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POLAROGRAPHIC STUDY OF MITOCHONDRIAL SUSPENSIONS

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SUMMARY

1. Mitochondrial suspensions exhibit a characteristic polarographic wave which is independent of tissue source. This same wave was found in the mitochondria of two species of animals.

2. This polarographic wave is anodic in character, irreversible, and diffusion controlled.

3. The anodic process results in the formation of a mercury mercaptide. The source of mercaptan in fresh mitochondrial preparations is sulfhydryl groups within the mitochondrial membrane. These appear to be due to sulfhydryl groups contained in the protein structure of the mitochondrial membrane.

4. Aging of the mitochondrial preparation results in loss of the early wave and the appearance of a second wave. This latter wave originates from the contents of the mitochondria.

INTRODUCTION

This investigation arises from a continuing study of possible means by which trace metals may be bound and utilized in essential biochemical reactions. Previous work had indicated that a possible means of cation transfer is through chelation with sulfhydryl groups in biochemical systems[1-3]. These observations have prompted further investigation into the essential role of thiol groups in making required metallic ions available for enzyme-controlled reactions. The mitochondria were chosen as a model system for this study since many enzymic processes have been studied in detail using mitochondria. In addition, COOPER[4] has demonstrated the sulfhydryl activity of mitochondrial preparations by use of sulfhydryl-binding reagents.

EXPERIMENTAL METHODS

Reagent-grade chemicals were used with the exception of sucrose which was purified by deionization to remove all trace metals. A 60% suspension of rat-liver mitochondria in 0.25 M deionized sucrose was prepared by the method of NEUBERT AND LEHNINGER[5]. 1-2 ml of this suspension were placed in 5 ml of 1 M potassium nitrate supporting electrolyte.

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The effects of 1 M potassium nitrate electrolyte on mitochondrial swelling was determined spectrophotometrically according to the method of LIPSETT AND CORY[6].

Mitochondria were treated with ultrasonic radiation at 10 kcycles for 30 sec and the membranes separated from the mitochondrial contents by centrifugation[7]. The membrane suspension in 0.25 M deionized sucrose represented a 50% preparation from liver tissue. The supernatant component was divided into two fractions. One fraction was treated in a water bath at 100° for 10 min to coagulate protein. Separate polarograms were run on the original mitochondrial washings, on intact mitochondria, on the mitochondrial membrane suspension, and on each of the two supernatant fractions described.

All polarographic measurements were made with a Sargent Model XV Polarograph. A Lingane H-cell fitted with a saturated calomel reference electrode (SCE) and a 3% agar–saturated potassium chloride salt bridge was employed. The mitochondrial suspension in the supporting electrolyte was placed in the sample compartment of the H-cell and flushed with nitrogen gas for 8 min prior to each run. During the course of the polarogram, a nitrogen atmosphere was maintained above the sample solution. "Seaford" nitrogen (Southern Oxygen Co.) was used throughout the investigation. All polarograms were obtained on a sample thermostatted at $23 \pm 0.2°$.

Reversibility of the electrode reactions was tested by determining the slopes of the log $(i_d - i)/i$ *versus* E plot. Values for the half-wave potentials were taken from these logarithmic plots. Data for these plots were obtained by manual operation of the polarograph. Applied voltages were determined by measurement with a student potentiometer.

RESULTS

The polarogram of a sample consisting of 2 ml of 60% mitochondrial suspension (in 0.25 M deionized sucrose) and 5 ml of 1 M potassium nitrate supporting electrolyte is shown in Fig. 1. The half-wave potential for this wave is approx. −0.290 V *versus* a saturated calomel electrode. The slope of the log $(i_d - i)/i$ *versus* E plot indicates an "n-value" of 0.6–0.7. The height of the wave is directly proportional to the concentration of the mitochondrial suspension and the current appears to be diffusion controlled (the wave height is directly proportional to the square root of the height of mercury). This same wave is obtained with mitochondrial suspensions prepared from rat liver, kidney, and brain, and from dog liver.

If the mitochondrial suspension is stored in ice for 45 min, the half-wave potential and the wave height remain unchanged. However, storage at room temperature for 15 min results in the rapid decrease in wave height coupled with the appearance of a second minor wave with a half-wave potential of −0.301 V and an n-value of 0.7. Further aging of the preparation results in complete disappearance of the original wave and the appearance of a new wave with a half-wave potential of −0.417 V an n-value of 0.99.

When the sample of fresh mitochondrial suspension is cooled to 8°, the original mitochondrial wave shifts to more negative potentials (−0.284 to −0.298 V) and the wave height decreases (0.156 to 0.084 μA). Warming the suspension to 38° results in a shift of the wave to more positive potentials (−0.284 to −0.276 V) and an increase in the wave height (0.156 to 0.210 μA). In both cases, "n" does not change

significantly. The temperature coefficient of the half-wave potential was found to be 0.7 mV/degree.

When the cooled solution was warmed to room temperature (23°), the original half-wave potential and wave height were restored. However, upon cooling of the warmed solution, the half-wave potential was restored but the wave height was increased over the original value (0.192 *versus* 0.156 μA).

An electrocapillary curve run on the sample solution exhibited a broad plateau extending from —0.200 to —0.600 V *versus* a saturated calomel electrode (Fig. 2).

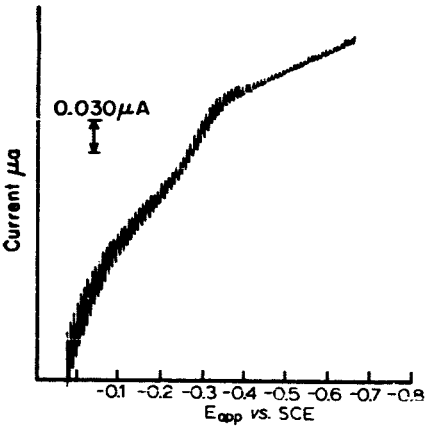

Fig. 1. Polarogram of mitochondrial suspension. Sample contains 4 ml of 60% mitochondrial suspension (in 0.25 M deionized sucrose) in 10 ml of 1.0 M potassium nitrate supporting electrolyte. Capillary constant 2.46 $mg^{\frac{1}{2}}$ $sec^{-\frac{1}{2}}$.

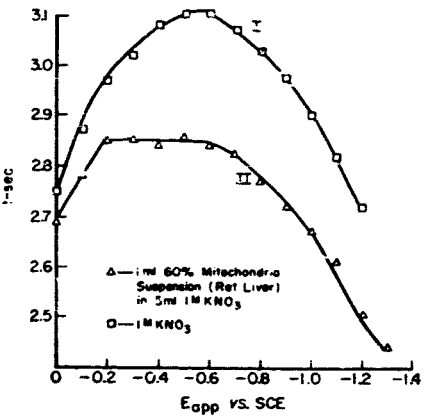

Fig. 2. Electrocapillary curves of mitochondrial suspension and supporting electrolyte. Curve I, 1.0 M potassium nitrate solution; Curve II, 1 ml of 60% mitochondrial suspension in 5 ml of 1.0 M potassium nitrate.

The origin of the mitochondrial wave

GREEN, ZIEGLER AND DOEG[8] state that the succinic dehydrogenase complex contains 3.5 mμmoles of cytochrome c_1, 1.0 mμmole of cytochrome c, 0.15 mμmole of cytochrome b, and 1.2 mμmoles of total flavins per mg of protein. These concentrations are well below those which can be measured polarographically. Mitochondrial preparations contain 68 μg of DPN + DPNH, 36 μg of TPN + TPNH, 36 μg of FAD + FMN, and 2.41 mg of GSH per g of liver[9]. A polarogram of DPN in 1 M potassium nitrate supporting electrolyte exhibits two waves with half-wave potentials at —0.914 and —1.360 V *versus* a saturated calomel electrode, respectively. The first wave is well defined (n = 0.6) and the second wave is ill defined (n = 0.5). The height of both waves appears to be proportional to the concentration of DPN.

When DPN is added to a sample containing mitochondrial suspension, the wave at —0.290 V shifts to more positive potentials. The "n-value" of this wave increases to 0.9. Upon addition of mitochondrial suspension to a sample containing DPN, the DPN waves approach one another. Similar studies with coenzyme Q_{10} indicated that the mitochondrial wave is not from this source. A polarogram of mitochondrial suspension in BRDICKA's solution[10] (10^{-3} M $CoCl_2$, 0.1 M NH_3, 0.1 M NH_4Cl) exhibits the typical protein double wave. The height of these waves is proportional to the mitochondrial concentration. Upon the addition of $7.2 \cdot 10^{-4}$ M iodoacetamide

solution, no change in the double wave was noted. However, on standing at room temperature for 12 h, the height of the double wave is reduced to one-third its original value. Since the double wave is caused by the presence of a sulfhydryl group[11] and since iodoacetamide is specific for sulfhydryl groups, the protein double wave must arise from sulfhydryl groups produced by the reduction of disulfide linkages. This reduction takes place at potentials more positive than the protein double wave[12].

The results in BRDICKA's solution suggest that the mitochondrial wave at -0.290 V arises either from the reduction of disulfide groups or the anodic formation of mercury mercaptides. The latter possibility is the more likely[13].

To a sample solution of mitochondrial suspension was added 10^{-3} M sodium sulfite. After thorough washing and resuspension of the mitochondrial preparation, no change in the mitochondrial wave was observed. When this experiment was repeated using $7.2 \cdot 10^{-4}$ M iodoacetamide in place of the sodium sulfite, the height of the wave at -0.290 V was diminished. Prolonged reaction between the mitochondrial suspension and iodoacetamide resulted in the disappearance of the wave at -0.290 V.

The definition of the anodic wave was made by use of an external galvanometer to determine the zero-current point. This was found to be -0.330 V *versus* a saturated calomel electrode. Since the wave for mitochondria in all cases was more positive than this value, the electrode reaction for mitochondria must represent an oxidative process. Evidence of the non-reversible nature of the anodic process supports the observation that this reaction is accompanied by the formation of a mercury mercaptide.

A 10^{-4} M solution of GSH in 1.0 M potassium nitrate, prepared and maintained under a nitrogen atmosphere, was found polarographically to be free of GSSG.

When this solution was added to the mitochondrial suspension and a polarogram was run, it was noted that the slope of the wave was unchanged although wave height increased immediately and with time. No shift of half-wave potential was noted immediately after the addition of GSH nor following incubation for 45 min; however, the wave height increased approximately two-fold. The slope of the mitochondrial wave and that of free GSH differ and in this experiment only that wave characteristic of mitochondria was observed. This evidence strongly suggests that the wave shown by the mitochondrial suspension is due to the reduced glutathione contained in the mitochondria.

The accumulated evidence indicated the presence of a sulfhydryl wave in the mitochondrial preparation which changed its nature in time following isolation; therefore, the source of this variation was investigated.

A swelling study of the effect of 1 M potassium nitrate electrolyte indicated that this medium causes a rapid swelling of mitochondria; however, the mitochondria do not burst, as indicated by the swelling curve which leveled off after a time. In those cases where rupture has been reported to occur, as in the swelling studies of LEHNINGER AND SCHNEIDER[14], the curve does not level off but continues its downward trend leading to almost complete transparency of the mitochondrial suspensions.

Polarographic study of the original sucrose washings from the mitochondrial preparation failed to show any response. When 1 ml of the mitochondrial membrane suspension was polarographed in 1 M potassium nitrate the original wave shown in a fresh mitochondrial preparation was observed. Increase of this amount to 4 ml resulted in a proportionate increase in wave height. The response of the membrane

suspension disappeared rapidly on standing at room temperature and the minor wave previously observed with aging mitochondria appeared.

The non-heat treated supernatant component following sonication gave a wave with a half-wave potential of —0.417 V and an *n*-value of 0.99. Treatment by heat reduced the original protein concentration of the supernatant fraction from 2.5 mg/ml to 0.5 mg/ml. The filtrate from this preparation was polarographed and produced a wave having the same slope, half-wave potential, and *n*-value as the untreated aliquot although the wave was diminished in height by approx. 30%. A study of this wave using iodoacetic acid for SH complex formation indicates that the wave is definitely sulfhydryl in origin, but differs considerably in slope and half-wave potential from glutathione. The origin of this sulfhydryl compound is not known at this time.

These latter observations indicate that the original variations within the mitochondrial preparations are dependent on the condition of the mitochondria. So long as the mitochondrial membrane remains intact the sulfhydryl wave is observed. The disintegration of the membrane results in the wave which is characteristic of the contents of the mitochondria.

These observations of variation between surface activity and mitochondrial contents suggests that the mitochondria can be utilized as a system to study membrane transport where chelate activity is to be observed.

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REFERENCES

1 E. C. KNOBLOCK AND W. C. PURDY, *Radiation Res.*, 15 (1961) 94.
2 E. C. KNOBLOCK AND W. C. PURDY, *J. Electroanal. Chem.*, 2 (1961) 493.
3 G. D. CHRISTIAN AND W. C. PURDY, *Biochim. Biophys. Acta*, 54 (1961) 587.
4 C. COOPER, *J. Biol. Chem.*, 235 (1960) 1815.
5 D. NEUBERT AND A. L. LEHNINGER, *J. Biol. Chem.*, 237 (1962) 952.
6 M. N. LIPSETT AND L. M. CORWIN, *J. Biol. Chem.*, 234 (1959) 2448.
7 W. W. KIELLEY AND J. R. BRONK, *J. Biol. Chem.*, 230 (1958) 521.
8 D. E. GREEN, D. M. ZIEGLER AND K. A. DOEG, *Arch. Biochem. Biophys.*, 85 (1959) 280.
9 C. LONG, *Biochemists' Handbook*, Van Nostrand, Princeton, N.J., 1961.
10 R. BRDICKA, *Research*, 1 (1947) 25.
11 A. G. STROMBERG, *Zhur. Fiz. Khim.*, 20 (1946) 409.
12 M. BREZINA AND P. ZUMAN, *Polarography in Medicine, Biochemistry and Pharmacy*, Revised English Ed., Interscience, New York, 1958, Chapter XXX.
13 E. C. CHEVALIER AND W. C. PURDY, *Anal. Chim. Acta*, 23 (1960) 574.
14 A. L. LEHNINGER AND M. SCHNEIDER, *J. Biophys. Biochem. Cytol.*, 5 (1959) 109.