Localization and Kinetics of Reduced Pyridine Nucleotide in Living Cells by Microfluorometry*

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On the basis of early studies of the blue fluorescence of living cells and tissues before chemical treatment, Sjöstrand (1) suggested its association with the mitochondrial bodies. Microspectroscopic observations of prepared tissue sections revealed emission bands of the fluorescent material of axons (1) and acid-treated groups of kidney cells; critical evaluations of available spectrograms of purified materials lead to the identification of thiamin and riboflavin, respectively. Although some of the kidney sections, before acid treatment, showed fluorescence bands in the spectrograms that are now regarded as suggestive of reduced pyridine nucleotide, the fluorescence of which was first observed by Warburg (2), insufficient data were available at that time to consider reduced pyridine nucleotide as a possible cause of the tissue fluorescence.

Recent studies by Boyer and Theorell (3) and Duy sens and Kronenberg (4) on alcohol dehydrogenase show clearly the great enhancement of DPNH fluorescence that is caused by a binding of the coenzyme to the enzyme surface. Furthermore, Duy sens and Ames (5) demonstrate that the intact yeast cell shows a fluorescence characteristic of bound reduced pyridine nucleotide. In more recent experiments, it has been found that intramitochondrional reduced pyridine nucleotide also exhibits the same characteristic fluorescence, calling attention to the possibility of a close relationship between this effect and the blue fluorescence of living cells and tissues (6). The fluorometric result agrees with the spectrophotometrically determined large RPN* content of mitochondria (7). Furthermore, its binding to a mitochondrial component has been suggested by kinetic studies (7). More recent data show that the fluorescence of intact muscle diminishes upon electrically induced contraction, in agreement with the spectrophotometrically observed oxidation of RPN (8). Thus, there is good evidence that a considerable amount of tissue fluorescence is due to this component.

To study the fluorescence of mitochondrial RPN independently of that of the cytoplasm, it has been desirable to develop a microfluorometric method, which, in conjunction with suitable biological materials showing isolation of the mitochondrial bodies, could be used to investigate cytoplasmic-mitochondrial interactions and also to permit the assay of RPN localized in different types of cells and under different metabolic conditions. This paper describes such an instrument and its application to the observation of mitochondrial RPN, particularly in highly localized mitochondrial bodies such as the nebennern of the grasshopper spermatid (11). It is now possible to investigate in vivo the independent changes of mitochondrial and cytoplasmic pyridine nucleotide in the aerobic-anaerobic transition. In other cells, where mitochondrial localization is not sufficient for independent characterization of cytoplasmic and mitochondrial components of the fluorescence, assays of the oxidation-reduction state of the total pyridine nucleotide in individual cells in different states of metabolism and growth are possible. The combination of this differential fluorometer with the spectrophotometer described elsewhere (12, 13) for the localization of activities of respiratory and glycolytic enzymes in cells affords a new approach to the dynamic aspects of metabolic reactions.

EXPERIMENTAL

Materials—Grasshoppers were grown in the laboratory from eggs provided by Dr. T. N. Tahmisian. The follicles were prepared for microscopic examination as described elsewhere (27). Ehrlich hyperdiploid ascites tumor cells were grown in a strain of mice injected with cells supplied by Dr. T. S. Hauschka. Bakers' yeast (National Yeast Company) was washed twice and treated with 0.1 mM ethanol. Pentaploid yeast, obtained from Dr. Cornelius Tobias, was grown by Dr. Nils-Erik Saris of this laboratory in a sucrose medium (14), supplemented with 1 per cent Bacto-yeast extract (Difco). Fly spermatids were obtained from a preparation of myofibroblasts in 0.25 M sucrose by Dr. Bertram Sacktor. The liver and kidney cells were teased from the tissue (15). Liver cells, separated by mechanical and chemical treatments, were also used.

Method

A 1000-watt mercury arc illumination is filtered by means of a Corning (597) water-cooled filter. This radiation is further filtered by an Eppendorf 366-ma multicomponent filter which has been found extremely effective for isolating the mercury line and rejecting unwanted emission bands of the arc lamp. The half width of this filter is 30 µm. This radiation is projected upon a cardiod condenser (Bausch and Lomb) and then through the usual optical system of a metallurgical-type microscope.

Recent studies with the electron microscope (9, 10) support the fact that the nebennern is a mitochondrial aggregate forming in the grasshopper spermatid under certain conditions.

W. J. Rutter, personal communication.

J. M. Marshall, Jr., personal communication.
microscope (Unitron BMEC) at a magnification of 1000 for visual observation and 300 for the camera attachment. In place of the camera attachment, an EMI 9524A Cs-Sb (S-11) surface photocell is equipped with a 60-c.p.s. vibrating diaphragm which has a circular hole corresponding to a 5-μ aperture and which sweeps through a distance of ~15 μ. The photo surface is protected from the 366-mu radiation by a Wratten 2A filter. An associated switch circuit (16) selects signals from the electrical output of the photomultiplier which correspond to the extreme excursions of the diaphragm. Half-maximal electrical response to a point source of fluorescence is obtained with a displacement of 5.6 μ (16).

The closure of the switch contacts and the wave form of the photocurrent and light intensity for an AC-operated light source (see below) are indicated in Fig. 1. The fluctuations of the light intensity (~100 per cent modulation) indicated on the top line cause synchronous variations which result in an asymmetrical wave form for the photocurrent, provided the fluorescent object coincides with the extremes of the excursions of the vibrating diaphragm. To measure the fluorescence intensity of the object (M) and that of a nearby "free space" (R), the switch circuit is adjusted so that it closes for a brief interval at the peaks of the photocurrent wave form (Fig. 1). The portions of the photocurrent selected by this switch are used to charge a condenser so that its potential represents the difference of the photocurrents at the two times. This potential is amplified by a "Millivac," type 17C, and by an Esterline-Angus 1-ma. recorder. The collector of the phototube is connected to a 1.5-megohm resistor in the grid circuit of a cathode follower. The gain from the cathode follower to the input of the "Millivac" is 0.46. The over-all gain is 1 mv = 10^{-16} ampere. The dark noise of the phototube, recorded with a time constant of ~0.8 second, is less than 2 x 10^{-18} ampere (16). The signal currents from the nebenphototube are freely transmitted through the Wratten 2A filter. As an additional precaution, the microscope studies are carried out with dark field illumination in which the background scatter into the optical system leading to the photocell is less than that obtained from the oblique illuminating system used in these "macro" studies. The cell suspensions are relatively concentrated (60 mg. per cc. for the yeast cell suspensions). The close correspondence of the amplitudes of the peaks is a consequence of adjusting the photocell dynode voltage appropriately (928).

Characteristics of Filter Combinations—The energy response of the Wratten 2A filter and the S-4 (Cs-Sb) surface has a peak at 440 μ and gives half-maximal responses at 420 and 540 μ. This compares very favorably with the energy distribution from bound DPNH which has a peak at 443 μ and half-maximal emission at 400 and 500 μ (4). Thus, the response approximates that of bound DPNH.

For an adequate test of the filter combination used, the fluorescence of cell suspensions that were later studied microscopically was measured by a recording fluorometer (Fig. 2). The same filter combinations were used, but a monochromator was interposed between the fluorescent suspension and the photocell. The object of this test was to determine whether the 436 μ and other mercury lines were transmitted through the filter combination and would thereby result in an illumination artifact.

Fig. 2 shows that neither the excitation wave length at 366 μ nor the 436- or 540-μ bands create an artifact when the monochromator is set at these wave lengths. (The 405-μ bands are eliminated by the Wratten 2A filter.) As an additional precaution, the microscopic studies are carried out with dark field illumination in which the background scatter into the optical system leading to the photocell is less than that obtained from the oblique illuminating system used in these "macro" studies. It is of interest that the danger of interference from background fluorescence of the solution is considerably less in the microscopic method, where a very thin layer of the solvent is involved. Thus, satisfactory observations of cellular fluorescence have been made even in a suspension medium for liver cells containing a considerable amount of riboflavin.
Studies of mitochondria treated with ADP to cause the disappearance of RPN fluorescence show that a relatively small contribution of the flavoprotein of the respiratory chain remains and that flavoprotein fluorescence does not measurably change with its oxidation-reduction state. Thus, it is felt justified in these preliminary studies to attribute the major portion of the fluorescence observed to RPN. Evidence in favor of this view is indicated below, where chemical transitions affecting the oxidation-reduction state and hence the fluorescence of reduced pyridine nucleotide show that most of the fluorescence localized in the mitochondria is affected by this transition and hence is not a "fixed" background fluorescence.

Optical Artifacts—The possibility of encountering optical artifacts when using the absorption method is treated in detail elsewhere (19, 27). This analysis indicates that conditions are rather favorable for measuring the absorption and fluorescence of the nebenkern of the grasshopper spermatid because of the small refractive index change (20) between the nebenkern and the surrounding cytoplasm ("NK − cytoplasm = 0.015; "NK/"cytoplasm = 1.01"). Thus, the nebenkern in the clear cytoplasm of the spermatid can probably be studied without significant optical artifacts. A nebenkern located near the nuclear membrane or the cell wall does not show appreciably different fluorescence signals, although some optical artifact might be expected in this case. However, the fact that the fluorescence light originates from the object being viewed may allow somewhat greater freedom with the fluorescence than with the absorption method. It is only in the case of the small diploid yeast cell with its highly refractile membrane that a possibility of artifact has been noted. Here it is found that the maximal fluorescence signal is obtained with a focus other than that giving clearest optical definition. In other objects tested (cf. Table I), the maximal signal coincides with the best visual focus.

RESULTS

Visual Observations—Before the development of the differential photoelectric circuit, a number of visual observations of grasshopper spermatid were made and the results may be summarized as follows: fresh preparations of grasshopper spermatid showed no localization of fluorescence at the nebenkern, but, in the course of ~60 minutes, a blue fluorescence appeared. Cells which showed a separation of the nebenkern into two positions visually showed two distinct blue spots in the same position with 366-nm excitation. Cells in which no nebenkern was detected showed no visually detectable localization of fluorescence. Visual observations of spermatocytes in telophase showed intense fluorescence associated with mitochondrial bundles. Also, the collected mitochondria of cells in meiotic prophase showed corresponding localization of fluorescence. However, visual observations, besides being of inadequate sensitivity, were unsuitable for quantitative recordings of the kinetics of changes of nebenkern intensity from aerobiosis to anaerobiosis and for quantitative comparison of the intensities of the cytoplasm and the nebenkern or of different cells. For this reason, the differential fluorometer recording instrument was developed. Experimental results achieved with it are summarized below.

Relative Intensities of Signals—A survey of various biological materials has been made to determine the relative intensities of the signals obtained and to demonstrate the feasibility of studies of their fluorescence. This study is largely incomplete, but the preliminary results summarized in Table I are rather encouraging. These fluorescence intensities range from a small value for the aerobic nebenkern of the grasshopper spermatid to a large value for the anaerobic pentaploid yeast cell. The larger currents give a signal-to-noise ratio of such magnitude that delicate indications are given, not only of the magnitude of the fluorescence, but also of changes that may occur in different metabolic states or in different parts of the cells. At higher currents, accuracies >100:1 are possible.

Localization of Fluorescence—The inadequate resolution of the optical microscope and the uniform distribution of the mitochondria throughout the cytoplasm of such cells as bakers' or pentaploid yeast or ascites tumor cells offer little possibility for localizing the mitochondrial fluorescence as opposed to the cytoplasmic fluorescence. However, two approaches are available for the independent study of these two types of fluorescence associated with pyridine nucleotide. First, the cell may be centrifuged to aggregate the mitochondria in a particular portion of

### Table I

Summary of fluorescence intensities for various cell types and metabolic states

<table>
<thead>
<tr>
<th>Material</th>
<th>Portion of cell</th>
<th>Experimental conditions</th>
<th>Substrate</th>
<th>Increment of photo-current relative to adjacent free space</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakers' yeast (diploid)</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Ethanol</td>
<td>2–5</td>
<td>925</td>
</tr>
<tr>
<td>Pentaploid yeast</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Growth medium</td>
<td>10–30</td>
<td>925</td>
</tr>
<tr>
<td>Pentaploid yeast</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Growth medium</td>
<td>8</td>
<td>922c</td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>(3 of anaerobic)</td>
<td>929c</td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>10–35</td>
<td>914</td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>42</td>
<td>929, 931</td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>6</td>
<td>927c</td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>6</td>
<td>927a</td>
</tr>
<tr>
<td>Ascites tumor cells</td>
<td>&quot;Bright cytoplasm&quot;</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>7</td>
<td>932</td>
</tr>
<tr>
<td>Grasshopper spermatid</td>
<td>Nebenkern</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>4</td>
<td>926b</td>
</tr>
<tr>
<td>Grasshopper spermatid</td>
<td>Nebenkern</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>1</td>
<td>922</td>
</tr>
<tr>
<td>Grasshopper spermatid</td>
<td>Cytoplasm</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>2.5</td>
<td>922</td>
</tr>
<tr>
<td>Grasshopper spermatid</td>
<td>Mitochondrial aggregate</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>5</td>
<td>925</td>
</tr>
<tr>
<td>Fly muscle</td>
<td>Sarcomasses</td>
<td>Anaerobic</td>
<td>Endogenous</td>
<td>1</td>
<td>922e</td>
</tr>
</tbody>
</table>
the cytoplasm (18). Second, natural biological structures in which there is an aggregation of mitochondria (e.g. the grasshopper spermatocyte and spermatid) afford a unique opportunity for the study of mitochondrial fluorescence independent of that of the cytoplasm. Fig. 3 indicates qualitatively the fluorescence signal obtained from the cytoplasm and nebenkern, which are represented schematically at the bottom of the figure. The crosses mark the measuring and reference apertures of the scanning microfluorometer. Where both apertures are on the free space (FS-FS) surrounding the cell, a negligible signal is obtained when the photocell is either uncovered (1) or covered (4). If, however, one of the apertures is placed in the cytoplasm of the cell (CY-FS), a deflection of 1 scale division is obtained due to the difference between cytoplasm and free space. If the cell is now moved so that the fluorescence light from the nebenkern falls on the measuring aperture (NK-FS), a signal of 6 scale divisions is obtained. Last, both apertures are moved onto the cell so that the measuring aperture is on the nucleus and the reference aperture is on the nebenkern (Nuc-NK). Since this is a differential instrument and the nebenkern emits more energy than the nucleus, the deflection is in a downward direction to the extent of 3 scale divisions. These results indicate a high degree of localization of fluorescence which is associated with the nebenkern body of the cell.

Measurements of different spermatids having nebenkern are summarized in Table II. The ratios of the fluorescence signal measured when the aperture is placed on the nebenkern and upon the cytoplasm (top row) are found to vary from 1.7:1 to 6:1, an average of 3.3:1. The ratios of the nuclear to the cytoplasmic fluorescence (bottom row) range from 1.7:1 to 3.0:1, an average of 2.4:1. The high fluorescence of the nucleus cannot surely be attributed to RPN. Also of interest is the fact that the nucleus of this cell gave no detectable cytochrome absorption bands (13).

Intensity-Distance Relationship—By the use of the quartz plate technique (19) for obtaining reproducible motions of the cell with respect to the differential apertures, we have obtained an intensity distribution for a spermatid with nebenkern, indicated in Fig. 4. In this figure, the width of the rectangles represents the half power width of the aperture in microns and the heights represent the intensity of the fluorescence. In agreement with Fig. 2, we find a high localization of the fluorescence at the position of the cell identified in the dark field as the nebenkern.

Localization of Fluorescence in Primary Spermatocytes—A primary spermatocyte has been observed to show an intense fluorescence of mitochondria-like bodies near the nuclear membrane, characteristic of meiotic prophase (20) (see Table I). This intensity exceeds that of the nebenkern of the spermatid, possibly because of the greater surface area of this mitochondrial aggregate.

Recordings of "mitochondrial sheaves" which are characteristic structures of meiotic telophase, show a fluorescence 1.6-fold higher than that of the neighboring cytoplasm. Here again the fluorescence is in accord with the cytological evidence on mitochondrial distribution.

Stability of Nebenkern Fluorescence—During the first or second minute of exposure to the excitation light of a previously unexposed cell, a decrease of fluorescence intensity occurs, particularly when the arc is operated on direct current (see below). An example of this is indicated in Fig. 5. The trace begins approximately 20 seconds after the cell has been illuminated and shows a decrease of intensity for 90 seconds, after which the value becomes constant. Extrapolation of the trace back to \( t = 0 \) shows that a 40 per cent decrease of fluorescence has occurred. This response is to be expected in view of the ultraviolet damage to mitochondria that has been reported elsewhere (21).

Fig. 5 shows that the fluorescence intensity does not decrease to 0 but, for a reason not fully understood, reaches a steady
its value and remains stable thereafter (921). Is seen that the fluorescence diminishes to approximately one-half fluorescence of a previously unilluminated nebenkern. Continuous rather than intermittent illumination (cf. Fig. 1) is used. It is possible, especially in view of the high signal-to-noise ratio of the fluorescence measurements which permit an even further reduction of the input power.

In Fig. 7A, the ratio of nebenkern to cytoplasmic fluorescence, plotted as determined by records similar to those of Fig. 6. The numbers in the diagram refer to the cell studied. The abrupt upward discontinuity of the record at approximately 45 minutes occurs when anaerobiosis is expected. B, the individual measurements of the cytoplasmic (●—●) and nebenkern (○—○) fluorescence. The number of the cell used for measurement is also indicated along the scale of the abscissa (922a, b).

Although this effect is largely avoided by the technique of Fig. 1, brief exposures of cells to ultraviolet light are used. Such measurements are possible because the response time of the recorder is on the order of a second. Alternatively, a series of cells may be studied, as in Fig. 6.

Biochemical Response of Fluorescence Intensity—It is possible to demonstrate a response of the fluorescence of the nebenkern to the metabolic state of the cell. Whereas the fluorescence intensities of aerobic cells, it is found that one grasshopper sperm follicle placed under a paraffin wax-sealed coverslip in a glucose-free medium of several microliter's volume will be maintained in an essentially aerobic state for about 45 minutes.8 Recordings of cytoplasmic and nebenkern fluorescence at 40 and 60 minutes are given in Fig. 6. In contrast to Fig. 2, the deflection (Fig. 6A) corresponding to the nebenkern fluorescence at 40 and 60 minutes is plotted as a function of time. The graph indicates that the increase of fluorescence measurement has been used (see "Methods") to make measurements over a prolonged interval without a further decrease of intensity.

In an effort to avoid this diminution of nebenkern fluorescence caused by high intensity ultraviolet radiation, a method of illuminating the cell with high intensity radiation only at the time of the fluorescence measurement has been used (see "Methods"); this technique decreases the average power input to the specimen to such a degree that prolonged illumination of the nebenkern causes a much smaller decrease of fluorescence than that illustrated by Fig. 5. Further improvements along this line are possible, especially in view of the high signal-to-noise ratio of the fluorescence measurements which permit an even further reduction of the input power.

8 The smaller coverslip used with the fluorometer encloses about half the liquid volume enclosed by the larger coverslip used in the microspectrophotometer (127). With the latter, 90 minutes are required for anaerobiosis.
flourescence intensity is due exclusively to the nebenkern and that little change of fluorescence occurs in the cytoplasm. This record is of particular significance in indicating whether or not there is a mixture of oxidizing and reducing equivalents between the mitochondrion and the cytoplasm. According to our data, a change in the oxidation-reduction state of pyridine nucleotide in the mitochondrion has no detectable effect upon that in the cytoplasm (see Discussion).

Aerobic-Anerobic Transition in Ascites Tumor Cells—In ascites tumor cells, a clear distinction between the mitochondrial and the cytoplasmic fluorescences is not possible. Nevertheless, measurement of the kinetics of reduction of mitochondrial plus cytoplasmic pyridine nucleotide can be made by fluorometric observations of the cell. In this case a prediction of the time for anaerobiosis is afforded by respiratory measurements of the cell suspension before dilution for microscopic observation. In a particular experiment, a stock solution of ascites cells (freshly withdrawn from the mouse and washed once in a saline phosphate solution) gave a time of 4 minutes for expenditure of the oxygen dissolved in the saline phosphate solution. In order to give a 20-minute time for anaerobiosis, a 5-fold dilution of the cells was used and 5 μl. were quickly placed under the coverslip. The preparation was sealed with paraffin and then observed with the differential microfluorometer. The experimental record is shown in Fig. 8 where successive measurements of the fluorescence intensity indicated by the downward sweep of the trace at periodic intervals illustrate the increase of fluorescence intensity of the ascites cell as a function of time after sealing off the preparation. A graphical representation of all the experimental points is given in Fig. 9, where the time for anaerobiosis calculated from the platinum microelectrode experiment (20 minutes) is compared with the time required for the fluorescence to reach its maximal intensity (~24 minutes). The agreement is considered very satisfactory. The same phenomenon is observed with a suspension of cells which show a considerable increase of fluorescences in the transition from the aerobic steady state to anaerobic conditions. Thus the kinetics of reduced pyridine nucleotide based upon the observation of a single cell agrees with those of cells observed in suspension.

Similar studies have been carried out on pentaploid yeast cells, where an increase of fluorescence intensity can be observed at a time appropriate to the exhaustion of oxygen in the covered preparation. In addition, yeast cells afford an opportunity for comparison of fluorescence of mother and daughter cells in the budding yeast. It may be of some interest that, early in the budding process, the fluorescence measured with the 5-μ aperture for the daughter cell is approximately equal to that observed when the 5-μ aperture is placed on the mother cell. This suggests that the mitochondrial material may be transferred in large quantities at an early stage of cell division. This may be correlated with the peculiarity of yeast cells, that chromatin is transferred last.

**DISCUSSION**

The data presented lead us to associate a fluorescence with the mitochondria of the grasshopper spermatid in the following cytologically identified locations: (a) in mitochondrial concentrations at the nuclear membrane of the primary spermatocyte; (b) in mitochondrial bundles which are present in the meiotic telophase; and (c) in mitochondrial concentrations in the nebenkern.

In addition, it has been found that the nebenkern fluorescence is small compared to that of the cytoplasm in aerobicity and becomes large compared to it in anaerobiosis. The kinetics of the aerobic-anaerobic transition follows what would be expected from known kinetics of this transition in cell suspensions (23) and from independent observations of the rate at which oxygen is utilized by the cell preparation. The aerobic-anaerobic transition, which has a large effect on the RPN of the mitochondria, has no measurable effect on the cytoplasmic component. Just how much of the cytoplasmic fluorescence is due to RPN associated with glycogenolytic enzymes cannot be determined without further biochemical studies. An explanation for the lack of a cytoplasmic effect is afforded by a compartmentalization of mitochondrial pyridine nucleotide in vivo. This has been discussed in considerable detail on the basis of experiments in vitro (24-26). One uncertainty of the earlier results has been the possibility that impermeability of the mitochondria may have been acquired during isolation, for example, by development of the mitochondrial membrane to cytoplasmic RPN. The results reported here suggest, at least for the grasshopper spermatid, that the unreactivity of the mitochondrial membrane to cytoplasmic RPN may be a reality and not an artifact, an important conclusion in the study of metabolic permeability.

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material (22, 23). In bakers' yeast, for example, the kinetics of respiratory initiation and cytochrome reduction upon glucose addition suggests that the pyridine nucleotide reduced by glycerol-aldehyde 3-phosphate dehydrogenase is readily accessible to the mitochondria. It is not known, however, whether or not mitochondria of yeast cells are more permeable to cytoplasmic RPN than are those of other tissues. Further investigation of the type described for the grasshopper spermatid is desirable, although the technical obstacles seem formidable at present.

There is a question as to whether there occurs a redistribution of RPN between free and bound forms, with an attendant change in fluorescence intensity. This is partially answered by studies of liver mitochondria (6) which show that the wave length maximum of the fluorescence intensity does not vary greatly, despite considerable changes in the oxidation-reduction level. It may, therefore, be concluded that the fluorescence intensity changes recorded here are caused by oxidation-reduction changes.

An anaerobic suspension of grasshopper spermatid follicles shows a 340-nm absorption band indicating a relatively large amount of RPN. This RPN can also be oxidized by the respiratory chain (12, 27). However, a measurable 340-nm absorption band is not observed with the microspectrophotometer, possibly because of technical difficulties or because of its localization at the surfaces of the mitochondrion (13). Thus the fluorimetry and spectrophotometric methods complement each other in the study of localized respiratory enzymes.

SUMMARY

A fluorescence with spectral characteristics that are similar to those of reduced pyridine nucleotide of isolated mitochondria has been demonstrated to be localized in three cell configurations which cytologically show mitochondrial aggregation. The oxidation and reduction of mitochondrial pyridine nucleotide without a measurable change of cytoplasmic fluorescence suggest that compartmentalization of mitochondrial and cytoplasmic pyridine nucleotide occurs in vivo, at least in the grasshopper spermatid.

Studies of other material, particularly pentaploid yeast cells and ascites tumor cells, indicate that similar changes of fluorescence of the single cell are observed in the aerobic-anaerobic transition. In such cells, optical resolution does not permit localization of mitochondrial bodies. Nevertheless, the state of pyridine nucleotide in the individual cell can be investigated in its response to changes of metabolism and growth.

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