide. Had he done so, he undoubtedly would have observed the band to shift to the 430 nm peak of Atmungsferment in the thorax of the living bee, an almost indisputable correlation of Warburg's work with that of Keilin's original paper of 1925. Maybe he did not hope for a correlation! – who knows?

Nevertheless, it was the work of Keilin himself using the famed heart muscle preparation prepared in collaboration with Hartree in which he observed the shift of the 445 nm band of the reduced form of indophenol or cytochrome oxidase to 430 nm upon addition of carbon monoxide, thereby bridging the gap between his work and that of Otto Warburg. There was, however, one flaw, namely, the strong photolysis light did not affect the CO compound of cytochrome oxidase as formed in the heart muscle preparation. Such a flaw would be troublesome to Warburg: a CO compound that could not be photodissociated could not be the CO compound of Atmungsferment. But this was apparently of little interest to him at that time; his fascination with anaerobic fermentation in tumor cells, as sparked by the collaboration with Dean Burk, had already diverted his interest from Atmungsferment while his young colleague, Th. Bücher, who had indeed remained with Warburg for part of the war (Bücher, Th. (1983) in Biological Oxidations. 34. Colloquium (Mosbach; Sund, H. and Ullrich, V. eds), Springer, Berlin), himself took up energy transfer from aromatic amino acids to the heme but remained steadfast to carboxymyoglobin for, indeed, only meager materials were available in war-torn Germany (personal communication). This paper stimulated Warburg to the point of a printed comment, but neither Bücher nor Warburg reopened the study of cytochrome oxidase, even though the tools were now at hand to observe the photodissociation of the a_3-CO compound. Keilin and Hartree had in all probability saturated the heart muscle preparation with CO and thus could only observe the a_3-CO compound; no photolysis at room temperature was observed, even though the most intense sources were employed in their study. However, in a later study at liquid N_2 temperature, the opposite was true, no a_3-CO compound could be observed. Keilin and Hartree, as quoted from their seminal paper (Nature 1949, 164, 254) and reprinted in the History of Cell Respiration, stated that "on cooling the preparation containing a_3CO, its absorption band disappears completely, whereas that of a is intensified. This phenomenon, which is perfectly reversible is difficult, to explain. It may be due either to the dissociation at low temperatures and light of a_3CO, to the shift of its band towards the red, or else to the fact that, not being greatly intensified by cooling, it is masked by the strongly intensified band a." It seems obvious that had no other pressing matters taken their attention from this problem, they would have proceeded further and presumably with more sophisticated techniques to verify that they had indeed completely closed the ring of their work and Warburg's. Did they really want to? Who knows?

During the war, most of those who were able to do so dedicated their time to war research projects and it was only after the end of World War II that this research work on photodissociation was resumed. With the fresh impetus of the radar technology, the dual-wavelength spectrophotometer soon emerged and with it the capability of precise optical observations in highly scattering materials such as the Keilin and Hartree heart muscle preparation on the one hand, and suspensions of bacteria on the other. In fact, the method had adequate resolution to clearly identify the difference between cytochrome c and cytochrome c_1 or e as predominant in the cytochrome-c-deficient preparations, even before it was noted by Keilin himself. In both cases, the research was greatly accelerated by the collaborations with Lucile Smith, Helen Conrad Davies and Thomas Devlin, just to mention a few. Soon the dual-wavelength system was operating satisfactorily and churning out point by point difference spectra of the oxidized-reduced states of many of the respiratory enzymes of bacteria, mammalian cells, and tissues (Chance, B. (1954) Science 120, 767–775). At the same time, the CO difference spectra, particularly the technique of recording the fully reduced spectrum in a split-beam machine, were developed in a collaborative project with C.C. Yang. Bubbling one of the two cuvettes containing the reduced cytochromes with CO provided information on the CO difference spectra of many cytochromes, known and unknown, particularly cytochromes a_1, a_2, a_3 and a hint of a hitherto unobserved cytochrome o. It is interesting that this one has turned out to be a protoporphyrin IX cytochrome oxidase, i.e., resembling cytochrome a_3 as a binuclear oxidase except for the chemistry of the heme.

With the dual-wavelength technique, similar CO difference spectra were generated if, instead of adding CO to the reduced form, the already combined CO compound was photolyzed and the absorption difference measured. As it turned out, indeed, cytochrome oxidase was recalcitrant to photolysis and succumbed only at lowered temperatures of 4° C, which adequately slowed the rate of recombination. Titration of dilute cytochrome oxidase (about 1 μM) with the addition of aliquots of a saturated solution of carbon monoxide increased the photochemical response to an optimum, i.e., with insufficient combination with carbon monoxide, the optical response was small; with excess carbon monoxide the photolysis was undetectable (no doubt the condition used by Keilin and Hartree). The technology that was required for this development was that of the crossed filter technique, where the wavelengths of the photolysis light were isolated from those of the

measuring light, so that negligible cross-talk occurred, i.e., negligible displacement of the dual-wavelength trace occurred when the photolysis light was employed. One of the great advantages of the dual wavelength technique and the transformer-coupled phase-sensitive detector was that the signal from the steady photolysis light was largely rejected. The photolysis light source itself was a fortunate happenstance; the Johnson Research Foundation oscillograph, an excellent photokymograph designed by Rawson and Hervey who employed a graphite carbon arc lamp which required a significantly high direct current (but fortunately the JF was wired for 110 volts DC as well as AC). Such arc lamps were also used by Warburg and gave an intense source which was focused onto the cuvette via appropriate heat-absorbing and spectral filters which eliminated most of the cross-talk between the optical measurement and the photolysis.

Last but not least, it was a fortunate choice to photolyze the alpha band region because of the excellent gelatin filter (Wratten) for observation in the Soret band. The dark/light difference spectra show clearly the loss of the 445 nm band and the increase of the 430 nm band. Furthermore, the extinction coefficient of the oxidase in Keilin and Hartree's heart muscle preparation should indeed be the same as that obtained by Otto Warburg's study of *Torula utilis*. Taking myoglobin as a standard with a quantum yield of 1, it was readily possible to show from kinetics of recombination that cytochrome oxidase had an extinction coefficient of the CO compound of 11 $cm^{-1} \cdot mM^{-1}$ at 590 nm. Thus the identity of the oxidase in the two systems, *T. utilis* and Keilin and Hartree's heart muscle preparation, was established qualitatively from the photolysis spectrum and quantitatively by the extinction coefficients.

The intensive studies of the low temperature kinetics of the CO compounds of both myoglobin and cytochrome oxidase showed not only the recombination kinetics with CO, but also the ligand exchange kinetics, particularly from carbon monoxide to oxygen in cytochrome oxidase. This was avidly taken up by Quentin Gibson during most of his scientific career. Generally, the study of low-temperature kinetics of recombination of myoglobin with carbon monoxide has become the principal endeavour of this laboratory and that of Frauenfelder at Urbana. The reaction of CO is finely tuned to structural modifications of the oxidase and most recently the effect of subunit composition upon the reactivity of cytochrome oxidase at low temperatures ($-70\,^{\circ}C$) has become a principal study of this laboratory. In addition, this laboratory employs low-temperature photolysis in the presence of oxygen as a standard procedure to evaluate the oxygen reactivity and character of intermediates of oxygen reduction of cytochrome oxidases of mammalian and microbial origin.

All in all, the study of low-temperature photolysis of cytochrome oxidase has not only bridged the gap between the work of Warburg in the late '20s and the work of Keilin in the late '30s, but has provided a stable and reproducible method for precise evaluation of reactivity of oxidases towards ligands on the one hand, and the finest details of reactivity of hemoproteins towards ligands on the other. It was obviously a worthwhile scientific pursuit.

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NEW METHODS FOR THE STUDY OF THE CARBON MONOXIDE COMPOUNDS OF RESPIRATORY ENZYMES*

by

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In the past few years it has been possible to improve considerably and to extend the range of three basic methods for the study of respiratory enzymes, especially their carbon monoxide compounds. Visual spectroscopy—used first by MACMUNN in 1885[1] to discover the histohemins, by KEILIN in 1925[2] to identify and to study the cytochromes in detail, and by WARBURG and his co-workers to identify the CO compound of the respiratory enzyme in *Acetobacter pasteurianum*[3]—until recently has had no successful competitor for the study of the absorption bands of the respiratory enzymes in suspensions of intact cells or in muscle tissue. But improved photoelectric surfaces and electronic techniques have in our hands and more recently in the hands of others[4] brought sharply into focus the absorption spectra that could at best be only dimly perceived by earlier photoelectric techniques such as were used by WARBURG AND CHRISTIAN to show the presence of flavoprotein in *Bacterium delbrückii*[5] and by HAAS to measure the speed of reduction of cytochrome *c* in *Torula utilis*[6]. It is now possible to observe the reduction of respiratory enzymes in the range 320 to 660 mμ in many types of respiring cell suspensions. In bakers' yeast, for example, the reaction kinetics and spectra of the pyridine nucleotides, flavoproteins and cytochromes of types *a*, a_3, *b* and *c* can be separately studied by rapid and sensitive spectroscopic methods[7,8]. In some cases the sensitivity exceeds that achieved by highly skilful visual observers since we can regularly record the 590 mμ band of cytochrome a_1 in cultures of *Azotobacter chroococcum*. More recently these spectroscopic methods have been improved so that they are suitable for measuring the changes in optical density caused by the formation of the carbon monoxide compounds[9] of the respiratory enzymes[10] or cytochrome oxidases[11] of the cell suspensions.

About ten years ago BÜCHER AND NEGELEIN developed an "optical method" for the study of the kinetics of photodissociation of the CO compounds of the soluble pigments myoglobin and hemoglobin[12]. By introducing new electronic techniques, we now have developed a more sensitive method for use with turbid cell suspensions, first, for demonstrating that the photodissociation of the cytochrome a_3–CO compound actually occurs[13], secondly, for obtaining "photodissociation spectra" of the CO compounds of respiratory enzymes[13], and thirdly, for obtaining accurate values for the molecular extinction of the α-bands of the CO compounds of the respiratory enzymes by direct meas-

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urements of the kinetics of photodissociation and recombination of the CO compounds[14]. The latter method is much more direct than the manometric method[15,16] which responds too slowly to permit a direct measurement of the kinetics of photodissociation. We find the molecular extinction coefficient of the α-band of cytochrome a_3–CO to be $\varepsilon = 12$ cm$^{-1} \times$ mM^{-1} for bakers' yeast cells and for heart muscle homogenates[14].

The classical manometric method for determining the relative photochemical action spectrum for the reversal of carbon monoxide inhibition of respiration that was developed twenty-five years ago[17] has been until now the only method so far available. Although this manometric method has given excellent spectra, there seem to be large changes in the heights of the major absorption bands and in the details of the subsidiary bands when the temperature is lowered[18,19]. Also, MELNICK'S action spectrum for yeast[20] does not agree in detail with that of KUBOWITZ AND HAAS[18] nor does his 450 mμ peak for heart muscle preparation agree with any of the other data on cytochrome a_3[13,19]. Thus a new method that permits monochromatic illumination of the sample over a wide range of wavelengths is highly desirable. We can report here preliminary experiments with an apparatus for measuring photochemical action spectra in a drop of cell suspension[21] with the aid of the platinum microelectrode[22,23]. We have not yet perfected the fourth and logical development of these techniques—the plotting of the photochemical action spectra from data on the direct measurement of the photodissociation kinetics for a number of wavelengths of monochromatic photodissociating light, but such an apparatus appears feasible.

We have surveyed the respiratory pigments of various materials with these sensitive methods[24] and have recently focussed our attention upon a rather different "CO-binding pigment" found in *Staphylococcus albus* and in other bacteria. Our absorption spectrum (difference spectrum) for this CO compound shows peaks at 416 mμ[9], 535 and 570 mμ[25], and the photodissociation spectrum shows close agreement with the absorption spectrum; the peak of the Soret band lies at 415 mμ[13]. We here present quantitative data on the kinetics of photodissociation of this CO-binding pigment and find that it is considerably less light-sensitive at 589 mμ than the enzyme of yeast or muscle, but that it has a distinctive band at 546 mμ. Our preliminary value for the molecular extinction of the CO-binding pigment at this wavelength is $\varepsilon = 5$ cm$^{-1} \times$ mM^{-1}. We have also determined the relative photochemical action spectrum for this pigment and find that the action spectrum in the Soret region has a peak at 418 mμ, in agreement with our absorption and photodissociation difference spectra and distinctly different from the 430 mμ peak for yeast and muscle[21]. Our results suggest that this "CO-binding pigment" is a new respiratory enzyme that has a prosthetic group closely allied to that of the protohemin enzymes and distinctly different from the dichroic hemin enzymes, and should therefore be classed as a completely new type of respiratory enzyme.

*Absorption difference spectra**

One of the more useful methods for obtaining difference spectra is illustrated by Fig. 1. This method utilizes the light chopping and demodulating system developed by CHANCE for a double-beam spectrophotometer[8] and an automatic gain control circuit (agc) developed by R.C.A.[26], together with a number of ingenious improvements devised by Dr. C. C. YANG[27]. The apparatus consists of a source of high intensity monochromatic light that is split into two paths by a vibrating mirror (60 cps) and illuminates

* *Footnote see page 292.*

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two suspensions of respiring cells of equal concentration. Cuvette A is taken to be the reference cell and the photocurrent obtained upon illuminating that cuvette is maintained at a predetermined level by an automatic gain control circuit that receives its signal from the contacts of the first demodulator and adjusts the dynode voltage of the photomultiplier to the appropriate value for constant photocurrent regardless of the intensity of the light illuminating the cuvette, the transmission through the sample, or the sensitivity of the photosurface. This constitutes the "100% transmission" signal. When cuvette B is illuminated, a signal representing the actual transmission is received from the first demodulator and is measured by a second demodulator. In order to record optical densities, the percent transmission is converted into logarithms by a segmented logarithmic characteristic consisting of ten diodes[28, 29]. The optical density values are then plotted by a servo recorder (Leeds and Northrup Speedomax). In order to have a linear wavelength scale on the chart, an appropriately loaded potentiometer operates in the feedback circuit of the chart drive servomechanism[30].

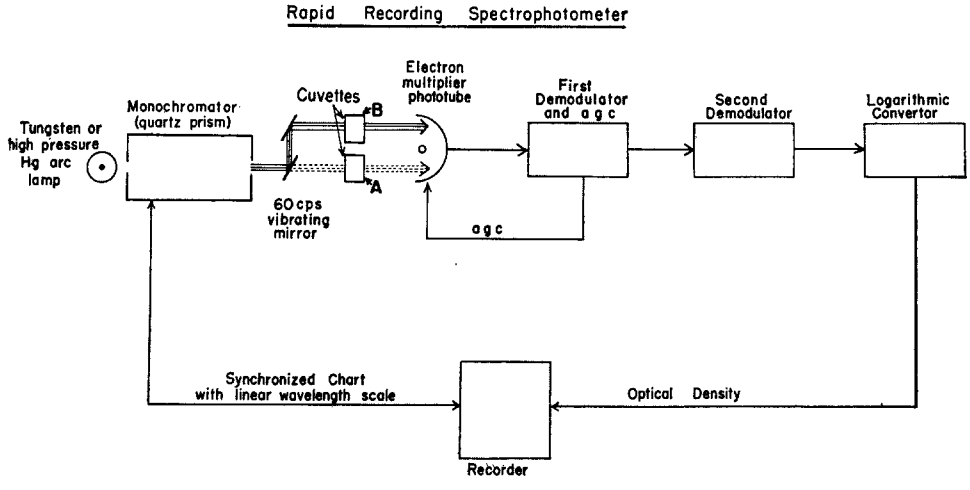

Fig. 1. A schematic diagram of the operation of a spectrophotometer suitable for recording the spectra of the respiratory pigments of cell suspensions and tissue homogenates. agc represents "automatic gain control" (MD-25).

The apparatus has a noise level of $\sim 1 \cdot 10^{-4}$ in optical density and operates with a spectral interval of 2 mμ or less when the cuvettes are filled with a turbid suspension of respiring cells. The spectrum is plotted at the rate of a few millimicrons per second.

In studies with turbid cell suspensions, it is important to gather both the transmitted and the scattered light from the cell suspension in order to obtain adequate sensitivity. This we accomplish by placing the phototube near the cell suspension and thereby avoid the lens and prism as were used earlier by WARBURG AND CHRISTIAN[6].

In actual use, a "base-line" is plotted with cuvettes A and B filled with equal concentrations of oxidized cells. Then the substrate is added to the cell suspension in cuvette B so that the oxygen is consumed and the absorption bands of the reduced cytochromes are recorded. Next the substrate is added to cuvette A and a second base-line is drawn. CO is finally bubbled through cuvette B to form the CO-reduced compound and the spectrum of the CO compound is plotted. (For further details see reference 9.)

Thus this apparatus is especially useful for plotting difference spectra, for example,

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the spectrum representing the difference between the reduced and oxidized forms of the respiratory pigments of *S. albus* illustrated by the trace labelled "reduced" in Fig. 2. And the trace labelled "reduced + CO" represents the difference between the CO compounds and the reduced forms of the respiratory pigments. In this case one observes a distinct peak at 416 mμ and a trough at 432 mμ, as contrasted to the peak and trough of the corresponding spectrum for the yeast cells which lie at 430 and 445 mμ respectively.

Similar studies can be carried out for *S. albus* in the visible region of the spectrum and peaks at 535 and 570 mμ are reported by SMITH[25].

Fig. 2 when compared with Fig. 3B of reference 9 shows that cultures of *S. albus* may be treated so as to increase considerably their relative content of this CO-binding pigment.

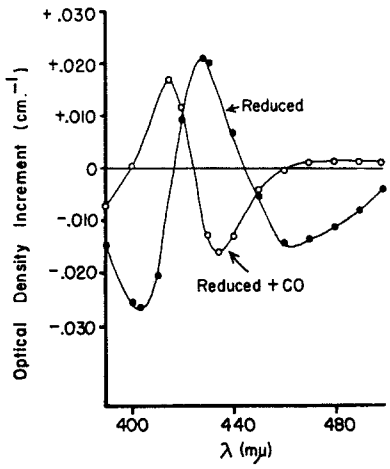

Fig. 2. The absorption difference spectra for a culture of *Staphylococcus albus* obtained by means of the apparatus of Fig. 1. The trace labelled "reduced" represents the differences between the reduced and oxidized cytochromes and the trace labeled "reduced + CO" represents the difference between the CO compound and the reduced form. Similar data can readily be obtained in the visible region (o-37).

Photodissociation difference spectra

Because their experimental conditions were inappropriate, KEILIN AND HARTREE were not able to demonstrate the photochemical dissociation of the CO compound of cytochrome a_3. We have recently been able to accomplish this by a differential spectrophotometric method that is suitable for the observation of changes in absorption due to the photodissociation reaction within the respiring cell.

This experiment is considerably more difficult than that carried out by BÜCHER AND NEGELEIN on clear solutions of hemoglobin and myoglobin carbon monoxide. It is not possible to use here the favorable optical geometry that they used—a short optical path for the photodissociating light and a long path for the measuring of light. With turbid cell suspensions, we require a fairly large surface area of the suspension near the measuring photosurface (see p. 291, 2nd paragraph under Fig. 1). Thus we have used a square cuvette in which the photodissociating and the measuring paths are both equal to one cm.

Another novel feature of the method that we use is the ability to vary the measuring wavelength and thereby to obtain a "photodissociation difference spectrum"* of the CO compound. Since the turbid suspensions scatter photodissociating light of very high intensity in the direction of the measuring phototube, our method has three features that avoid interference with the spectrophotometric measurement by the photodissociating light.

* In order to distinguish between the three types of spectra of the CO compounds that are discussed in this paper, it is useful to define the three terms:

Absorption difference spectrum. This is a spectrum representing the change of light absorption caused by a chemical change of the pigment, for example, from oxidized to reduced (a reduced-oxidized spectrum), or from reduced to the CO compound (a CO-reduced spectrum).

Photodissociation difference spectrum. This is a spectrum representing the change of light absorption caused by a photochemical reaction, for example, the photochemical dissociation of a CO compound of a reduced cytochrome in which case a CO-reduced spectrum is obtained.

Photochemical action spectrum. This is an absolute (not difference) spectrum. The ordinates are inversely proportional to the quantum intensity required at each wavelength to produce a given rate of photochemical decomposition of the CO compound.

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First, the photodissociation is accomplished by illuminating the cell suspension with yellow light, for example, the 589 mμ line of the Na arc or the 578 mμ of the Hg arc, other portions of the spectrum of these line sources being readily eliminated by appropriate filters having the characteristics shown in Fig. 3. The observation of the photodissociation of CO compounds is made in the region of the Soret band (410-480 mμ) and the photocell is rendered insensitive to the yellow light not only by the nature of its surface (Cs–Sb) but also by the blue colour filter combination shown by the solid curve of Fig. 3.

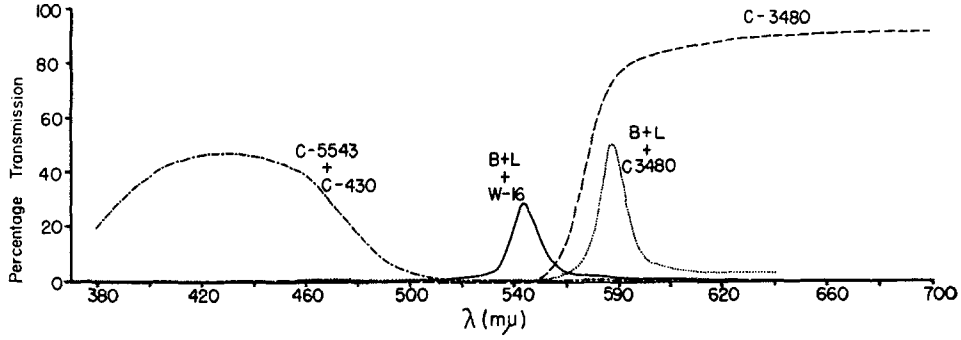

Fig. 3. Transmission curves for optical filters used to isolate photodissociating light (above 520 mμ) from spectrophotometric wavelengths (410–480 mμ). C represents Corning glass filters, W represents Wratten filter, and B + L represents Bausch and Lomb interference filters (MD-27).

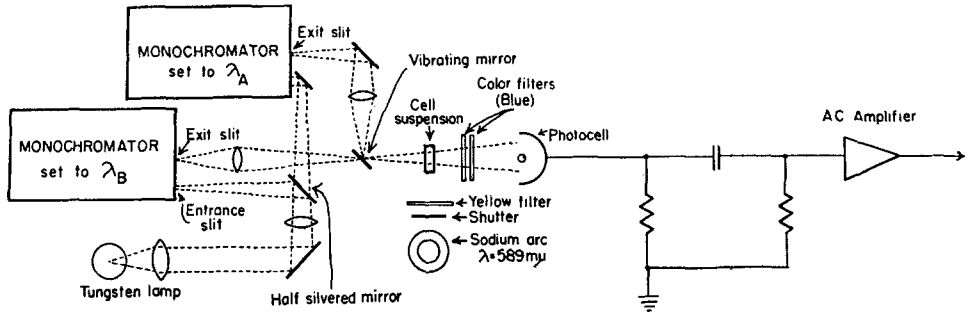

Fig. 4. A schematic diagram of a double-beam spectrophotometer suitable for measuring the kinetics of the photochemical decomposition of the CO compounds of the respiratory enzymes within intact cells. The characteristics of the optical filters are given in Fig. 3. The wavelengths λ_A and λ_B are set in the region 410–480 mμ (MD-28).

Secondly, as Fig. 4 shows, the differential double-beam spectrophotometer employs two wavelengths of light, one a reference wavelength, for example 480 mμ where no appreciable optical density changes due to photodissociation are to be expected, and the other an adjustable wavelength (in the region 410–480 mμ). The photoelectric circuit measures the differences of the light transmission changes at the two wavelengths, and this difference is not affected by leakage of yellow light through the colour filters.

Thirdly, to discriminate further against light leakage, the two beams of monochromatic light are chopped by a vibrating mirror at 60 cps so that first one and then the other is incident upon the sample and the photocell. Thus the output current from the photocell consists of a square 60 cps wave, the amplitude of which represents the difference of light transmission at the two wavelengths of light[8]. Since the arc lamps for

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causing photodissociation are operated on well-filtered direct current, there is no alternating component of the yellow light that leaks through the filters.

By means of these three design factors, the filtered light from a 100 watt Na or Hg lamp a few inches from the sample cell causes no deflection of the trace of the spectrophotometer. The only detectable effect of the light leakage is an increase in the shot noise output of the photoelectric circuit.

A typical record of the photodissociation and recombination of the CO compound in bakers' yeast cells is shown in Fig. 5. Starting with the steady-state oxidized yeast cells in the presence of alcohol, reduction of cytochrome a_3 causes the abrupt increase of optical density at 445 mμ (with respect to 480 mμ) as indicated by the downward sweep of the trace. Illumination of the cells with the Na arc at this time results in no

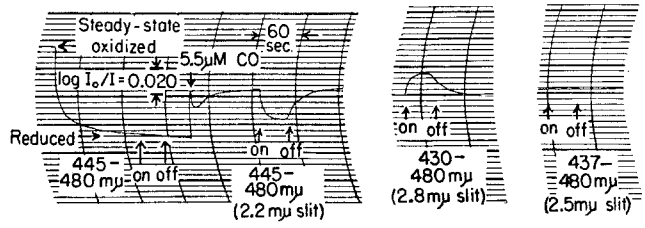

Fig. 5. An example of the measurement of a photodissociation difference spectrum of the cytochrome a_3–CO compound of bakers' yeast cells with the apparatus of Fig. 4. The points "on" and "off" represent the moments at which the photodissociating light is turned on and off. Illuminating light is 589 mμ. (25° C) (Expt. 145e).

deflection of the trace. When reduction is complete and the cells are substantially anaerobic, a solution of CO is added to give a final concentration of 5.5 μM, causing the formation of the cytochrome a_3–CO compound. Illumination of the cells now causes the dissociation of the CO compound while darkness allows its reformation, the latter change corresponding to a decrease of optical density and to a trough in the difference spectrum. If now the wavelength is shifted to 430 mμ, illumination causes the opposite sign of optical density change to occur, corresponding to a peak in the difference spectrum. And if a wavelength of 437 mμ is used no change at all occurs; this is an isosbestic point between the reduced and CO-reduced spectra providing a good control against possible artifacts.

These deflections, plotted as a function of wavelength, form a "difference spectrum" that represents the differences between the absorption of the CO compound and the reduced form of cytochrome a_3. The peak of this difference spectrum would be expected to lie very close to that of the absolute photochemical absorption spectrum of the respiratory enzyme at 430 mμ[17] because, as KEILIN AND HARTREE already have shown[11], the respiratory enzyme has many of the properties of cytochrome a_3. The result obtained with direct methods affords a conclusive proof of the identity of the respiratory enzyme of *T. utilis* and cytochrome a_3 of heart muscle preparations.

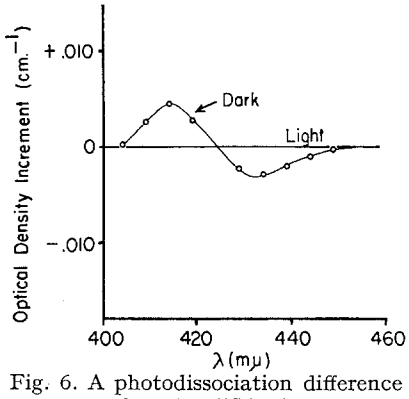

Fig. 6. A photodissociation difference spectrum for the "CO-binding pigment" of *Staphylococcus albus* obtained with the apparatus of Fig. 4 (948 c).

S. albus shows a rather different pigment with a peak at 415 mμ and a trough a 433 mμ as shown in Fig. 6 [13].

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In order to demonstrate that our method for measuring the photodissociation spectrum gives a result that agrees accurately with the actual absorption spectrum, we compare in Fig. 7 the spectrum obtained by subtracting the ferromyoglobin–CO spectrum from that of ferromyoglobin (BEZNAK[31]) with a photodissociation spectrum obtained with this apparatus. The agreement of the data shows that the photodissociation method gives nearly as accurate results as the direct measurement of the absorption spectra.

It should be noted that exact coincidence of the peaks of the absorption photodissociation difference spectra with those of the relative photochemical action spectra is not to be expected. Fig. 7 (Curve A) clearly shows that for protohemin pigments the peak of the difference spectrum lies 2.5 mμ below that of the absolute spectrum and, in the case of the dichroic hemin enzyme lactoperoxidase, the displacement is 1.5 mμ[13]. Thus the displacement is small, but significant.

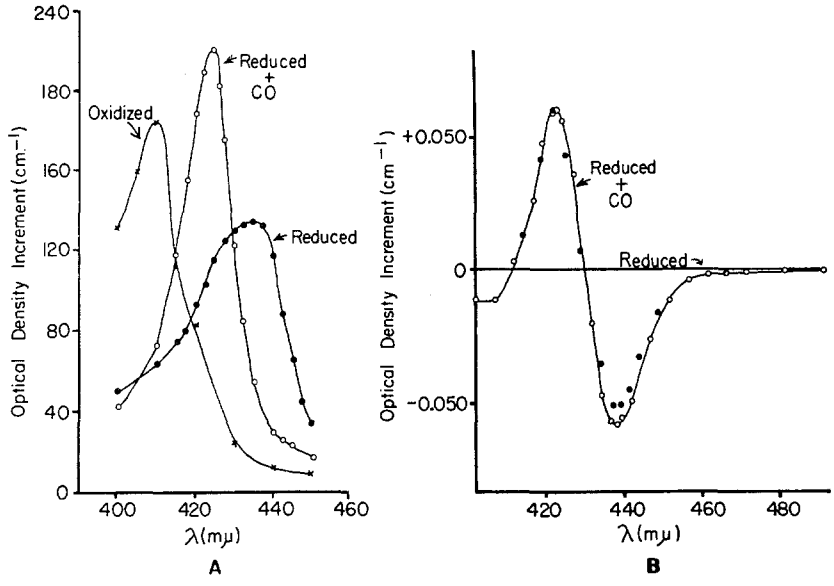

Fig 7. (A), the oxidized, reduced, and reduced-CO spectra for myoglobin (from BEZNAK[31]) and (B), (open circles), the difference spectrum of the CO compound. Solid circles of (B) show experimental data on the photodissociation spectrum of myoglobin-CO obtained by the method of Fig. 4. In order to facilitate the comparison, the ordinates of the photodissociation curve were multiplied by a constant factor to cause the two sets of data to match at the peak of the curve (1 μM Mgb, pH = 7.0, 2.9 μM CO) (Expt. 143a).

Calculation of the molecular extinction coefficients

By measuring the kinetics of photodissociation and recombination of the CO compound on a faster time scale as in Fig. 8, the molecular extinction coefficient may be calculated in a manner similar to that used by BÜCHER AND KASPERS[33] provided the intensity of the photodissociating light is known. But instead of a bolometer we use myoglobin–CO as a standard and thereby avoid the need for an accurate measurement of the light intensity as well as the distribution of intensities in the cuvette. To simulate light scattering when myoglobin is used, *Escherichia coli* are added to give the same scattering effects as the yeast cells (see reference 14 for details). On this basis, we have computed the values of the molecular extinction coefficients for the CO compounds in heart muscle

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homogenates, bakers' yeast cells, *A. pasteurianum*, and *Bacillus subtilis*[14], and find $\varepsilon_{589} = 12\ cm^{-1} \times mM^{-1}$ for the cytochromes of type a_3. (Reference 14 gives detailed data on the experimental controls and also the method of calculating the results.)

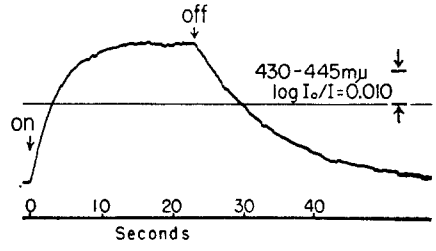

Fig. 8. The kinetics of photodissociation and recombination of the cytochrome a_3–CO compound in bakers' yeast cells as measured by the method of Fig. 4 (Expt. measured by the method of Fig. 4. Illuminating light is 589 mμ (Expt. 144).

In our previous studies the sensitivity of the apparatus was insufficient to give any quantitative idea of the molecular extinction coefficients of the "CO-binding pigment" of *S. albus*. We now have increased the sensitivity, and satisfactory kinetic data may be obtained as in Fig. 8. A preliminary value can be given for the molecular extinction coefficient of the band at 546 mμ, $\varepsilon = 5\ cm^{-1} \times mM^{-1}$, about half that of the value for myoglobin–CO at 580 mμ ($10.6\ cm^{-1} \times mM^{-1}$)[32]. The extinction coefficient at 589 mμ is very small compared to that of the yeast enzyme and this emphasizes the difference between this CO-binding pigment and cytochrome a_3.

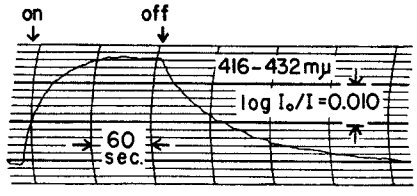

Fig. 9. The kinetics of photodissociation and recombination of "CO-binding pigment" in a suspension of *Staphylococcus albus* cells as measured by the method of Fig. 4. Illuminating light is 578 mμ (Expt. 144).

The photochemical action spectrum

One of us (L.C.) has recently developed an apparatus for measuring photochemical action spectra in a drop of bacterial suspension by means of the platinum microelectrode[21]. This method utilizes the steady-state system developed by CONNELLY AND BRINK[23] in their studies of the respiration of nerve. A steady-state in oxygen tension results from the balance between inward diffusion of oxygen and utilization of oxygen by the nerve tissue. The same conditions obtain in a drop of bacterial or yeast suspension respiring in a CO–O_2 atmosphere. The size of the drop, the number of cells, and the substrate concentration are adjusted so that the steady-state oxygen concentration for maximal effectiveness of the photochemical reaction is obtained. Illumination of the drop will displace this steady-state and the change of oxygen concentration is sensitively recorded by the platinum microelectrode.

Our results for *S. albus* show a Soret band at 418 mμ definitely displaced from the 430 mμ peak measured for yeast cells with the same apparatus. A preliminary action spectrum for the respiratory enzyme in *S. albus* in the Soret region is shown in Fig. 10.

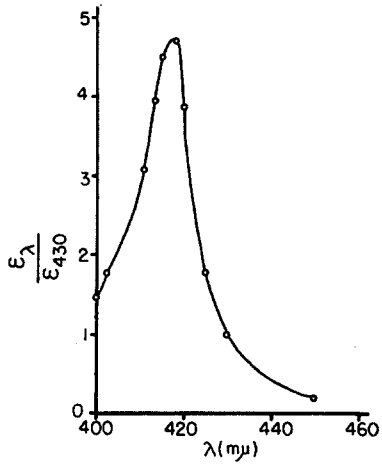

Fig. 10. A relative photochemical action spectrum of the CO-binding pigment in *Staphylococcus albus*. Similar data are readily obtained in the visible region of the spectrum (25°) (0–38).

In the visible region of the spectrum, the peaks of the CO compound are found to lie at 535 and 566 mμ in fairly good agreement with the peaks of the absorption difference spectrum that lie at 535 and 570 mμ[25]. These values are similar to those for hemoproteins

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that have protohemin as their prosthetic group and lead us to suggest that this new respiratory enzyme has a protohemin prosthetic group instead of a dichroic hemin and that it should be classified as a completely new type of respiratory enzyme.

SUMMARY

Several highly refined physical methods have been described for the study of the CO compounds of the respiratory pigments of living cell suspensions. These methods reveal significant differences in the respiratory enzymes of different bacterial cells and our results suggest that *Staphylococcus albus* contains a new respiratory enzyme that has a prosthetic group closely related to the protohemin enzymes and that this respiratory pigment should be classified as a completely new type of respiratory enzyme.

RÉSUMÉ

Plusieurs méthodes physiques très fines pour l'étude des composés oxycarbonés des pigments respiratoires de cellules vivantes en suspension ont été décrites. Ces méthodes révèlent des différences significatives entre les enzymes respiratoires de différentes bactéries et nos résultats suggèrent que *Staphylococcus albus* renferme un nouvel enzyme respiratoire dont le groupement prosthétique est très voisin des enzymes protohémines. Ce pigment respiratoire doit être rangé dans un groupe complètement nouveau des enzymes respiratoires.

ZUSAMMENFASSUNG

Mehrere höchst verfeinerte physikalische Methoden für die Untersuchung der Atmungspigmente von Suspensionen lebender Zellen werden beschrieben. Diese Methoden erlauben bedeutsame Unterschiede zwischen Atmungsfermenten verschiedener Bakterien aufzufinden. Unsere Versuche machen es wahrscheinlich dass *Staphylococcus albus* ein Pigment enthält dessen prosthetische Gruppe mit den Protohäminfermenten nahe verwandt ist, aber als ein ganz neuer Typ von Atmungsferment angesehen werden muss und entsprechend eine Klasse für sich bildet.

REFERENCES

1 C. A. MACMUNN, *Phil. Trans. Royal Soc. London*, 177, (1885) 267.
2 D. KEILIN, *Proc. Roy. Soc. London, Series B*, 98 (1925) 312.
3 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 262 (1933) 237.
4 H. LUNDEGÅRDH, *Arkiv. Kemi*, 5 (1952) 97.
5 O. WARBURG AND W. CHRISTIAN, *Biochem, Z.*, 266 (1933) 399.
6 E. HAAS, *Naturwissenschaften*, 22 (1934) 207.
7 B. CHANCE, *Nature*, 169 (1952) 215.
8 B. CHANCE, *Rev. Sci. Instruments*, 22 (1951) 619.
9 B. CHANCE, *J. Biol. Chem.*, 202 (1953) 383.
10 O. WARBURG, *Schwermetalle als Wirkungsgruppen von Fermenten*, Chapter 8, Freiburg (1949).
11 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. London, Series B*, 127 (1939) 167.
12 T. BÜCHER AND E. NEGELEIN, *Biochem. Z.*, 311 (1942) 163.
13 B. CHANCE, *J. Biol. Chem.*, 202 (1953) 397.
14 B. CHANCE, *J. Biol. Chem.*, 202 (1953) 407.
15 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 202 (1928) 202.
16 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 214 (1929) 64.
17 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 193 (1928) 339.
18 F. KUBOWITZ AND E. HAAS, *Biochem. Z.*, 255 (1952) 247.
19 D. KEILIN AND E. F. HARTREE, *Nature*, 171 (1953) 413.
20 J. L. MELNICK, *J. Biol. Chem.*, 141 (1941) 269.
21 L. N. CASTOR AND B. CHANCE, to be published.
22 P. W. DAVIES AND F. BRINK, *Rev. Sci. Instruments*. 13 (1942) 524.

[23] C. M. CONNELLY AND F. BRINK, *Rev. Sci. Instruments*, August, 1953.
[24] L. SMITH, to be published.
[25] L. SMITH, *Federation Proc.* 12 (1953) 270.
[26] Radio Corporation of America, personal communication on a normalizing amplifier (1949).
[27] C. C. YANG, *Proc. Institute of Radio Engineers*, 40 (1952) 220.
[28] B. CHANCE, V. HUGHES, E. MACNICHOL, D. SAYRE AND F. C. WILLIAMS, *Waveforms*, Chapter 8, New York (1949).
[29] B. CHANCE, F. C. WILLIAMS, C. C. YANG, J. BUSSER AND J. HIGGINS, *Rev. Sci. Instruments*, 22 (1948) 683.
[30] I. GREENWOOD, J. HOLDAM AND D. MACRAE, Electronic Instruments, p. 94, New York (1948).
[31] M. BEZNAK, *Acta Chem. Scand.*, 2 (1948) 333.
[32] H. THEORELL, *Biochem. Z.*, 268 (1934) 55.
[33] T. BÜCHER AND J. KASPERS, *Biochim. Biophys. Acta*, 1 (1947) 21.

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