*FLAVOPROTEINS OF MITOCHONDRIAL FATTY ACID OXIDATION**

BY PETER B. GARLAND,† BRITTON CHANCE,‡ LARS ERNSTER,§ CHUAN-PU LEE,‡ AND DAVID WONG‡

UNIVERSITY OF BRISTOL, UNIVERSITY OF PENNSYLVANIA, AND UNIVERSITY OF STOCKHOLM

Communicated May 29, 1967

One of the difficulties in spectroscopic studies of the flavin components of the respiratory chain is that, unlike the cytochromes, which possess sharp absorption maxima each at different wavelengths, the flavoproteins have absorption maxima that are superimposed in a broad band in the wavelength region of 450–475 mμ. In a previous paper, we have described a method which combines fluorescence and absorption measurements to allow a distinction between the flavoproteins of the respiratory chain on the basis of their relative fluorescence efficiencies.[1] Flavoproteins involved in NADH (Fp_{D1} and Fp_{D2}) and succinate (Fp_S) linked oxidations have been so identified.

An unsolved problem in previous work has been to identify the function of the flavoprotein component of rat liver mitochondria that has been heretofore reducible only by dithionite.[2] This flavoprotein was shown to consist of material of a very low (approximately one eighth) fluorescence efficiency compared to that of the flavoprotein reduced by succinate.[3] In this paper, the weakly fluorescent flavoprotein is identified by spectrophotometric and fluorometric techniques with components of the fatty acid oxidizing enzyme system, and is designated as Fp_A and Fp_{ETF}. The mitochondrial oxidation of fatty acids is functionally related to the respiratory chain in the manner shown in Figure 1.

Materials and Methods.—The techniques for split-beam[8, 9] and double-beam[1] spectrophotometry, flavoprotein fluorescence measurements,[1, 2, 10] rapid kinetic studies,[11] and the preparation of rat liver mitochondria[12] have been described. Acyl-CoA and acyl-carnitine compounds were kindly provided by Drs. J. R. Williamson and J. L. Lowenstein.

Each of the experiments of this paper was preceded by a preincubation of the mitochondria for two minutes at 25°C in the presence of 3.3 μM FCCP[13] or 7 μM PCP, in order to establish experimental conditions in which Fp_A and Fp_{ETF} are fully

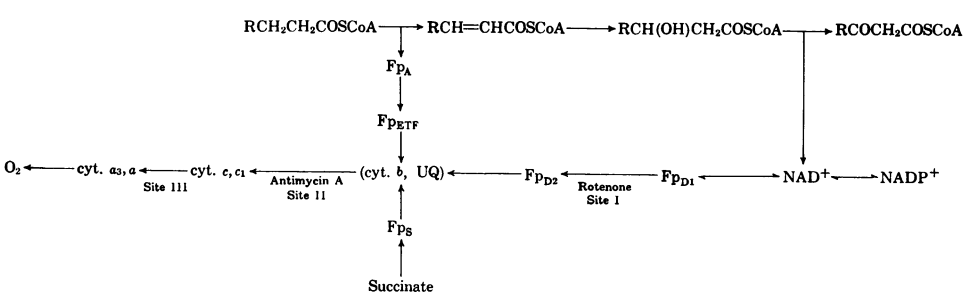

FIG. 1.—Relationship of the β-oxidation of fatty acids to the respiratory chain. The segment from NAD to cytochrome *b*, UQ is derived from Chance *et al.*[1] Fp_A represents acyl-CoA dehydrogenases,[3] Fp_{ETF} represents electron-transferring flavoprotein.[3] β-Oxidation is shown at one chain length only, and the reactions catalyzed by acyl-CoA ligases,[4, 5] acyl-CoA carnitine acyltransferase,[6] and thiolase[7] are not included.

oxidized and the oxidation of endogenous substrates, particularly fatty acids, is minimal. The uncouplers promote the oxidation of the respiratory chain and inhibit the operation of the ATP-dependent acyl-CoA ligase. In addition, in most experiments the preincubating medium contained 1.3 mM arsenate (to inhibit the GTP-dependent acyl-CoA ligase[4, 14]), 0.5 mM arsenite (to inhibit the oxidation of endogenous 2-oxoglutarate), and 0.5 mM malonate (to inhibit the oxidation of endogenous succinate).

After the preincubation, antimycin A was added, in order to inhibit electron transport in the respiratory chain (Fig. 2); a fluorescence change on the addition of antimycin A is largely attributable to the small amount of remaining endogenous substrate. Upon the further addition of β-hydroxybutyrate there occurs a flavin reduc-

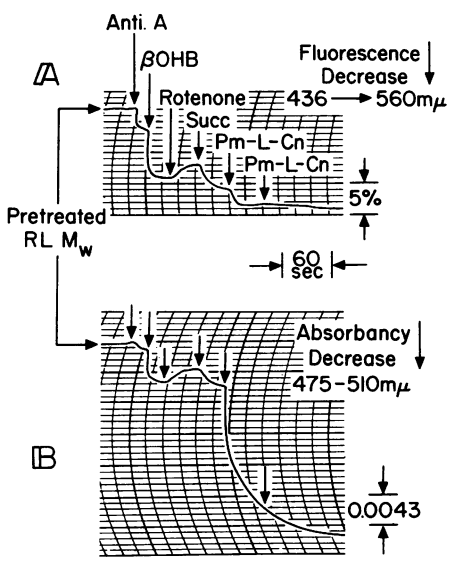

FIG. 2.—Simultaneous recording of flavoprotein fluorescence and absorbance. Trace *A* is fluorescence, trace *B* absorbance. Before antimycin A (1.7 μg/ml) was added, the rat liver mitochondria (2.3 mg/ml) had been incubated in 100 mM KCl, 50 mM tris-chloride, pH 7.4, at 25° for 3 min with 7 μM PCP, 0.7 mM arsenite, 1.7 mM arsenate, and 0.5 mM malonate. Addition of 3.3 mM βHOB causes extensive reduction of Fp_{D_1}, Fp_{D_2}, and Fp_S. After the addition of 2.5 μM rotenone, Fp_{D_2} and Fp_S become oxidized but are reduced again by the addition of 5 mM succinate (the succinate:malonate ratio is 10). Finally 8.3 μM palmitoyl-L-carnitine reduces Fp_A + Fp_{ETF}. Further addition of 8.3 μM palmitoyl carnitine is without effect. The light path was 1 cm.

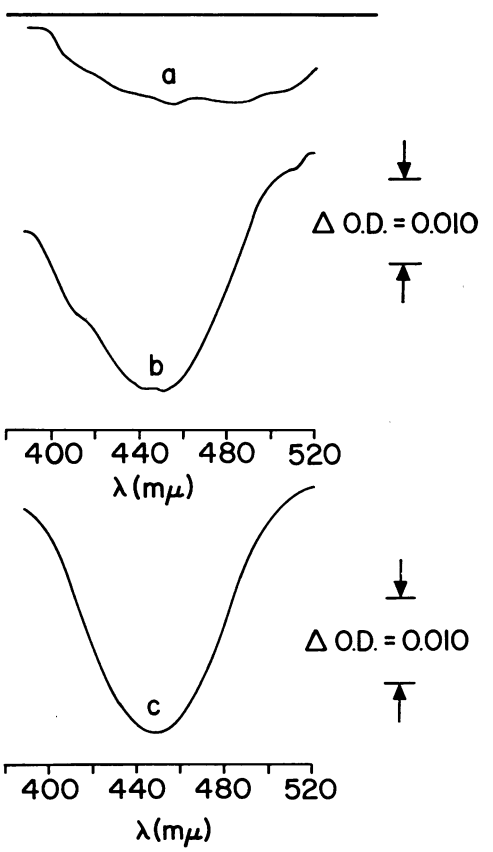

FIG. 3.—Difference spectrum for Fp_A + Fp_{ETF} reduction. Initially both the reference and sample cuvettes contained mitochondria (4 mg/ml) that had been brought to the state just preceding palmitoyl carnitine addition in Fig. 2, and a base line (*a*) was recorded. Twenty-five μM palmitoyl-L-carnitine was then added to the sample cuvette and trace *b* was recorded. Trace *c* is the difference spectrum after correction for the base line. The slit width was 1 mμ, and the cuvette was of 1 cm light path.

tion. As previously concluded,[1] this involves both a low- and a high-potential component of the NADH dehydrogenase, Fp_{D1} and Fp_{D2}, as well as succinate dehydrogenase, Fp_S. Addition of rotenone causes a partial oxidation of flavoprotein (Fp_{D2} and Fp_S). The reduction of Fps and Fp_{D2} is then restored by the addition of succinate. At this point, all the components of the respiratory chain on the substrate side of the antimycin A site (i.e., Fp_{D1}, Fp_{D2}, Fp_S, cytochrome *b*, and ubiquinone) are largely reduced, and the contribution of fatty acid oxidation to the reduction of the mitochondrial flavin pool can be evaluated. The large absorbancy change now caused by the addition of 8.3 μM palmitoyl-L-carnitine is approximately three times that caused by β-hydroxybutyrate and is attributed to Fp_A and Fp_{ETF} as defined in Figure 1.

This material can be identified as a flavoprotein by comparing split-beam spectrophotometric recordings of the mitochondrial suspensions taken at points just before and after the addition of acyl-carnitine in Figure 2. Twenty-five μM palmitoyl carnitine is added; the difference spectrum (Fig. 3) is free of interference from the Soret band of cytochrome *b* and has a broad trough at 450 mμ. Calculated on the basis of an extinction coefficient[15] for $flavin_{ox.\ -\ red.}$ of 10.6 $mM^{-1}\ cm^{-1}$ at 450 mμ, this change corresponds to 0.67 mμmole flavin per mg protein. A similar difference spectrum is obtained with octanoyl carnitine as substrate.

Rapid kinetics: Experiments similar to those of Figure 2*B* were performed with the stopped flow apparatus. Mitochondria were first brought to the stage corresponding to that just before the addition of acyl-carnitine in Figure 2 and then mixed in the apparatus with a solution of palmitoyl carnitine. Figure 4 shows that

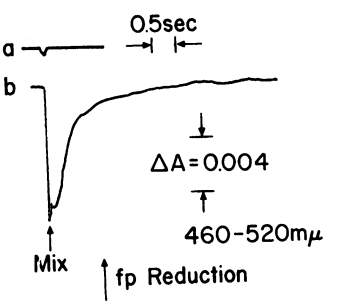

FIG. 4.—Reaction kinetics of the reduction of $Fp_A + Fp_{ETF}$ by palmitoyl carnitine. Trace *a* is the syringe plunger velocity, trace *b* is absorbance. Mitochondria (4 mg/ml) in 100 mM KCl, 50 mM tris chloride, pH 7.4, were pretreated for 3–4 min at 25° with 30 μM FCCP. After adding antimycin A (3 μg/mg protein), 10 mM succinate, and 6 mM β-hydroxybutyrate, the mitochondrial suspension was mixed with an equal volume of 147 μM palmitoyl-D,L-carnitine in 100 mM KCl, 50 mM tris-chloride, pH 7.4. The light path was 1 cm.

the characteristic features of the reduction of $Fp_A + Fp_{ETF}$ by palmitoyl carnitine consist of an induction period and a rapid rise to the complete reduction. In this experiment in the stopped flow apparatus, the induction period is 0.1 second. This is probably due to a prior need to allow intramitochondrial palmitoyl-CoA to accumulate, since palmitoyl carnitine itself is not a substrate for the acyl-CoA dehydrogenase. The reduction rate thereafter reaches 100 mμmoles/min/mg of mitochondrial protein, in excess of the steady-state oxygen utilization in the presence of palmitoyl carnitine (28 mμmoles of flavoprotein/min/mg[16]). The maximal rate at which palmitoyl carnitine can acylate intramitochondrial CoA is 17–18 mμmoles/min/mg.[17, 18] This rate could support the reduction of $Fp_A + Fp_{ETF}$ at 119–126 mμmoles/min/mg (the oxidation of palmitate to acetyl-CoA produces 7 $FADH_2$ and 7 NADH). It is thus concluded that the rate-limiting step causing the induction period is the palmitoyl-CoA carnitine acyltransferase activity. In fact, the rate of

reduction of Fp_A + Fp_{ETF} by octanoyl carnitine was similar to that observed for palmitoyl carnitine, but the induction period was shorter.

The fluorescence yield: The fluorescence of FMN or FAD is considerably altered on combination with their particular apoproteins.[2] In most instances there is a quenching of fluorescence, the extent of which varies between different flavoproteins. Under a given set of experimental conditions the ratio of fluorescence change to absorbance change (f/a ratio) that occurs upon the reduction of an oxidized flavoprotein is a measure of the fluorescence yield of the flavoprotein concerned, and can be used to characterize different flavoproteins.[19] This is illustrated in the experiment of Figure 2, where the simultaneous recording of flavoprotein fluorescence and absorbance shows that the f/a ratios for Fp_{D1}, Fp_{D2} + Fp_S, and Fp_A + Fp_{ETF} differ from each other. [The low value of f/a ratio for Fp_S demonstrated by the spectrophotometric and fluorometric responses to TTFA (Fig. 2 in ref. 1) was incorrectly interpreted as being due to Fp_S instead of to Fp_{D2}.] Values for the absolute content and f/a ratios of the various flavoproteins are summarized in Table 1. For the pur-

TABLE 1

AMOUNTS AND RELATIVE FLUORESCENCE YIELDS OF FLAVOPROTEINS OF BEEF HEART AND RAT LIVER MITOCHONDRIA

Flavoprotein	Amount (mμmole flavin/mg) BHMw	RLMw	F/a Ratio BHMw	RLMw
Fp_{D1} (βHOB-rat)	0.15	0.078	1	1
Fp_{D2} + Fp_S	0.135	0.065	0.57	0.66
Fp_S (reduced c̄ TTFA[34])	0.04		0.54	
Fp_{D2} (oxidized c̄ TTFA[34])	0.094		0.16	
Fp_A + Fp_{ETF}		0.65		0.05

The amount of each flavoprotein in intact mitochondria was calculated from the decrease in absorbance at 475–510 mμ resulting from reduction by the appropriate substrate.

pose of comparison the f/a ratio for Fp_{D1} has been made unity. The contribution of nonheme iron to the absorbancy changes attributed to Fp_A + Fp_{ETF} is presumably zero, since they are not presently regarded as metalloflavoproteins.[5] Possible contributions to the absorbancy changes of Fp_{D1}, Fp_{D2}, and Fp_s were discussed previously.[1]

Location of Fp_A and Fp_{ETF}: The effects of atractylate and carnitine on the adenine nucleotide translocase[20–23] and fatty acid activation,[21] the latency of the mitochondrial enzymes of β-oxidation toward the added acyl-CoA substrates,[6] the location of palmitoyl-CoA carnitine acyltransferase on the inner mitochondrial membrane,[17, 24] and the measurements of mitochondrial spaces for adenine nucleotides,[20] NAD,[20] and CoASH[17] all point to the location of the enzymes of β-oxidation and their cofactors within the confines of the inner mitochondrial membrane and matrix.[18] On the other hand, it has been proposed that the enzymes of β-oxidation are associated with the outer mitochondrial membrane.[25] Reduction of the respiratory chain (on the inner membrane) by the acyl-CoA dehydrogenases (on the outer membrane) was envisaged as occurring through Fp_{ETF} acting as a mobile carrier. It was therefore relevant to study the effects of atracylate and carnitine on the reduction of Fp_A + Fp_{ETF} by fatty acid oxidation. The results are summarized in Table 2. In all respects they are essentially similar to those obtained when fatty acid oxidation is assessed by the reduction of NAD(P).[18, 21] The salient points are as follows. (*a*) The reduction of Fp_A and Fp_{ETF} by added palmitoyl-CoA required carnitine. (*b*) The reduction of Fp_A and Fp_{ETF} by palmitate required ATP, and the rate of reduc-

TABLE 2

EFFECTS OF CARNITINE AND ATRACTYLATE ON THE REDUCTION OF $Fp_{A, ETF}$ BY FATTY ACID OXIDATION

Fatty acid	Addition	Rate of flavoprotein reduction (mμmoles flavin/min/mg)
Palmitate (13 μM)	ATP (0.167 mM)	1.3
"	ATP, D,L-carnitine (1 mM)	>4.0
"	ATP, carnitine, atractylate (0.2 mM)	0.8
"	ATP, carnitine, atractylate, palmitoyl carnitine (20 μM)	>4.0
Palmitoyl-CoA (10 μM)		0.2
"	Carnitine	>4.0
Octanoate (0.1 mM)	ATP (0.167 mM)	>4.0
"	ATP, atractylate (0.2 mM)	0.3
"	ATP, atractylate, octanoyl carnitine (70 μM)	>4.0

In each experiment rat liver mitochondria (4 mg/ml) were incubated at 25° in 100 mM KCl, 50 mM tris-chloride, pH 7.4, for 2 min with 1.67 μM FCCP, 5 mM malonate, and 2 mM arsenate before the addition of antimycin A (3 μg/ml), succinate (6 mM), D,L-3-hydroxybutyrate (2 mM), and oligomycin (2 μg/ml). Flavoprotein reduction was measured at 456–520 mμ. Initial rates greater than 4 mμmoles flavin/min/mg could not be estimated because of the rapidity of the reaction compared with the speed of mixing by hand (cf. Fig. 4).

tion was stimulated by carnitine. (*c*) Atractylate inhibited the ATP- and carnitine-dependent reduction of Fp_A and Gp_{ETF}. The inhibition was not observed when the substrate was palmitoyl carnitine or octanoyl carnitine. (*d*) The reduction of Fp_A and Fp_{ETF} by octanoate required ATP but not carnitine (cf. ref. 27). In addition it has been found[26] that rotenone-inhibited rat liver mitochondria do not oxidize palmitoyl carnitine in the presence of added NAD^+ and cytochrome *c*, i.e., under conditions when added NADH is oxidized via the cytochrome b_5 reductase–cytochrome b_5 system associated with the outer membrane.[28] These data are consistent with the idea that the enzymes of β-oxidation including Fp_A and Fp_{ETF} are located with the inner mitochondrial membrane and matrix rather than the outer membrane of rat liver mitochondria.

Discussion.—The oxidized-minus-reduced difference spectrum for $Fp_A + Fp_{ETF}$ lacks the broad peak between 500 and 600 mμ that is attributed to the semiquinone form of flavin.[3] In this respect the spectrum resembles that obtained when purified Fp_{ETF} is reduced by acyl-CoA and purified Fp_A.[3] By contrast, reduction of purified Fp_A by acyl-CoA causes a semiquinoid type of flavin spectrum.[3] This suggests that most of the absorbance change attributable to $Fp_A + Fp_{ETF}$ in intact mitochondria is due to Fp_{ETF}. It is likely that Fp_A and Fp_{ETF} have different fluorescence yields, and rapid kinetic measurements of both fluorescence and absorbance during the reduction of $Fp_A + Fp_{ETF}$ may assess the relative contributions of the individual flavoproteins. The maximal observed rate of reduction of $Fp_A + Fp_{ETF}$ was four times that required for the state 3 rate of oxidation of palmitoyl carnitine. $Fp_A + Fp_{ETF}$ was not reduced by succinate or NAD-linked substrates,[29] and its redox potential is therefore probably lower than that of Fp_S and Fp_{D2}.

The considerable amount and metabolic activity of $Fp_A + Fp_{ETF}$ may well have complicated previous attempts at spectrophotometric studies of respiratory chain flavoproteins in intact mitochondria.[30] Although Fp_{ETF} transfers to the respiratory chain at the region of cytochrome *b*, it operates in close dependence on events occurring between NAD and cytochrome *b*. This is because the total amount of mitochondrial CoASH is less than 2.0 mμmoles/mg,[31] and deacylation of the intermediates of β-oxidation does not occur at a significant rate.[16] Interruption of β-oxidation at

the NAD-linked step (for instance, by NADH accumulation due to rotenone or state 4 inhibition of the respiratory chain[30]) also inhibits the reduction of $Fp_A + Fp_{ETF}$. Mitochondrial preparations usually contain endogenous fatty acids, and the availability of ATP alone can result in fatty acid activation and reduction of $Fp_A + Fp_{ETF}$ even in the absence of added carnitine. This is distinguishable from an energy-linked reduction of Fp_{D1}[1] by its insensitivity to oligomycin and to rotenone. The rotenone-insensitive "energy-dependent" reduction of flavoprotein observed in locust flight-muscle mitochondria[32, 33] (which oxidize fatty acids) may be due to $Fp_A + Fp_{ETF}$ rather than respiratory chain flavoproteins.

The variations in the absolute amounts and the f/a ratios of the flavoproteins (Table 1) show that $Fp_A + Fp_{ETF}$ are best detected by absorbance measurements whereas Fp_{D1} and Fp_{D2} are most specifically and easily measured by fluorescence. In either case better characterization is obtained by measuring both flavoprotein fluorescence and absorbance during redox transitions that have been devised to select only one or two of the many flavoproteins of intact mitochondria.

Summary.—The flavoproteins involved in the oxidation of fatty acids in rat liver mitochondria have been identified and quantitatively estimated by spectrophotometric and fluorometric measurements. These flavoproteins, which constitute approximately 80 per cent of the total flavin of rat liver mitochondria, are distinguished from those involved in the oxidation of NADH and succinate by a low fluorescence. They are not reduced by NADH or succinate. Evidence is presented that the fatty acid oxidizing flavoproteins are associated with, or located inside, the inner membrane of liver mitochondria.

Abbreviations: NADH, nicotinamide adenine dinucleotide, reduced form; Fp_{D1} and Fp_{D2} are the low- and high-potential components of the respiratory chain-linked NADH dehydrogenase; Fp_S, succinate dehydrogenase; Fp_A, acyl-CoA dehydrogenases; Fp_{ETF}, electron-transferring flavoprotein; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone; PCP, pentachlorophenol; FAD, FADH, flavin adenine dinucleotide and its reduced form; FMN, flavin mononucleotide; GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; βHOB, D,L-3-hydroxybutyrate; TTFA, thenoyltrifluoroacetone; BHMw, intact beef heart mitochondria; and RLMw, intact rat liver mitochondria.

* Supported by USPHS grant G.M. 12202; a preliminary note of this work has been submitted to the Fourth Meeting of Federation of European Biochemistry Societies, Oslo, Norway, July 3–7, 1967.

† Department of Biochemistry, University of Bristol, Bristol, England.

‡ Johnson Research Foundation, University of Pennsylvania, Philadelphia.

§ Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden.

[1] Chance, B., L. Ernster, P. B. Garland, C-P. Lee, P. A. Light, T. Ohnishi, C. I. Ragan, and D. Wong, these PROCEEDINGS, **57**, 1498 (1967).

[2] Chance, B., and B. Schoener, in *Flavins and Flavoproteins*, ed. E. C. Slater (Amsterdam: Elsevier, 1966), p. 498.

[3] Beinert, H., in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (New York: Academic Press Inc., 1963), vol. 7, pp. 447 and 467.

[4] Rossi, C. R., and D. M. Gibson, *J. Biol. Chem.*, **239**, 1694 (1964).

[5] Kornberg, A., and W. E. Pricer, *J. Biol. Chem.*, **204**, 329 (1953).

[6] Fritz, I. B., and K. T. N. Yue, *J. Lipid Res.*, **4**, 279 (1963).

[7] Lynen, F., *Federation Proc.*, **12**, 683 (1953).

[8] Yang, C. C., and V. Legallais, *Rev. Sci. Instr.*, **25**, 801 (1954).

[9] Chance, B., *Rev. Sci. Instr.*, **22**, 634 (1951).

[10] Chance, B., and B. Schoener, in *Flavins and Flavoproteins*, ed. E. C. Slater (Amsterdam: Elsevier, 1966), p. 510.

[11] Chance, B., B. Schoener, and D. DeVault, in *Oxidases and Related Redox Systems*, ed. T. E. King, H. Mason, and M. Morrison (New York: John Wiley, 1965), p. 907.

[12] Lardy, H. A., and H. Wellman, *J. Biol. Chem.*, **195**, 215 (1952).

[13] Heytler, P. G., *Biochemistry*, **2**, 357 (1963).

[14] van den Bergh, S. G., *Biochim. Biophys. Acta*, **98**, 442 (1965).

[15] Mahler, H. R., *J. Biol. Chem.*, **206**, 13 (1954).

[16] Garland, P. B., and C. I. Ragan (unpublished).

[17] Yates, D. W., and P. B. Garland, *Biochem. Biophys. Res. Commun.*, **23**, 460 (1966).

[18] Garland, P. B., and D. W. Yates, in *Structure and Compartmentation of Mitochondria*, ed. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (Bari, Italy: Adriatica Editrice, in press).

[19] Wong, D., *Federation Proc.*, **26**, 732 (1967).

[20] Klingenberg, M., and E. Pfaff, in *Regulation of Metabolic Processes in Mitochondria*, ed. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (Amsterdam: Elsevier, 1966), p. 180.

[21] Chappell, J. B., and A. R. Crofts, *Biochem. J.*, **95**, 707 (1965).

[22] Brierley, G., and D. E. Green, these PROCEEDINGS, **53**, 73 (1965).

[23] Kemp, A., and E. C. Slater, *Biochim. Biophys. Acta*, **92**, 178 (1964).

[24] Farstad, M., J. Brenner, and K. Norum, *Biochim. Biophys. Acta*, **132**, 492 (1967).

[25] Allman, D. W., L. Galzigna, R. E. McCaman, and D. E. Green, *Arch. Biochem. Biophys.*, **117**, 413 (1966).

[26] Ernster, L., and K. Nordenbrand, unpublished results.

[27] Lehninger, A. L., *J. Biol. Chem.* **157**, 363 (1944).

[28] Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand, *J. Cell Biol.*, **32**, 415 (1967).

[29] Frisell, W. R., M. V. Pativardhan, and C. G. MacKenzie, *J. Biol. Chem.*, **240**, 1829 (1965).

[30] Chance, B., and G. R. Williams, *Advan. Enzymol.*, **17**, 65 (1956).

[31] Garland, P. B., D. Shepherd, and D. W. Yates, *Biochem. J.*, **97**, 587 (1965).

[32] Klingenberg, M., in *Flavins and Flavoproteins*, ed. E. C. Slater (Amsterdam: Elsevier, 1966), p. 522.

[33] Meyer, H., B. Preiss, and S. L. Bauer, *Biochem. J.*, **76**, 27 (1960).

[34] Zeigler, D. M., in *Biological Structure and Function*, ed. T. W. Goodwin and O. Lindberg (New York: Academic Press, Inc., 1961), vol. 2, p. 253.