MICROSPECTROPHOTOMETRY OF CYTOCHROMES IN THE SINGLE CELL AT ROOM AND LIQUID NITROGEN TEMPERATURES

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ABSTRACT

By increasing further the sensitivity of microspectrophotometry, it is now possible to measure, under favorable conditions, the smaller absorption bands of the respiratory pigments of single cells in the visible region of the spectrum. A considerable aid in the distinction between cytochromes is afforded by liquid nitrogen microspectrophotometry. Under favorable conditions, the height of the peaks is increased over 8-fold at low temperatures. In diploid yeast, characteristically sharpened components not resolvable at room temperature are observed at low temperature; and in pentaploid yeast, a hitherto unrecognized pigment is observed at 583 mμ. These preliminary results indicate the feasibility and the value of low temperature microspectrophotometry of biological materials.

Using the microspectroscope and cooling the preparation down to liquid nitrogen temperature, Keilin and Hartree (1) were able to observe more clearly the absorption peaks in the region of the α bands of the cytochromes. At room temperature some of these pigments of the respiratory chain are indistinguishable from one another. The development of much more sensitive spectrophotometric methods (2, 3) has largely extended such observations, quantitatively as well as qualitatively, as indicated by the work of Estabrook, who has extended these methods to the macrospectrophotometric study of cytochromes at liquid nitrogen temperature (4). The essential features of the absorption characteristics revealed at liquid nitrogen temperature in comparison with those at room temperature are narrowing or sharpening of the bands, and an increase of the specific absorbancies up to 10-fold.

Microspectrophotometric localization and assay of the respiratory pigments in the single intact cell has demonstrated the complexity and compartmentalization of various metabolic units *in vivo* (5). To obtain a more precise biochemical definition of these subcellular components, it seems necessary to attain a better microspectroscopic resolution. The present paper describes the application of the low temperature technique to the microspectrophotometry of the single cell.

The first problem is a technological one, namely, to construct a suitable low temperature chamber for the cooling of microscopic objects on the microspectrophotometer stage. In the actual microspectrophotometric measurements, the main question has been: do the respiratory pigments display the same spectra at low temperature in the medium of the cell as in cell suspensions and in purified preparations? Another question is: to what extent will the complex light-scattering properties of the cell cause an enhancement of the specific absorption, especially in the region of the α bands?

METHODS

Low Temperature Attachment

The low temperature attachment was intended to maintain the specimen at approximately the tem-

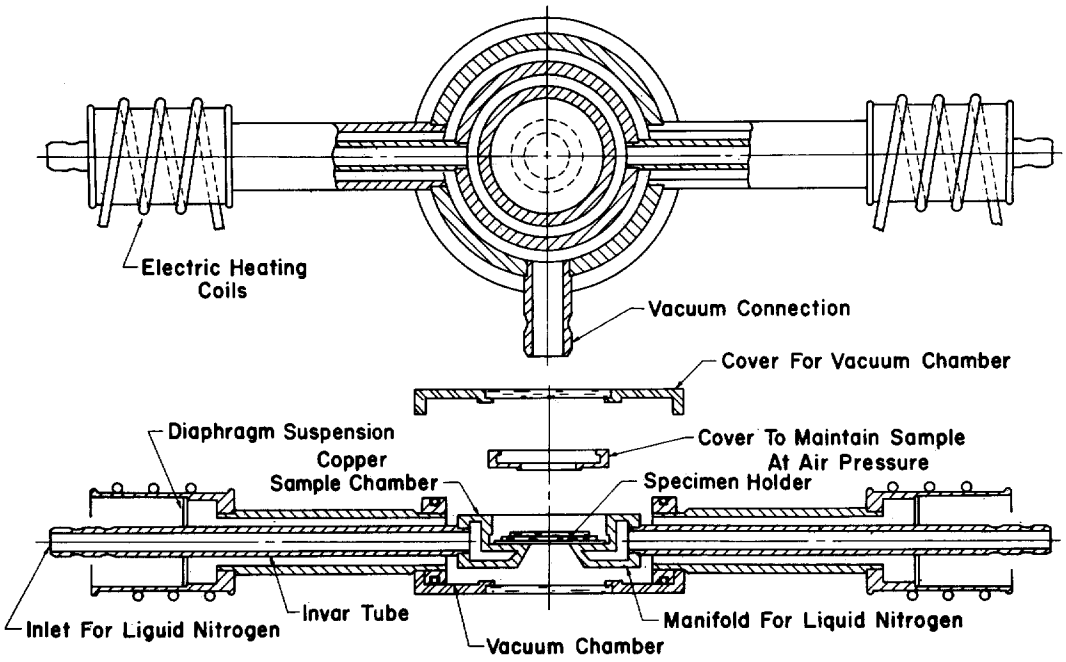

FIGURE 1 Diagram of the low temperature attachment. (FA 62.)

perature of liquid nitrogen and mechanically stable against motion due to the temperature gradient along the tube carrying the liquid nitrogen (Fig. 1). This was accomplished by suspending the tube by a diaphragm at the end of a vacuum closure surrounding the specimen holder. The tube was made of Invar, to obtain a low coefficient of thermal expansion. In addition, electric heating coils were placed over the ends of the rod and were differentially adjusted to allow a precise positioning of the rod. The specimen was placed in a vacuum chamber so that the conduction of heat away from the sample chamber to the ends of the chamber would be negligible. The sample chamber itself was arranged so that the liquid nitrogen did not enter the chamber but was contained in a manifold surrounding the chamber. The chamber was made of copper, to give good heat conduction from the liquid nitrogen to the specimen. Since the liquid nitrogen flowed continuously past the chamber, the temperature of the sample was taken to be that of the liquid nitrogen. The temperature was also controlled by a thermocouple. The specimen itself was held on a coverslip 8 mm in diameter in close contact with the cold metal. The inner chamber could be evacuated so that the sample was "frozen and dried." Alternatively, a vacuum-tight cover was provided so that the sample might be maintained at air pressure. The vacuum was pumped continuously with an Eimac oil diffusion pump.

Modifications and General Performance of the Spectrophotometer

The apparatus previously described (6) was equipped with a Cooke 40× long working (1 cm) objective and a 20× condenser. The over-all magnification was 40 × 3.5. With the low temperature chamber in place and using a measuring aperture of 8 μ, a noise level of 0.02 per cent in absorption (8×10^{-5} in terms of optical density) has been achieved with a time constant of 5 seconds and a spectral interval of 5 mμ. A sensitivity of 0.02 per cent per small scale division was adequate to record clearly the spectra presented here, and the noise scarcely shows in the traces except when the spectral interval is 1.5 mμ at liquid nitrogen temperature (Fig. 3 *a*, *b*).

It is generally observed that the light scattering along the optical path in the free space outside the cell differs greatly from that through the cell, particularly in the frozen state at liquid nitrogen temperature. Thus, a number of the spectra observed here are recorded on a slanting base line. Cytochrome concentrations can, nevertheless, be measured by taking advantage of the known isosbestic points for cytochrome difference spectra at 540 and 580 mμ. Thus, the main assumption in the estimate of the absorbancy changes is whether or not the base line is straight over this 40-mμ interval. In these experiments, it is assumed to be straight.

Enhancement is calculated here by the ratio of the absorption band of a particular cytochrome at low temperature to that measured at room temperature (26°C), the base line being drawn as described above. The enhancement is due to the sharpening of the band at low temperature and to the increased optical path due to multiple scattering of light. Our enhancement values are purely operational and indicate the usefulness of the low temperature method in increasing the detectability of the material. Only under highly controlled conditions is it permissible to use the absorbancy changes measured for a calculation of cytochrome concentrations. This procedure is surely not recommended at this early stage in the development of low temperature microspectrophotometry.

Preparation of Sample

The most convenient method of preparation of isolated proteins consisted in quick drying of a few microliters of the protein solution (with or without dithionite as required) in the low temperature cell by means of the vacuum pump. Usually the sample was scratched in order to provide a convenient clear space for the reference light beam of the spectrophotometer. Cells were spread on a coverslip and treated with a minute quantity of dithionite as necessary. After partial drying, a drop of paraffin oil or 70 per cent glycerol was added and a coverslip was placed over the drop. The 8-μ aperture is sufficiently small to enable observation of spectra in single yeast cells and in portions of kidney and liver cells.

RESULTS

Experiments with Isolated Cytochrome c

Thin films of cytochrome *c*, dried in the manner described above, provided an excellent material for testing the performance of the apparatus at low temperature, because of the characteristic sharpening of the cytochrome *c* absorption band (4). Fig. 2*a* illustrates the room temperature absorption spectrum of dithionite-reduced cytochrome *c* of bonito heart.[1] It is seen that there is a 3 per cent absorbancy change between the peak and the trough of the α band (550 to 540 mμ. If the temperature is lowered by passing liquid nitrogen through the chamber, the absorbancy peak (Fig. 2*b*), which was at 550 mμ at room temperature, is now observed at 548 mμ, and its amplitude is 4.3 per cent, a 40 per cent increase over the value measured at room temperature. In view of the fact that the band is considerably sharper at low temperature, the

[1] Courtesy of B. Hagihara.

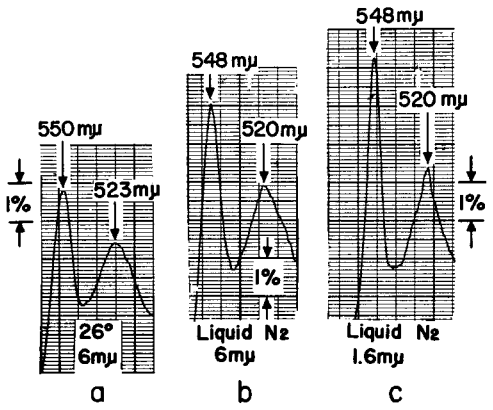

FIGURE 2 Room and low temperature spectra of crystals of cytochrome *c* of bonito heart, air-dried with dithionite in the low temperature chamber. (36A, B, C.)

spectral intervals of 6 mμ in Fig. 2 *a* and *b* are diminished to 1.6 mμ in the record in Fig. 2 *c*, with the result that the magnitude of the band has increased to 5.5 per cent, an 80 per cent increase over that at room temperature. There is a slight indication of the α_2 shoulder on the short wave side of the peak (4).

Though the magnitude of the absorption band for purified cytochrome *c* increases significantly at low temperature, the extent is not nearly so great as is observed with cytochrome *c* in the respiratory chain in various cells, as described below. Since this increase is largely due to an increase in the effective optical path due to multiple scattering, the difference may be at least partly attributed to the differences in the medium in which the cytochromes are frozen.

It is significant to note that at the low temperature the noise level is very nearly equal to that at room temperature, indicating the establishment of a satisfactory mechanical stability. Since the signal is somewhat larger, a net over-all improvement of performance has been achieved.

Intracellular Cytochromes

KIDNEY CELLS: Cells were teased from rat kidney cortex and were treated with a small amount of dithionite and then immersed and covered in paraffin oil. The cells, which had a total absorption at 550 mμ of approximately 7 per cent (the bottom of Fig. 3 *a* is zero absorption), showed distinct peaks at 551.5 and 603 mμ. The magnitude of the peak at 551.5 mμ with

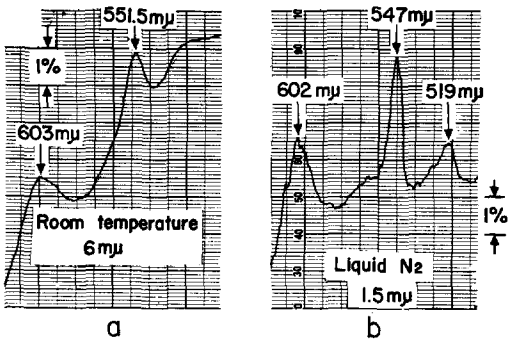

FIGURE 3 Room and low temperature spectra of a cell from rat kidney cortex. Cell treated with dithionite and covered with paraffin oil. (30 III.)

reference to a base line tangent at 580 and 540 mμ is approximately 2 per cent. Fig. 3 *b* illustrates the effect of lowering the temperature by cooling the specimen with liquid nitrogen. The bands are at 547 and 602 mμ, and the amplitude of the 550-mμ band is increased to about 4 per cent, a 2-fold increase. The β bands are observed fairly prominently in this experiment, and the $c_{\alpha 2}$ shoulder can be seen. It is noteworthy, however, that no significant absorption appears at 560 mμ. This is in agreement with our results on microspectrophotometry of small (~1 μ) areas in intact cells (7), where Soret bands devoid of cytochrome *b* were reported. Though cytochrome *b* has clearly been identified in mitochondria isolated from rat kidney (8), this component is slowly reduced in anaerobiosis (9), and it is probable that cytochrome *b* is not reduced under the present experimental conditions.

DIPLOID YEAST: A thin film of diploid bakers' yeast, treated with dithionite, dried, and immersed under a coverslip of paraffin oil, showed at room temperature (Fig. 4 *a*) a band at 552.5 mμ which is about 0.6 per cent in magnitude, computed as for Fig. 2 *a*. Actually the spectrum shown in Fig. 4 *a* was obtained after the cells were warmed to 26°C subsequent to measurement at the low temperature. At liquid nitrogen temperature, two and possibly three peaks can be observed. The prominent peaks are at 547.5 and 557.5 mμ, and are probably due to cytochrome *b* and *c*. The small peak in between is probably due to cytochrome c_1. The height above the apparent base line is 5 per cent. Although the estimate of the peak height at room temperature is difficult, the low temperature has probably increased the height of the absorption band as much as 8-fold.

LIVER CELLS: A study was made of liver cells under some variety of conditions because of the considerable interest in the mitochondrial and cytoplasmic cytochromes of this material. The best cytological definition of internal structure was afforded by cells which had been frozen-dried and sectioned to a thickness of approximately 10 μ. Interestingly enough, these specimens gave a large and characteristic absorption band, which is shown in Fig. 5. Here a cell shows a band with an amplitude of approximately 1.4 per cent having an α peak at 560.5 mμ at room temperature. There is no evidence of cytochromes of the respiratory chain, as indicated by the lack of a 550-mμ or a 603-mμ peak. When this specimen is cooled with liquid nitrogen, the peak is considerably sharpened and has slight evidence of a shoulder on the long wave side. The maximum is at 558.5 mμ and the absorbance change is 2.5 per cent, approximately twice that at room temperature. These absorption bands are not identical with those of purified cytochrome b_5. Though there is a possibility of hemochromogen formation due to the freeze-drying procedure, treatment of this specimen with carbon monoxide did not cause an appreciable shift of the 558.5-mμ absorption band. However, the lack of any evidence for cytochromes *a* and *c* in this spectrum

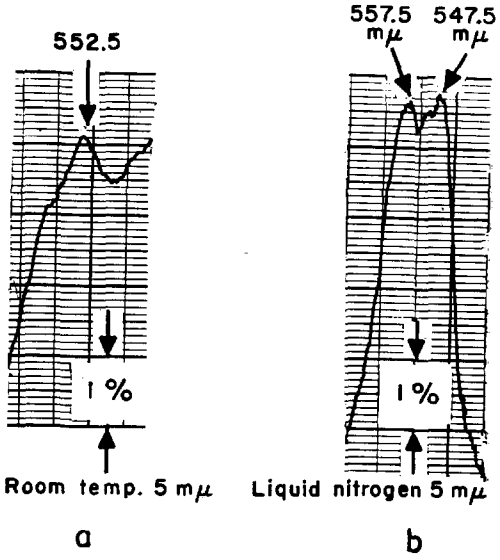

FIGURE 4 Room and low temperature spectra of diploid bakers' yeast cell treated with dithionite and immersed in paraffin oil. (29A III.)

and in those taken from other locations within the cell raises some questions as to the possibility of damage during the histotechnical procedure.

In order to determine whether cytochrome bands would be more distinctive in a cell treated less drastically, some cells were teased from liver, dried rapidly with a trace of dithionite, treated with a drop of paraffin oil, and closed under a coverslip. A room temperature spectrum, given in Fig. 6 *a*, shows a broad maximum peaking at 558 mμ. When the same specimen is cooled to liquid nitrogen temperature, only suggestions of peaks are obtained (Fig. 6 *b*), and one is at 551.5 mμ. This is what can be expected from our earlier microspectroscopic measurements on teased liver cells (7), in which the Soret absorption band during anaerobiosis showed a peak of only slightly above 1 per cent.

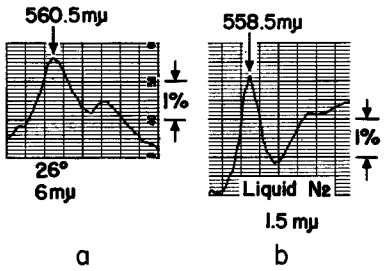

FIGURE 5 Room and low temperature spectra of frozen-dried and sectioned liver cell treated with crystals of dithionite and covered with 70 per cent glycerol. (31A.)

In order to obtain a larger group of mitochondria for spectrophotometric measurements, a piece of liver was centrifuged (7) and cells were teased from it in the manner described for Fig. 6. The results at room temperature are shown in Fig. 7 *a*, where again a broad maximum ranging from 560 to 550 mμ is indicated by the wavelength markers. When the specimen is cooled to liquid nitrogen temperature (Fig. 7 *b*), some resolution of peaks is obtained, and what appears to be the characteristic cytochrome *c* band at 548 mμ is observed. A *b* band is observed at a somewhat longer wavelength and appears to be a composite of cytochrome *b* from the respiratory chain and the endoplasm. It is not possible to estimate the increase of absorption at low temperature, but it is apparently very large.

PENTAPLOID YEAST: An interesting example of the increase in the discernibility of the absorption bands at low temperature is indicated in Fig. 8, where a dithionite-treated pentaploid yeast cell is under observation at room temperature (Fig. 8 *a*). Only a slight peak in the region of cytochrome *c* (549 mμ) is observable. On cooling with liquid nitrogen, a distinct peak is observed at 553 mμ (Fig. 8 *b*). A peak that was not observed at room temperature is now observed at about 583 mμ. The nature of this pigment in the pentaploid cells requires further investigation. No clear bands of cytochrome *a*, *b*, or *c* are discerned.

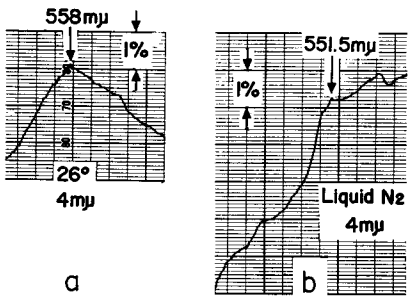

FIGURE 6 Room and low temperature spectra of liver cells dried with dithionite and covered with paraffin oil. (32 III.)

DISCUSSION

The first objective of these experiments was to determine whether the α bands of the cytochromes could be measured in single cells by microspectrophotometric methods at room temperature; our previous studies permitted the measurement of the Soret bands of cytochromes. With larger cells containing high concentrations of cytochromes, the location, the wavelength, and the amplitude of the α bands can be measured.

The second principal object of these experiments was to determine whether or not the absorption bands of the cytochromes could be dedetected when the temperature of the sample was reduced by means of liquid nitrogen. With the present design of the chamber, in which the thermal movements are largely compensated, this has been possible. The advantages achieved by this method are (*a*) a better distinction between cytochromes, and (*b*) more intense absorption bands.

The most successful application of the low temperature method is to diploid yeast, where a single peak at room temperature showed three peaks at low temperature, due to cytochromes of types *b*, c_1, and *c*. Also, the intensification at low temperature was approximately 10-fold. It is a matter of

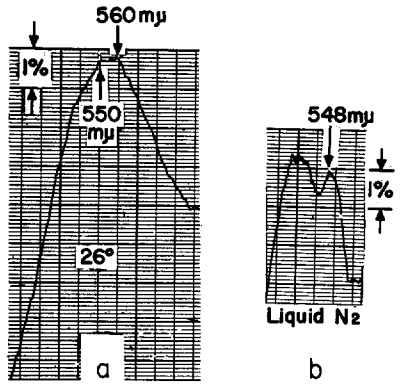

FIGURE 7 Room and low temperature spectra of cells teased from centrifuged liver. Cytochromes were reduced with dithionite and covered with paraffin oil. (33 III.)

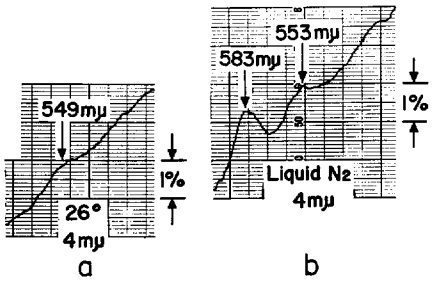

FIGURE 8 Room and low temperature spectra of pentaploid yeast cells. The yeast is mixed with dithionite, rapidly dried, and covered with paraffin oil. (31 III.)

interest that the cytochrome *c* band, which showed up clearly in diploid yeast, was not clearly observed in liver. However, it is recognized that different cytochromes show different intensifications at low temperatures. It would be expected that a cytochrome such as c_1 would be intensified as much in the yeast cell as in the liver and kidney cells. At the present time, we have no further explanation for the observed phenomenon.

Not surprisingly, it was found that certain types of tissue are more satisfactory than others for the purposes of these investigations. For example, excellent results can be obtained with cells that contain a relatively high concentration of cytochrome, such as diploid yeast and kidney cells. In the latter, owing also to favorable optical conditions, the Soret absorption clearly reveals the cytochrome complex at room temperature (7), although the low temperature spectra still give considerably higher resolution.

For further development, it is highly desirable to compare the results on absorption measurements of the cytochromes with the results obtained by fluorescence methods. The fluorescence method affords a quick and effective means of scanning the cells for the regions where the highest concentration of bound pyridine nucleotide is located. It has recently been possible to record fluorescence in tissues at liquid nitrogen temperature, and thus microlocalization of this component in the frozen state is possible (10). A correlation of the pyridine nucleotide and the cytochrome concentrations of such regions would be a matter of considerable importance. For example, it might allow a distinction between concentrations of pyridine nucleotide associated with glycolysis, in which no cytochromes could be observed, and concentrations of pyridine nucleotide associated with respiration, in which strong bands of cytochromes would be observed.

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