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CHARACTERIZATION OF THE OXYGEN PROBE PYRENEBUTYRIC ACID IN RABBIT HEART MITOCHONDRIA

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SUMMARY

The fluorescence of pyrenebutyric acid is quenched by oxygen and the quenching is a linear function of the oxygen concentration. In addition, pyrenebutyric acid has been shown to be readily taken up by the cell with a partition coefficient of approx. 200. Results are presented on the effect of pyrenebutyric acid on oxidative metabolism in rabbit heart mitochondria.

1. Pyrenebutyric acid is readily taken up by heart mitochondria: 0.843 ± 0.087 nmol pyrenebutyric acid taken up by M_r/mg dried weight.

2. Pyrenebutyric acid does not alter the steady-state redox levels (State 1, 2, 4 or 5) of any member of the respiratory chain.

3. Pyrenebutyric acid does not alter the response of cytochrome *b*, cytochrome *c* or NADH in the State 4–3–4 transition induced by the addition of ADP. Thus pyrenebutyric acid would appear to be non-toxic to oxidative metabolism.

INTRODUCTION

In any discussion of tissue performance, the availability of oxygen is of prime concern, since oxidative phosphorylation is the major source of energy for the cell [1]. It is therefore not surprising that considerable effort has been expended in developing techniques to monitor intracellular oxygen concentration [2–7]. Unfortunately most techniques presently employed suffer from two major shortcomings. First, in order to determine intracellular oxygen, the tissue is physically disrupted by insertion of the oxygen electrode. The effect of this injury is not precisely known [8]. The second shortcoming with traditional polarographic oxygen electrodes is the fact that they consume ($O_2 + 4H^+ + 4e^- \rightarrow 2HOH$) the very substance they are trying to measure [9]. In large volumes, this is not a problem; but in attempting to determine cellular pO_2, this cannot be ignored [8]. Because of these shortcomings, there is a need for an oxygen probe which neither disrupts the integrity of the cell nor alters the intracellular pO_2.

With the discovery that oxygen quenched the fluorescence of pyrenebutyric acid [10], it became evident that such a probe might be available. In experiments conducted in non-biological solutions, the following properties of pyrenebutyric acid

were demonstrated [10]. The intensity of the pyrenebutyric acid fluorescence is related to the oxygen tension by the classical Stern-Volmer relation $F_0/F = 1+q$ $[pO_2]$ where: F_0 = fluorescence of pyrenebutyric acid at $pO_2 = 0$; F = observed fluorescence; q = quenching coefficient and $[pO_2]$ = partial pressure of oxygen. The relation of the collisional rate constant at a given $[pO_2]$ to changes in temperature indicate that the quenching of the fluorescence of pyrenebutyric acid by oxygen is due to collision rather than to a chemical reaction which would consume oxygen. The fluorescence lifetime of pyrenebutyric acid has been shown to be in the order of 200 ns. This is sufficiently short to permit accurate determination of the pO_2 for virtually all biologic processes. In addition, other experiments [11] indicated that the pyrenebutyric acid is rapidly and readily taken up by the cell. From the hydrophobic-lipophilic properties of the pyrenebutyric acid molecule [10], the most probable site of pyrenebutyric acid concentration is in or on the various membranes in the cell. Therefore, the fluorescence from pyrenebutyric acid should accurately indicate the pO_2 in the proximity of the cell cytoplasm.

Before utilizing this oxygen probe in biological preparations, it is first necessary to determine the impact of pyrenebutyric acid on basic metabolic processes. The present experiments were directed towards this goal. The metabolic effects of pyrenebutyric acid were determined by examining the redox levels under both steady-state and transient conditions of the members of the respiratory chain using various optical techniques. These techniques rest on the fact that each member of the respiratory chain (from NADH to cytochrome a–a_3) possesses different optical properties depending upon whether they are in the reduced or oxidized state [12, 14].

METHODS

Cardiectomies were performed on stunned female rabbits (6–8 lb) and the heart perfused with warm Ringer solution to remove the remaining blood. The isolation of the mitochondria was carried out using the technique described in ref. 13. The pyrenebutyric acid (Eastman Kodak) used in these experiments was first purified and then precipitated as the sodium salt before use. The concentration for all experiments was 5 μM unless otherwise indicated. The temperature of the mitochondrial suspension was maintained at 23 ± 1 °C by a recirculating bath. The mean weight ($\pm$S.D.) of the dried (5 h at 105 °C) mitochondrial pellet was 46.7 ± 8.4 mg for all experiments.

The determine the effect of pyrenebutyric acid on the steady-state redox levels of the members of the respiratory chain, a splitbeam spectrophotometer [15] was used. The techniques used to examine each of the four steady states (1, 2, 4 and 5) were as follows:

State 1. The final mitochondrial pellet was resuspended in substrate-free reaction medium. Aliquots of this suspension were placed in two quartz (10 mm path-length) cuvettes and placed in the spectrophotometer. Oxygen (100%) was blown onto the tops of both suspensions. After recording the baseline absorption spectrum, pyrenebutyric acid was added to one of the cuvettes and again the absorption spectrum recorded.

State 2. The final mitochondrial pellet was resuspended in substrate-free reaction medium containing 100 mM ADP. After obtaining the baseline, pyrenebutyric

acid was added to one of the cuvettes and the absorption spectrum recorded.

State 4. The final mitochondrial pellet was resuspended in reaction medium containing either 10 mM pyruvate or 10 mM α-ketoglutarate. The results were not affected by which substrate was used. The procedure used was the same as that described for State 1.

State 5. The final mitochondrial pellet was resuspended in reaction medium containing 10 mM substrate (pyruvate or α-ketoglutarate) and 100 mM ADP. After recording the baseline, the gas was changed to nitrogen (100%). After 15 min, the absorption spectrum was recorded; pyrenebutyric acid was then added to one cuvette and another spectrum obtained. Cyanide (10 mM) was then added to both cuvettes. After 10 min, the final absorption spectrum was recorded.

To determine the effect of pyrenebutyric acid on the State 4–3–4 transition, two sets of experiments were conducted. The first set was directed at observing the transition using the double-beam spectrophotometer [14, 15] in the region of cytochrome *b* (430–405 nm) and cytochrome *c* (419–405 nm). The final mitochondrial pellet was resuspended in reaction medium containing 10 mM pyruvate or α-ketoglutarate. Aliquots of the suspension were transferred to two quartz cuvettes, one of which contained pyrenebutyric acid. One of the cuvettes was randomly selected, placed in the double-beam, and oxygen (100%) blown onto the surface. A series of ADP additions were carried out and the resulting changes in absorbance recorded. After each addition of ADP, the trace was allowed sufficient time to return to the baseline or to a new steady-state level. At the completion of the series, the second cuvette was placed in the double-beam and the identical procedure repeated. To determine the effect of pyrenebutyric acid on the NADH response to State 4–3–4 transitions, a protocol as just described was employed. However, instead of the double-beam, a fluorimeter [14, 15] was used.

For the experiments dealing with the effect of pyrenebutyric acid on the State 4–3–4 transition, three quantities were measured. The first was the maximal change (P_{max}) from the steady-state level in absorbance or fluorescence due to the addition of ADP. The second was the rate of return of the optical signal to the steady-state level (State 4) from the maximum change. This return may be described by a simple exponential. The lifetime of this exponential was calculated from an average of 34 points per curve using a non-linear fitting program [16]. The third parameter measured was the total area of the transient curve. Where appropriate, the analysis of the resolution-data was done using a PDP 11/45 with a digitized platten (0.08 mm).

To get an approximate estimate of the amount of pyrenebutyric acid taken up by the mitochondria, the two absorbance peaks of pyrenebutyric acid at 326 and 342 nm were used. After separating the mitochondria as before, the resulting suspension was divided into two portions. These were centrifuged (10 000 × *g* for 10 min); the supernatants removed for spectrophotometric assay of the pyrenebutyric acid remaining and the residual pellet dried (105 °C at 5 h) and weighed.

RESULTS

Control

The first set of experiments was designed to determine the non-toxic concentration of pyrenebutyric acid by monitoring the NADH fluorescence response to the

addition of ADP. Starting at 100 μM, the pyrenebutyric acid concentration was reduced by factors of approx. 2 until no difference in the amplitude of NADH fluorescence response or in the rate of return to the resting (State 4) level was detected as compared to the control results with each preparation. At each concentration, two experiments were conducted. In the first, the NADH fluorescence response to additions of ADP was determined in an aliquot of mitochondrial suspension with no pyrenebutyric acid present; this was followed by monitoring the response with pyrenebutyric acid present in a second aliquot. In the second experiment, the order was reversed so that the first aliquot contained pyrenebutyric acid and the second did not. A detectable change in response was defined to have occurred if both experiments showed a difference due to the presence of pyrenebutyric acid. The significance level chosen was 0.05 (Walsh Test) [17]. The results of these preliminary experiments show that at concentrations greater than 20 μM, a decrease in the rate of return was evident; and at 10 μM or less, no change was observed. For the following experiments, the concentration of pyrenebutyric acid used was 5 μM. Although there was impaired function of the mitochondria at pyrenebutyric acid concentrations of 100 μM, electron micrographs (courtesy of Dr. Jocham Sommer) indicate no disruption of the morphology. During these experiments, an attempt was made to monitor changes in pO_2 as a result of the addition of ADP. While the data indicated that this will be possible with further refinements in technique, the present instrumentation did not allow a quantitative determination.

Pyrenebutyric acid uptake by mitochondria

Previous experiments [11] with liver cell suspensions have indicated that pyrenebutyric acid is preferentially sequestered in the cell with a partition coefficient of approx. 200. To insure that a similar partition occurs with mitochondria, experiments were conducted to determine the relative uptake of pyrenebutyric acid by mitochondria using an absorbance spectrophotometer. The results from a typical experiment are presented in Fig. 1. As is evident, the major fraction of the pyrenebutyric acid introduced into the mitochondrial suspension is taken up by the mitochondria. The average amount of pyrenebutyric acid taken up by the mitochondria in the eight experiments was 0.843 ± 0.087 nmol pyrenebutyric acid taken up by M_r/mg dried weight.

Spectrophotometric assay of redox states

Typical spectra from the steady-state experiments are presented in Fig. 2. The top trace (Fig. 2A) is the difference absorption spectrum of two mitochondrial suspensions in State 1 without pyrenebutyric acid. This serves as the baseline against which possible pyrenebutyric acid effects should be measured. The apparent curvature in the baseline is a result of inherent properties of the spectrophotometer. The middle trace (Fig. 2B) is the spectrum after the addition of pyrenebutyric acid to the sample cuvette. There is no change except in the region of pyrenebutyric acid absorbance peak at 342 nm. The bottom trace (Fig. 2C) is the result of the addition of 10 mM cyanide in order to locate the absorbance peaks of the members of the respiratory chain accurately and indicate their concentration. Similar experiments on State 2, produced by an excess of ADP, show clearly that no appreciable changes in the redox level of the State 2 mitochondria occur as a result of the addition of pyrenebutyric

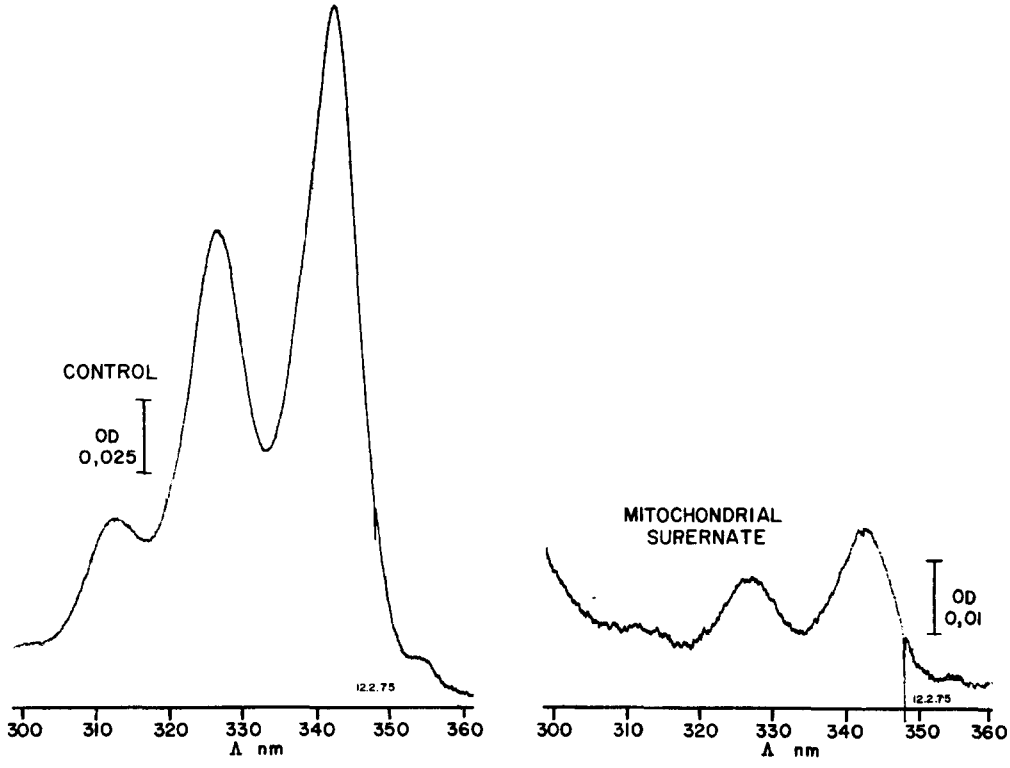

Fig. 1. Determination of rabbit heart mitochondrial uptake of pyrenebutyric acid by spectrophotometric assay. Left hand trace: absorbance from control suspension medium containing a known amount of pyrenebutyric acid. Right hand trace: absorbance from mitochondrial supernatant containing an unknown amount of pyrenebutyric acid.

acid. There was no effect of pyrenebutyric acid on the mitochondrial State 4 redox level with either pyruvate or α ketoglutarate as the substrate. Similarly, there was no observable effect of pyrenebutyric acid on the State 5 redox levels produced by exclusion of oxygen from the suspension. In another set of experiments the mitochondrial suspensions were placed directly in State 5, using cyanide, and the spectrum taken. Pyrenebutyric acid was then added and another spectrum taken. No effect due to pyrenebutyric acid in State 5 redox level was observed.

State 3 is the state of active respiration and phosphorylation. Because of the rapid exhaustion of O_2, steady-state spectra are difficult to obtain. Therefore, the effect of pyrenebutyric acid on State 3 was investigated by examining its effect on the three parameters (P_{max}, lifetime and total area under the transient curve) used to describe the State 4–3–4 transition. Since these parameters are closely linked to the kinetics of this transition, they provide a sensitive estimate of possible changes in turnover rate. In addition, these transitions closely approximate the physiologically-induced transient periods of work in intact cells. To induce this transition, limited amounts of ADP were added to the cuvette containing mitochondria in State 4. The three metabolic parameters were determined for each of the 120 State 4–3–4 transients of cytochromes *c* and *b*, and NADH. These data were used in a paired

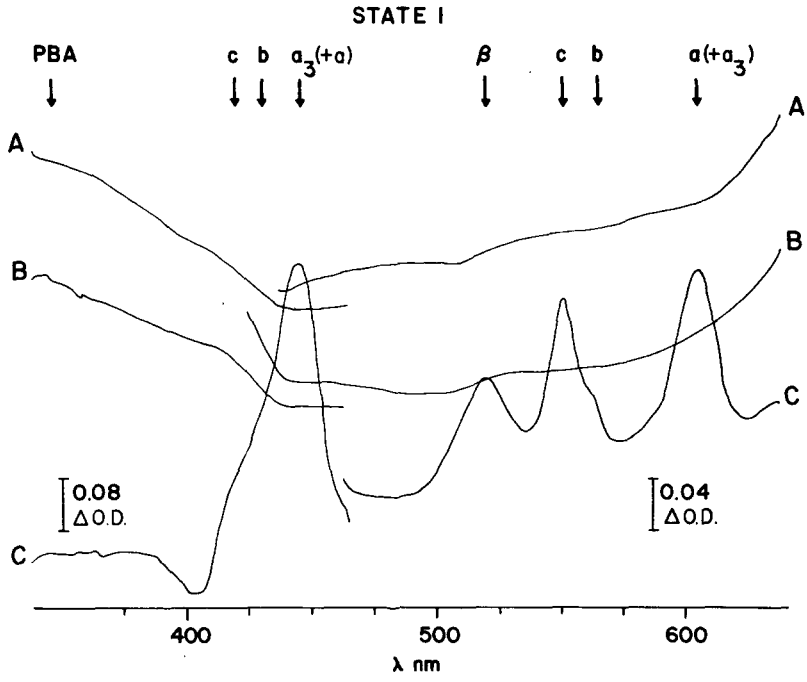

Fig. 2. Absorbance difference spectra of rabbit mitochondria from a splitbeam spectrophotometer. Trace A, spectrum with both suspensions in metabolic State 1. Trace B, spectrum after addition of pyrenebutyric acid (PBA) (5 μM) to one of the cuvettes. Trace C, spectrum after addition of cyanide (10 mM) to one of the cuvettes.

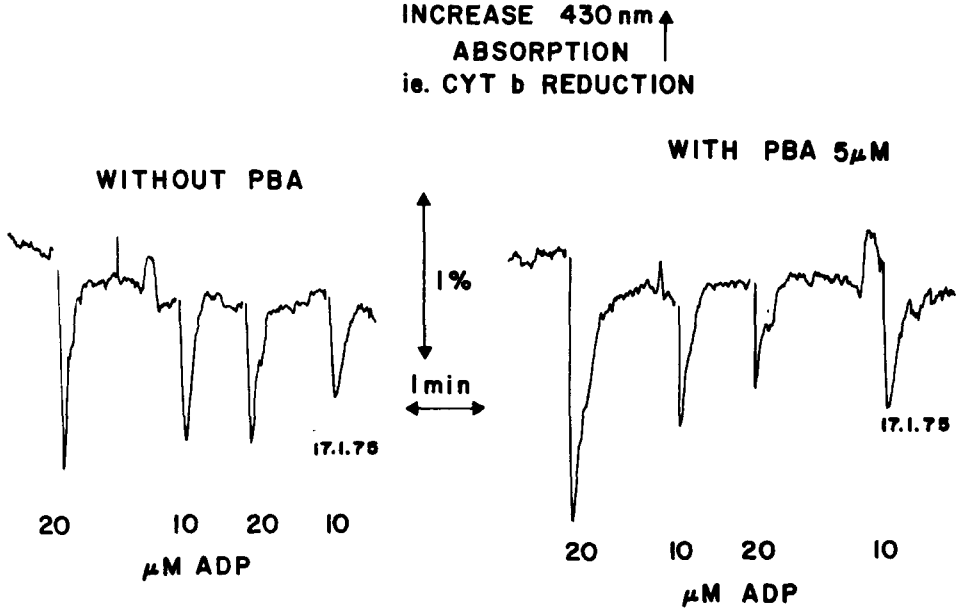

Fig. 3. See opposite page for legend.

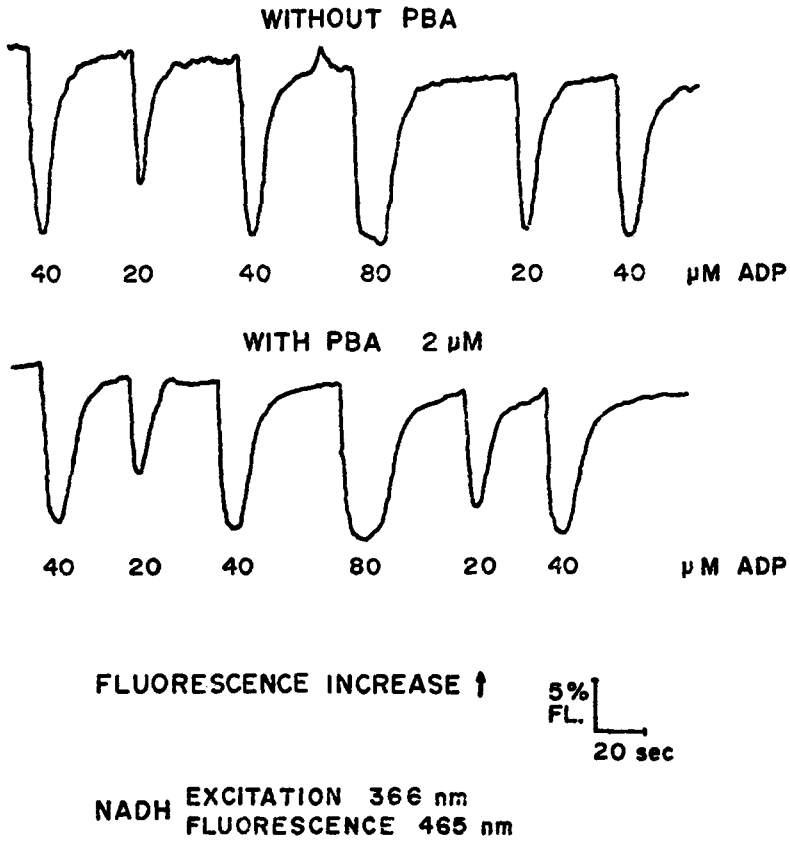

Fig. 3. Effect of pyrenebutyric acid (PBA) on the State 4–3–4 transitions in rabbit heart mitochondria. (A) Typical spectrum in the region of cytochrome *b* (430–400 nm) from a dual wavelength spectrophotometer. Transitions produced by the addition of ADP. (B) Typical NADH fluorimetric response (excitation 366 nm, fluorescence 465 nm) to additions of ADP.

fashion, to determine whether pyrenebutyric acid produced any change in the response to ADP.

The results of a typical experiment directed at determining whether pyrenebutyric acid alters this State 4–3–4 transition are presented in Fig. 3. The response of cytochrome *b* to the addition of various amounts of ADP is shown in Fig. 3A. Using the non-parametric Walsh Test [17], no significant ($P < 0.05$) difference in the three metabolic parameters was present as a result of the presence of pyrenebutyric acid. Fig. 3B compares the typical response of mitochondrial NADH to the additions of ADP with and without pyrenebutyric acid. Again using the Walsh Test, no difference ($P < 0.05$) was detected in the lifetime or the area. In this particular experiment, a difference in P_{max} was present. When the other six experiments were similarly analyzed, this difference was found to depend on which of the two samples (with or without pyrenebutyric acid) were studied first. The combined results indicate no change in the three parameters as a result of the presence of pyrenebutyric acid.

DISCUSSION

The purpose of the present set of experiments was to characterize pyrenebutyric acid in biological media. The results from the split beam spectrophotometer clearly indicate that pyrenebutyric acid does not alter the steady-state redox levels of any member of the respiratory chain. Thus pyrenebutyric acid would not appear to interfere with any of the enzyme systems which determine these levels.

The question of dynamic viability of pyrenebutyric acid-treated mitochondria was approached by examining the effect of pyrenebutyric acid on the State 4–3–4 transitions. The results show that pyrenebutyric acid does not alter the response of NADH, cytochrome *b* or cytochrome *c*. Considering the closely coupled nature of the members of the respiratory chain, it is reasonable to conclude that pyrenebutyric acid does not appreciably alter the response of the other members either. Thus the enzyme reactions and the kinetics of oxidative phosphorylation are not affected by the fluorochrome.

Pyrenebutyric acid has been shown to be metabolically non-toxic, altering neither steady-state redox levels of the members of the respiratory chain nor the mitochondrial response to ADP. In addition, the present experiments have shown that pyrenebutyric acid is readily taken up by mitochondria. From the solubility data, pyrenebutyric acid probably resides in or possibly on the surface of the membrane. Although the location is unknown, the data presented do demonstrate that pyrenebutyric acid does not alter mitochondrial oxidative metabolism. Since the quenching is a result of collision, there is no change in the intracellular pO_2 as a result of the presence of pyrenebutyric acid. Preliminary results have also shown that pyrenebutyric acid may be used to monitor pO_2 in cat brain and rabbit papillary muscle [18] with no apparent disruption of physiological function. In conclusion, pyrenebutyric acid has been shown to possess the required characteristics to be a valuable intracellular oxygen probe which neither disrupts oxidative metabolism nor affects the intracellular pO_2.

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