Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.
Seelig, J., and Hasselbach, W. (1971), *Eur. J. Biochem. 21*, 17.
Stoeckenius, W., and Rowen, R. (1967), *J. Cell Biol. 34*, 365.
Sudi, J. (1970), *Biochim. Biophys. Acta 212*, 213.
Sulkowski, E., and Laskowski, M. (1968), *J. Biol. Chem. 243*, 651.
Suzuki, H. (1967), Electronic Absorption Spectra and Geometry of Organic Molecules, New York, N. Y., Academic Press.
Tourtellotte, M. E., and Branton, D., and Keith, A. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 909.
Woldringh, C. L., and van Iterson, W. (1972), *J. Bacteriol. 111*, 801.

Respiration of Rat Lung Mitochondria and the Influence of Ca^{2+} on Substrate Utilization†

Aron B. Fisher,* Antonio Scarpa, Kathryn F. LaNoue, David Bassett, and John R. Williamson

ABSTRACT: Mitochondria were prepared from homogenates of rat lungs by modification of standard techniques. The mitochondrial preparation was characterized by its content of cytochromes, adenine and pyridine nucleotides, coenzyme A, and divalent cations. Rates of substrate oxidation were measured polarographically. Succinate was oxidized at a rate of 53 ± 9 nmol of O_2/min per mg of protein while other substrates were oxidized less rapidly. The Ca^{2+} content of the mitochondrial preparation was of major importance in determining rates of substrate oxidation. Addition of 100–200 μM Ca^{2+} during oxidation of NAD-linked substrates markedly decreased respiration rate; this effect was due to loss of free pyridine nucleotides from the mitochondria in the presence of Ca^{2+}. On the other hand, very low levels of Ca^{2+} (<1 μM) stimulated oxidation of α-glycerophosphate. This effect was due to increased glycerophosphate dehydrogenase activity in the presence of Ca^{2+}. Lung mitochondria were able to passively bind large amounts of Ca^{2+} and also actively accumulated Ca^{2+} from the suspending medium in the presence of exogenous ATP and during substrate oxidation in the presence of Mg^{2+} or rotenone. Although the effects on oxidation of NAD substrates may not be physiologically significant, cytosolic [Ca^{2+}] in the intact lung cell may be important as a determinant of mitochondrial glycerophosphate dehydrogenase activity. This activation may affect the rate of α-glycerophosphate incorporation into alveolar phospholipids or may regulate the activity of the α-glycerophosphate cycle for intramitochondrial transport of reducing equivalents.

Recent studies have shown the lung to be an actively metabolic organ with an oxygen consumption per unit weight almost as great as liver (Heinemann and Fishman, 1969; Weber and Visscher, 1969). Reiss and others have isolated mitochondria from lung homogenates (Reiss, 1966; Kyle and Riesen, 1970; Sayeed and Baue, 1971) and have suggested that these organelles are qualitatively similar to mitochondria derived from other tissues. However, basic information about the lung mitochondrial preparation such as content of cytochromes and nucleotides, factors affecting rates of substrate oxidation, and mechanisms for transport of reducing equivalents across the mitochondrial membrane have not been determined. Because this type of data obtained with mitochondria from other organs has provided important information with respect to control of tissue metabolism, we began similar studies with a lung mitochondria preparation. Although it is known that the lung is heterogeneous from a morphologic standpoint (Bertalanffy, 1964), preparation of mitochondria from the whole organ provided a reasonable starting point. Our initial results showed that respiration of lung mitochondria is very dependent on calcium ion content of the preparation and we have explored this area of lung mitochondrial metabolism in detail.

Materials and Methods

Preparation of Mitochondria. Eight to ten 180–220-g Sprague-Dawley male rats were killed by decapitation. The lungs were quickly removed, dissected free of large airways and blood vessels, and minced with scissors in ice-cold isolation medium containing 225 mM mannitol, 75 mM sucrose, and 2 mM EDTA. The minced tissue was transferred to isolation medium (approximately 1:10 tissue to medium ratio) containing in addition 5 mM Mops[1] (pH 7.3) and 1–2% (w/v) lipid-free bovine serum albumin (Chen, 1967). In some experiments, 20 mM EGTA was substituted for EDTA in the homogenization medium without demonstrable effect on characteristics of the mitochondrial preparations. The lung mince was

† From the Departments of Physiology, Biophysics and Medicine, University of Pennsylvania School of Medicine, and Philadelphia Veterans Administration Hospital, Philadelphia, Pennsylvania. *Received November 13, 1972.* Research support was provided by NIH Grant HL-15013-01, by American Medical Association Grant AMA ERF-39 (4-1315), and by the Veterans Administration Research Service. Preliminary reports of the findings presented in this paper have been published in *Physiologist 14*, 142 (1971), *Clin. Res. 19*, 740 (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 288 Abstr (1972), and *Amer. Rev. Resp. Dis. 105*, 1004 (1972).

[1] Abbreviations used are: Mops, morpholinopropane sulfate; EGTA, ethylene glycol bis(amino ethyl ether *N,N'*-tetraacetic acid); RCR, respiratory control ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; α-GP, α-glycerophosphate; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine.

dispersed with a mechanical disintegrator (Polytron Model PT10, Brinkmann, Inc.) for 10 sec with the rheostat set at 4. The dispersed tissue was homogenized in a Potter-Elvehjem vessel by two passes with a Teflon pestle of 0.16-mm clearance, and centrifuged for 5 min at 1500*g*. The supernatant was strained through single-layer cheesecloth and respun at 13,000*g* for 10 min to precipitate the mitochondrial pellet. The mitochondria were washed twice with cold isolation medium (without Mops or albumin), centrifuged at 13,000*g* for 10 min, and finally suspended in 1–2 ml of the same medium for use. For studies of Ca^{2+} uptake and Ca^{2+} binding, EDTA was omitted from the final wash and suspension solution.

Purity of the mitochondrial preparation during development of the isolation methods was monitored with electron microscopy. Mitochondria were fixed with 3% phosphate-buffered glutaraldehyde (pH 7.4) and postfixed in 2% osmium tetroxide. Thin sections were stained with uranyl acetate and lead citrate and examined with an RCA EMU 3-H electron microscope at 50 kV accelerating voltage.

Analytical Methods. Oxygen utilization was measured polarographically with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) in a 1.0-ml capacity vessel containing 145 mM KCl–5 mM KH_2PO_4–20 mM Tris-Cl (pH 7.2) saturated with room air and maintained at 28°.

Spectra of the reduced-minus-oxidized pigments of the mitochondrial respiratory chain were obtained with a split-beam wavelength-scanning spectrophotometer (built at the Johnson Foundation, University of Pennsylvania) with a 250-mm focus Bausch and Lomb monochromator and an effective band width of 1.6 nm. Samples in 250 mM sucrose were prepared in cuvets with either a 1-cm path length for room temperature spectra or 2-mm path length for low-temperature spectra by the trapped steady-state technique using liquid N_2 (Chance and Schoener, 1966). Cytochromes were reduced in one cuvet by adding dithionite. Cytochrome concentrations from room temperature spectra were calculated with the following millimolar extinction coefficients: cytochrome ($a + a_3$) = 24, cytochrome b = 22, and cytochrome c = 19 (Van Gelder, 1966; Wilson and Epel, 1968).

Changes in the oxidation–reduction state of mitochondrial pyridine nucleotides were followed with a filter fluorometer and samples were removed from the cuvette at intervals for determination of adenine and pyridine nucleotide content by standard enzymatic techniques using fluorometric analyses (Williamson and Corkey, 1969).

Mitochondrial glycerophosphate dehydrogenase (EC 1.1.-99.5) activity was measured by modification of the technique of Lee and Lardy (1965) using phenazine methosulfate and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-*p*-phenylmonotetrazolium in the presence of KCN. Incubation was carried out at room temperature and the reaction was started by the addition of α-glycerophosphate. The change in absorbance at 500 nm was continuously recorded and the reaction velocity was calculated from the initial linear portion of the tracing.

NAD-nucleosidase (EC 3.2.2.5) activity was assayed by modification of the technique of Yuan and Anderson (1971). Mitochondria were incubated at 38° in 1 ml of 0.1 M Tris-Cl (pH 8.0) with 0.3–0.6 mg/ml of mitochondrial protein. The reaction was started by the addition of NAD to give a final concentration of 0.7 mM. After 3 min, the reaction was stopped with 6 ml of 2 M KCN and the optical density was ready at 327 nm. Blanks were prepared by omitting the NAD. Zero time controls were obtained by adding KCN to the mitochondria prior to NAD addition.

^{45}Ca-binding by lung mitochondria was measured in the presence of 1.3 μM rotenone and 10 μg/ml of antimycin A. Mitochondria were incubated at room temperature for 5 min with increasing concentrations of $^{45}CaCl_2$ between 0.005 and 1.0 mM in 225 mM mannitol–75 mM sucrose–5 mM Mops (pH 7.3). After centrifugation, the pellet was washed twice with Ca^{2+}-free H_2O (<0.1 μM Ca^{2+}) and dissolved in 90% formic acid. Bound $^{45}Ca^{2+}$ was measured by scintillation counting.

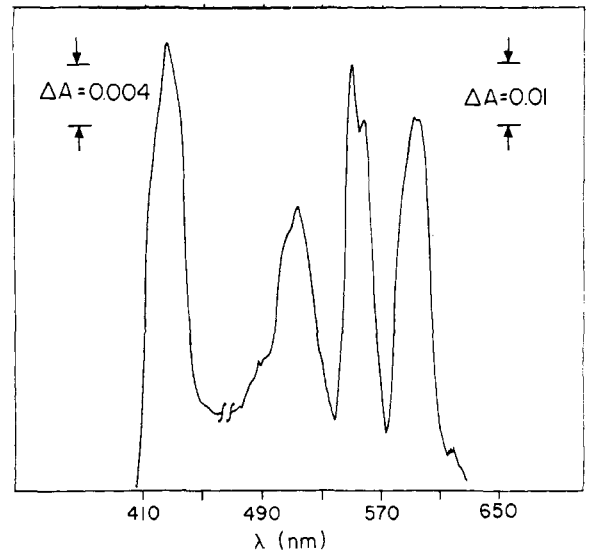

FIGURE 1: Reduced-minus-oxidized difference absorption spectrum of rat lung mitochondria at the temperature of liquid nitrogen. The mitochondria were suspended in aerated 250 mM sucrose at a protein concentration of 0.4 mg/ml and transferred to identical cuvets with a 2-mm path length. One sample was treated with dithionite before freezing. Spectra were recorded using a split-beam wavelength-scanning spectrophotometer. Absorption maxima in the visible region are seen at 602 (cytochromes $a + a_3$), 558 (cytochrome b), and 549 nm (cytochrome c). In the Soret region, an absorption maximum is evident at 428 nm (cytochrome b) with shoulders at 420 (cytochrome c) and 435 nm (cytochrome $a + a_3$). The sensitivity of the spectrophotometer was changed at 470 nm as indicated by the break in the line.

Uptake of Ca^{2+} and other divalent cations was measured spectrophotometrically in the presence of murexide, a metallochromic indicator of cation activity. The changes in absorbancy of murexide were followed at 540–507 nm in a dual-wavelength spectrophotometer (Scarpa, 1972).

Ca^{2+} and Mg^{+} contents of the mitochondrial preparation was measured with an atomic absorption spectrophotometer after samples were dissolved in 90% formic acid (Scarpa and Azzi, 1968). Protein content was determined by the biuret method (Gornall *et al.*, 1949) with crystalline bovine serum albumin as standard.

Results

Preparation and Characteristics of Lung Mitochondria. The standard preparative method generally yielded 20–30 mg of mitochondrial protein from the lungs of 8–10 rats. Electron microscopically, the preparation was rich in mitochondria but contained, in addition, parts of membranes, lamellar bodies, lysozomes, and other organelles. Further purification of the mitochondrial fraction was possible by centrifugation through a discontinuous sucrose gradient as described by Reiss (1965) but was not used for routine purposes.

The reduced minus oxidized spectra recorded at liquid N_2

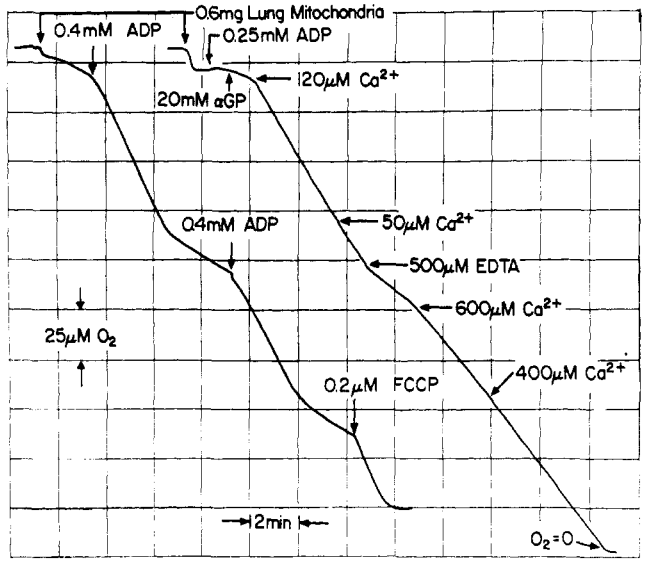

FIGURE 2: O_2 utilization by rat lung mitochondria as a function of time in 1 ml of buffer at 28° containing 145 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-Cl (pH 7.2), and 0.1 mM EDTA. The left hand trace shows respiratory control by the preparation; successive additions were pyruvate–malate, ADP (two additions), and FCCP, an uncoupler of oxidative phosphorylation. The respiratory control ratio (ADP-stimulated rate *vs.* rate after utilization of added ADP) was 3.8. The right-hand trace shows the effect of Ca^{2+} on oxidation of α-GP. Oxygen utilization in the presence of α-GP and ADP was stimulated fivefold by the addition of Ca^{2+}. A second addition of Ca^{2+} had no effect but the rate decreased 60% after addition of excess EDTA. Subsequent addition of Ca^{2+} again stimulated oxygen uptake although the rate was less rapid than after the first Ca^{2+} addition.

TABLE 1: Oxygen Utilization Rate and Respiratory Control Ratio of Rat Lung Mitochondria.

Substrate[a] (mM)	State 3 O_2 Uptake[b] (nmol of O_2/min per mg)	Respiratory Ratio Control
Pyruvate (5)	40 ± 5	2.9 ± 0.2
α-Ketoglutarate (3)	40 ± 5	2.8 ± 0.1
Isocitrate (4)	38 ± 4	2.5 ± 0.4
Malate (5)	20 ± 3	1.8 ± 0.1
Succinate (10)	53 ± 9	2.1 ± 0.1
Glutamate (10)	25 ± 3	3.0 ± 0.4
β-Hydroxy butyrate (1)	7 ± 2	1.4 ± 0.2
Acetoacetate (1)	7 ± 2	1.7 ± 0.3
Acetylcarnitine (1)	18 ± 3	1.8 ± 0.2
Octanylcarnitine (0.5)	24 ± 3	2.1 ± 0.2
Palmitylcarnitine (0.5)	26 ± 4	1.5 ± 0.2
α-Glycerophosphate (20)	22 ± 2[c]	1.3 ± 0.2
TMPD (0.5)–ascorbate (10)	50 ± 13	1.0
NADH (5)	1 ± 1	

[a] Substrate was added as the Tris-buffered salt (pH 7.2) to give the stated concentration. Incubation was carried out at 28° in a 1-ml cuvet containing 145 mM KCl, 5 mM $K_2H_2PO_4$, 20 mM Tris-Cl (pH 7.2), 0.2–0.4 mM ADP, 0.1 mM EDTA, and approximately 1 mg of protein. [b] Values are the mean ± (SE) of four to seven experiments. [c] Measured in the presence of 0.1 mM Ca^{2+}.

temperature demonstrated the usual cytochromes of the mitochondrial respiratory chain (Figure 1). The concentration of cytochromes in nanomoles per milligram of protein calculated from spectra obtained at room temperature was 0.17 ± 0.02 (SE) cytochromes ($a + a_3$), 0.31 ± 0.05 cytochrome *b*, and 0.33 ± 0.04 cytochrome *c*. These data give a cytochrome *b* to cytochrome ($a + a_3$) ratio of 1.8 and cytochrome *c* to cytochrome ($a + a_3$) ratio of 1.9.

The total pyridine nucleotide content of the mitochondrial preparation, calculated by summing the measured NAD(H) and NADP(H) contents, was 2.59 ± 0.29 (SE for four preparations) nmol/mg of protein with approximately two-thirds present as NAD(H) and one-third as NADP(H). Total adenine nucleotide content of mitochondria, calculated by summing the individually measured AMP, ADP, and ATP contents, was 6.40 ± 0.16 nmol/mg of protein. CoA content was 0.80 and 1.05 nmol per mg of protein with two preparations.

The mitochondrial content of Ca^{2+} and Mg^{2+} varied with the use of metal chelating agents in the isolation medium. When chelating agents were omitted during preparation, the Ca^{2+} content of the mitochondrial preparation was 213 ± 27 (SE) nmol/mg of protein and the Mg^{2+} content was 102 ± 27 nmol/mg of protein. The use of EDTA in the isolation medium reduced the Ca^{2+} content to 28 ± 4 (SE) nmol/mg and the Mg^{2+} content to 23 ± 2 nmol/mg. The Ca^{2+} content of rat liver mitochondria isolated with EDTA using similar methods was 7.0 nmol/mg of protein. These results and comparison with Ca^{2+} content of other mitochondrial preparations reported in the literature (Carofoli and Lehninger, 1971) suggested a relatively greater ability of the lung mitochondrial preparation to bind Ca^{2+}. This was confirmed by measurement of $^{45}Ca^{2+}$ binding in the presence of inhibitors of respiration. There was a progressive increase of Ca^{2+} bound with increasing concentration of Ca^{2+} in the suspending medium. Bound Ca^{2+} reached a plateau of 70.4 ± 11.0 (SE for five preparations) nmol of Ca^{2+}/mg of protein at 0.5–1.0 mM Ca^{2+} in the medium. The amount of $^{45}Ca^{2+}$ bound in the absence of respiration is almost twice as great as previously measured with liver mitochondria (Scarpa and Azzi, 1968).

Rates of Substrate Oxidation by Rat Lung Mitochondria. The ADP-stimulated rate of oxygen utilization (state 3 of Chance and Williams, 1956) was greatest with succinate as substrate and was also relatively rapid in the presence of pyruvate, α-ketoglutarate, isocitrate, and TMPD–ascorbate (Table I). Malate, glutamate, α-glycerophosphate, and the carnitine esters of plamitic, octanoic, and acetic acids were metabolized at a less rapid rate while acetoacetate and β-hydroxy butyrate were oxidized poorly. Respiratory control ratios (RCR) with NAD-linked substrates such as pyruvate were generally in the range of 2.5–4.0 (Figure 2) while lower RCR were observed with succinate and fatty acid substrates (Table I). Exogeneous NADH was not oxidized. Addition of 5 μM cytochrome *c* or 0.5% bovine serum albumin to the incubation chamber had no consistent effect on respiration and was not added routinely. Addition of 0.5 mM malate in combination with other substrates occasionally increased oxidation rates by 20–30%. The addition of FCCP, an uncoupler of oxidative phosphorylation, stimulated oxygen uptake to rates close to the state 3 value (Figure 2).

Effects of Ca^{2+} on α-Glycerophosphate Oxidation. With α-GP as substrate, the rate of oxygen uptake was stimulated 5- to 10-fold by added Ca^{2+} in the presence of either ADP

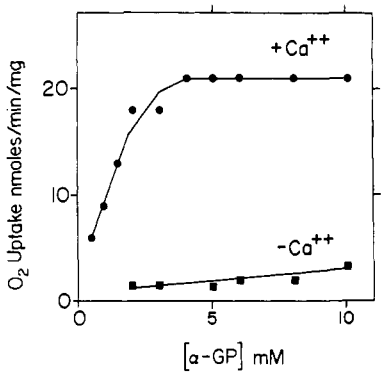

FIGURE 3: Rate of oxygen utilization as a function of α-glycerophosphate concentration. Mitochondria (1.1 mg/ml) were incubated with or without 0.3 mM Ca^{2+} at 28° in the buffer described in Figure 2 plus 0.5 mM ADP. Oxidation rate was obtained during sequential additions of α-glycerophosphate.

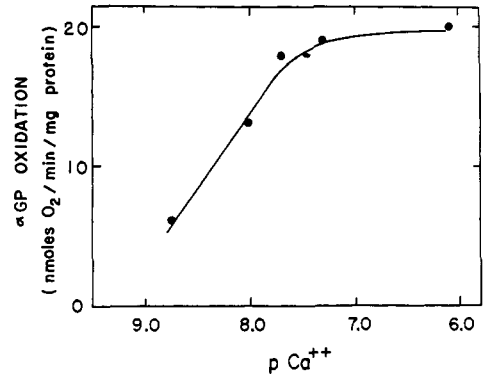

FIGURE 4: Relation of free Ca^{2+} concentration to rate of α-glycerophosphate oxidation. α-Glycerophosphate concentration was 3 mM. Free Ca^{2+} was measured with EGTA buffers (Portzehl *et al.*, 1964) and is plotted as minus log free Ca^{2+} (pCa^{2+}).

(Figure 2) or FCCP. With Ca^{2+} present, the rate of oxygen utilization increased as a function of added α-GP and reached a maximum at a concentration of approximately 4–5 mM α-GP (Figure 3). A double-reciprocal plot of these data from six experiments was linear and indicated a K_m of 1.8 ± 0.2 (SE) mM. Mg^{2+} (200 μM) also stimulated α-GP oxidation and gave a K_m of 4.3 ± 0.5 mM.

In these experiments, a variable concentration of EDTA, and hence free Ca^{2+}, was present in the incubation chamber. The actual Ca^{2+} concentration required for stimulation of α-glycerophosphate oxidation was determined with the use of Ca^{2+}–EGTA buffers in which the free Ca^{2+} could be calculated (Portzehl *et al.*, 1964). For these experiments, mitochondria were suspended in 0.2 mM EGTA. Six preparations showed a linear increase in the rate of α-glycerophosphate oxidation as a function of pCa (minus log Ca^{2+} concentration) with maximal stimulation occurring at less than 0.1 μM free [Ca^{2+}] (Figure 4). When free Ca^{2+} exceeded 1 μM, the rate of α-GP oxidation decreased by about 10–20%.

The stimulation of α-glycerophosphate oxidation by Ca^{2+} was further investigated by studying the effect of Ca^{2+} on mitochondrial glycerophosphate dehydrogenase activity measured with an artificial electron carrier system. Marked stimulation of glycerophosphate dehydrogenase activity by the addition of 3.2×10^{-8} free Ca^{2+} was observed (Figure 5). These experiments indicate that the Ca^{2+} effect on α-GP oxidation was independent of the mitochondrial electron transport chain.

Effect of Ca^{2+} on Oxidation of NAD-Linked Substrates. By contrast, quite different effects of Ca^{2+} were observed during oxidation of NAD-linked substrates: with pyruvate, malate, isocitrate, α-ketoglutarate, glutamate, or carnitine esters of fatty acids as substrates in the presence of either ADP or FCCP, addition of Ca^{2+} caused a progressive decrease in the rate of O_2 utilization (Figure 6). During state 4 respiration with these same substrates, an initial stimulation of oxygen uptake was followed by a progressive decrease (Figure 6). Generally, the addition of 100–200 μM Ca^{2+} completely inhibited subsequent O_2 uptake but oxidation was restored by addition of α-glycerophosphate (Figure 6) or succinate to the inhibited mitochondria.

The effect of Ca^{2+} on oxidation of NAD-linked substrates was also studied by using mitochondrial fluorescence changes as an indication of pyridine nucleotide oxidation–reduction state. Addition of Ca^{2+} to mitochondria in state 4 condition resulted in a rapid decrease of fluorescence indicating oxidation of NAD(P)H (Figure 7). Ca^{2+} added to the mitochondria at the start of incubation prevented the reduction of nucleotides upon subsequent addition of substrate.

In order to quantitate the effect of Ca^{2+} on mitochondrial nucleotides, the total free pyridine nucleotide content of the mitochondrial preparation was measured after incubation for 3 min with approximately 0.2 mM Ca^{2+}. In two experiments, Ca^{2+} addition resulted in a decrease of free NAD(P)(H) from a control value of 2.3 to 0.3 nmol per mg of protein. When mitochondria were prepared without metal chelating agents in the isolation medium, the NAD(P) plus NAD(P)H content was only 0.5 nmol/mg of protein. These results indicate that addition of Ca^{2+} to mitochondria did not merely lead to pyridine nucleotide oxidation but resulted in actual disappearance of free pyridine nucleotides from the preparation. This effect provides an explanation for the observed decreased oxygen utilization of the NAD-linked substrates in the presence of Ca^{2+}.

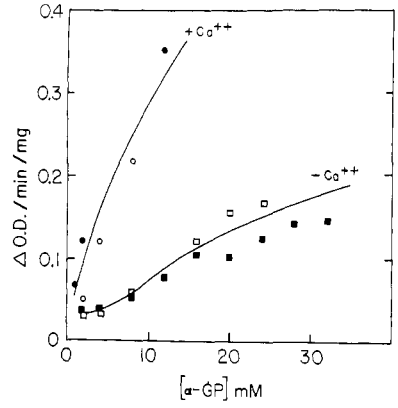

FIGURE 5: Effect of Ca^{2+} on α-GP activity of rat lung mitochondria. α-GP activity was measured using phenazine methosulfate and a tetrozolium salt in the presence of KCN as an artificial electron carrier system. The initial rate of reaction is plotted versus added α-glycerophosphate concentration. Incubation was carried out with 160 mM EDTA either without added Ca^{2+} (minus Ca^{2+}) or with added 80 μM Ca^{2+} (plus Ca^{2+}) which gave a free [Ca^{2+}] of 3×10^{-8} M. The solid and open symbols are results from two separate preparations.

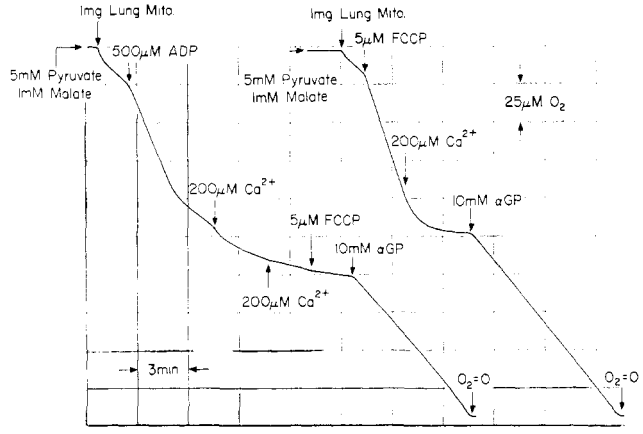

FIGURE 6: Effect of Ca^{2+} on oxidation of pyruvate–malate by rat lung mitochondria. Incubation conditions and representation of data is similar to Figure 2. The tracing at the left shows the effect of Ca^{2+} on mitochondria in state 4 condition (after utilization of added ADP). With addition of 200 μM Ca^{2-}, there is an initial slight stimulation of oxygen utilization followed by subsequent decrease to approximately one-third of the state 4 rate. O_2 uptake is further depressed by a subsequent addition of Ca^{2+}. Addition of FCCP, an uncoupler of oxidative phosphorylation, fails to stimulate the respiration rate but oxygen utilization resumes with the addition of α-GP. The right-hand trace indicates the effect of Ca^{2-} on mitochondria respiring in the presence of FCCP. Oxygen utilization is completely suppressed by Ca^{2+}, but resumes after the addition of α-GP.

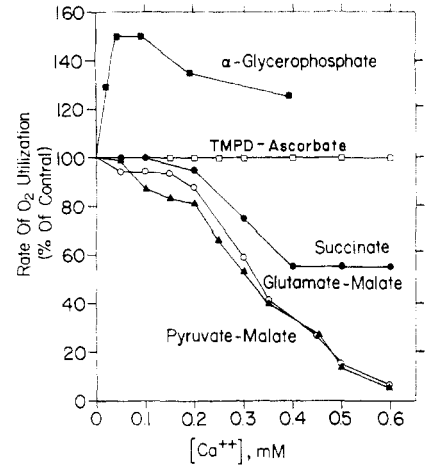

FIGURE 8: Summary of Ca^{2+} effects on respiration of rat lung mitochondria obtained with one preparation. Oxygen utilization, measured polarographically under conditions described in Figure 2, is plotted *vs.* concentration of added Ca^{2+}. Note that the incubation medium contained 0.1 mM EDTA. There is a progressive decrease in the rate of oxidation of the NAD-linked substrates while succinate respiration is depressed to a lesser extent and TMPD–ascorbate oxidation is unaffected. The oxidation of α-glycerophosphate is stimulated above the initial rate followed by slight depression at higher levels of added Ca^{2+}.

Nucleosidase Activity of Lung Mitochondria. The decrease of total pyridine nucleotides during incubation of the mitochondrial preparation with Ca^{2+} suggested the presence of NAD-nucleosidase activity. Measurement with five lung mitochondrial preparations showed hydrolysis of 370 ± 75 (SE) nmol of NAD/min per mg of protein. The addition of Ca^{2+} to the preparation did not affect NADase activity although the activity was inhibited 80% by 10 mM nicotinamide. However, nicotinamide did not prevent Ca^{2+} effects on NAD-linked substrate oxidation.

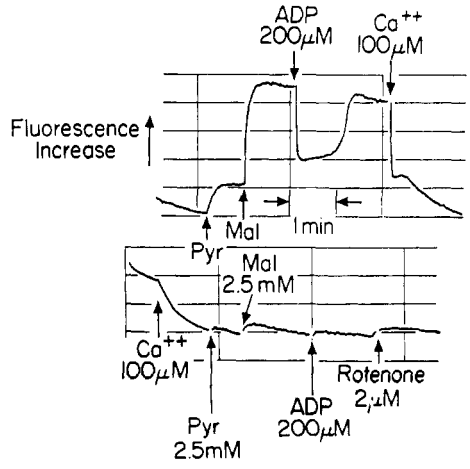

FIGURE 7: Effect of Ca^{2+} on fluorescence of pyridine nucleotides in rat lung mitochondria. Mitochondria (2.5 mg/ml) were incubated in 2 ml of buffer (145 mM KCl–5 mM KH_2PO_4–20 mM Tris-Cl (pH 7.2). The excitation wave length was 340 nm and the measurement was at 465 nm. Increased fluorescence indicates reduction of the pyridine nucleotides. The upper panel shows sustained reduction of pyridine nucleotides after the addition of substrate followed by a cycle of oxidation–reduction after ADP. Following addition of Ca^{2+}, there is first a rapid decrease of fluorescence followed by a slower decline. The lower panel shows that Ca^{2+} added to the mitochondria at the start of incubation led to decreased fluorescence and then prevented the fluorescence changes usually observed with addition of substrate, ADP, and rotenone.

Other Effects of Ca^{2+} on Substrate Oxidation. Ca^{2+} also inhibited the rate of succinate oxidation although compared with the NAD-linked substrates, higher concentrations of Ca^{2+} were required and the degree of inhibition rarely exceeded 60%. On the other hand, the oxidation of ascorbate plus TMPD remained unaffected by Ca^{2+} even up to 1 mM. The results of Ca^{2+} on substrate utilization are summarized in Figure 8. The illustrated pattern of effect of Ca^{2+} on substrate oxidation is a typical result although a slightly higher than usual concentration of Ca^{2+} was required to produce the effects.

Influence of Endogeneous Ca^{2+} on Substrate Oxidation. Mitochondria prepared without EDTA in the isolation medium in three experiments had a high endogeneous Ca^{2+} content (see above). Pyruvate, α-ketoglutarate, and isocitrate were oxidized poorly by these high Ca^{2+} mitochondria with an O_2 uptake of less than 2 nmol/min per mg of protein while succinate oxidation was 15–20 nmol/min per mg. Oxygen uptake with α-glycerophosphate as substrate was 18–20 nmol/mg per min and was not stimulated by addition of Ca^{2+}. The pattern of substrate utilization in these mitochondria with high endogeneous Ca^{2+} was similar to that observed in the routine preparation after Ca^{2+} was added exogenously. The addition of EDTA to the incubation chamber did not affect respiration rates of these high Ca^{2+} mitochondria.

Uptake of Divalent Cations by Rat Lung Mitochondria. Data for cation uptake were obtained with ten rat lung mitochondria preparations. Like other mitochondria, lung mitochondria accumulated Ca^{2+} from the medium in the presence of added ATP (Figure 9, upper left). Substrate oxidation supported active Ca^{2+} uptake in the absence of exogeneous ATP provided that Mg^{2+} was present (Figure 9, upper right). Rotenone could be substituted for Mg^{2+} in the case of flavine-

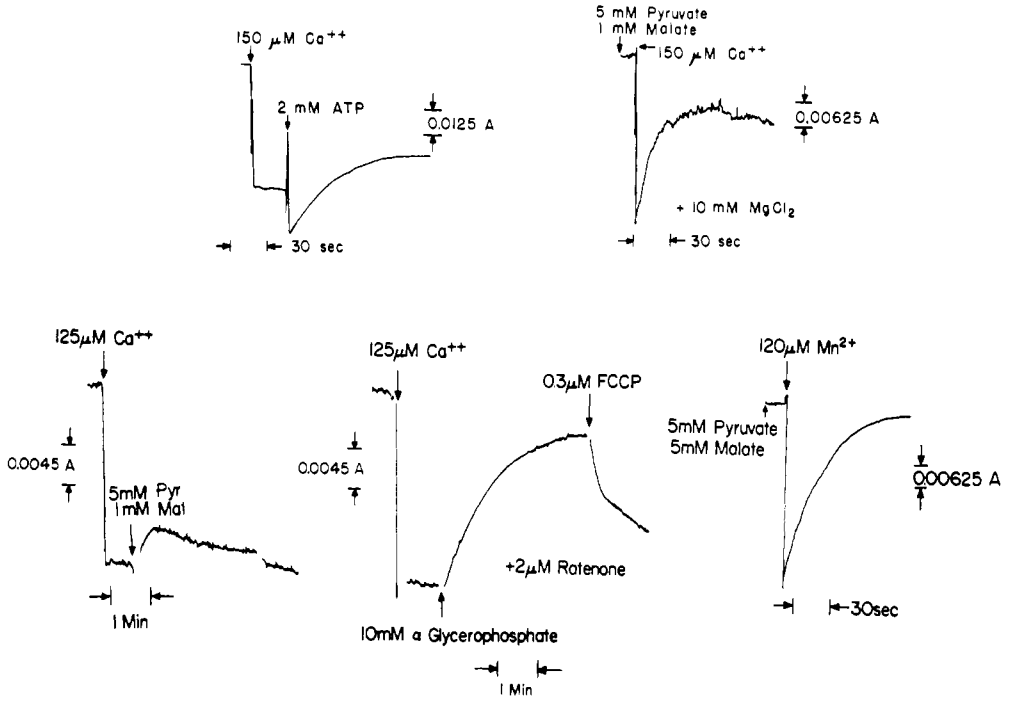

FIGURE 9: Energy dependent uptake of cations by rat lung mitochondria measured with a dual-wavelength spectrophotometer (Scarpa, 1972). Mitochondria were incubated in 145 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-Cl (pH 7.2), 35 μM murexide, and 1.5–2.1 mg/ml of mitochondrial protein. An upward deflection of the trace indicates increased absorbancy at 540–507 nm due to a decrease in Ca^{2+} or Mn^{2+} bound to murexide. The decrease of murexide-bound cation is due to cation uptake by mitochondria. Upper left: effect of ATP on Ca^{2+} uptake by mitochondria. Note the change in absorbancy after ATP was added, possibly due to Ca^{2+} contained in the ATP solution. 10 mM $MgCl_2$ was present in the incubation medium. Under these conditions Ca^{2+} is actively accumulated by the mitochondria. Upper right: Ca^{2+} transport during oxidation of puruvate–malate. Ca^{2+} is accumulated in the presence of 10 mM $MgCl_2$. Lower left: Ca^{2+} movements during pyruvate–malate oxidation in the absence of added Mg^{2+}. Ca^{2+} is taken up in only small amounts under these conditions showing Mg^{2+} requirements for Ca^{2+} transport. Lower center: Ca^{2+} uptake during oxidation of α-glycerophosphate in the presence of rotenone. Under these conditions, Ca^{2+} is actively accumulated. Succinate supported Ca^{2+} uptake equally well in the presence of rotenone. When rotenone was omitted from the incubation medium, neither α-glycerophosphate nor succinate supported significant accumulation of Ca^{2+}. Active Ca^{2+} accumulation also occurred when Mg^{2+} was substituted for rotenone. Lower right: Mn^{2+} uptake during oxidation of pyruvate–malate. Mn^{2+} was actively accumulated without the requirement for added Mg^{2+} or rotenone. Sr^{2+} also was transported under these same conditions by lung mitochondria.

linked substrates (Figure 9, lower center). Release of the accumulated Ca^{2+} occurred after addition of uncouplers of oxidative phosphorylation as is seen with other mitochondrial preparations (Scarpa, 1972). In the absence of Mg^{2+} or rotenone, only small amounts of Ca^{2+} were transported during substrate oxidation and this transported Ca^{2+} subsequently leaked out of the mitochondria (Figure 9, lower left). Lung mitochondria were able to accumulate Mn^{2+} (Figure 9, lower right) as well as Sr^{2+} from the medium in the presence of pyruvate and other substrates without the requirement for Mg^{2+} or rotenone.

We were not able to adequately explain the requirement for Mg^{2+} or rotenone to support accumulation of Ca^{2+} by lung mitochondria since similar requirements were not found for rat liver and heart mitochondria (Scarpa, 1972). One interpretation of the results is that the addition of Ca^{2+} to respiring mitochondria in the absence of Mg^{2+} or rotenone caused rapid dissipation of energy required for active Ca^{2+} accumulation. This hypothesis was supported by measurements which showed that the addition of Ca^{2+} to respiring mitochondria resulted in marked decrease in ATP levels, an effect partially prevented by the addition of rotenone (Figure 10).

Discussion

The isolation of lung mitochondria posed difficulties in preparation due to the collagenous nature of lung tissue and the high tissue content of lipids and lysozomal enzymes. Despite these difficulties, we were able to isolate a mitochondria-rich fraction with reproducible rates of substrate oxidation and respiratory control ratios. The mitochondrial content of cytochromes and nucleotides was characteristic of mitochondria in general, although actual levels were somewhat lower when expressed on a protein basis than values obtained with mitochondria isolated from organs such as rat liver and heart (Williams, 1968; Klingenberg *et al.*, 1959). The cytochrome content of our lung mitochondrial preparation was three to four times as high as observed by Matsubara and Tochino (1971) using rabbit lung mitochondria. This difference between cytochrome content of the preparations may reflect in part differences in contamination with nonmitochondrial protein since the succinate oxidase activity of our preparation was also considerably higher. The substrate oxidation rates measured in the present study were of the same order of magnitude as results obtained by other investigators with lung mitochondrial preparations from rabbits (Reiss, 1966), guinea pigs (Kyle and Riesen, 1970), and rats (Sayeed and Baue, 1971). But all investigators have found that a high concentration of EDTA in the isolation medium is necessary for the isolation of lung mitochondria with maximal respiratory rates. The present study has shown that in the absence of metal chelating agents, large quantities of Ca^{2+} are bound by the lung mitochondrial preparation resulting in slow respiration rates with NAD-linked substrates. On the

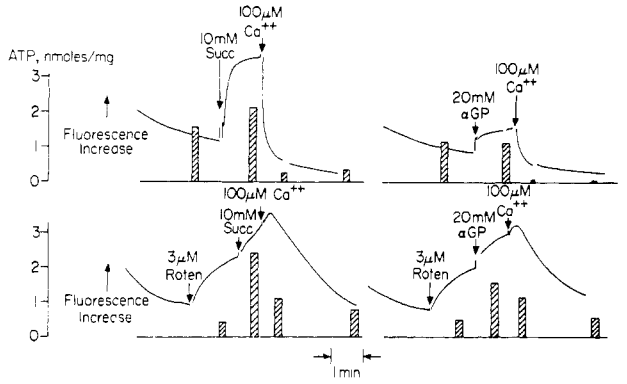

FIGURE 10: Effect of substrate and Ca^{2+} on ATP content of rat lung mitochondria. The solid line is the fluorescence trace indicating changes of pyridine nucleotide redox state as in Figure 7. Samples for ATP analysis were taken at the times indicated by placement of the vertical bars. The height of the bars indicates the ATP content of the mitochondria. Note the increased ATP level after addition of succinate (upper left panel). The ATP level increased with both succinate and α-glycerophosphate as substrates when rotenone was present in the incubation cuvet (lower panels). Within 1 min after addition of Ca^{2+}, there was oxidation of pyridine nucleotides and marked decrease in ATP concentration. In the presence of rotenone, pyridine nucleotide oxidation and ATP disappearance were less rapid.

other hand, the high mitochondrial Ca^{2+} content stimulated oxidation of α-glycerophosphate. These effects of Ca^{2+} on respiration are mediated through several different mechanisms. Results using the artificial electron carrier system indicate that stimulation of α-glycerophosphate oxidation by Ca^{2+} was due to activation of mitochondrial glycerophosphate dehydrogenase. While this effect of Ca^{2+} on α-glycerophosphate oxidation has not been reported with mitochondria isolated from other vertebrate organs, blowfly flight muscle mitochondria have a similar Ca^{2+} requirement (Hansford and Chappell, 1967).

The mechanism of Ca^{2+}-induced depression of NAD-linked substrate oxidation is not as readily apparent but depletion of intramitochondrial pyridine nucleotides appears to be a major factor. Our studies demonstrated disappearance of free pyridine nucleotides from the whole preparation (mitochondria plus suspending medium) after addition of Ca^{2+} to the lung mitochondria. A possible explanation for disappearance of NAD after addition of Ca^{2+} is that Ca^{2+}, NAD, and structural protein form a complex that is not detected by the analytical techniques for NAD assay. Alternatively, the pyridine nucleotides may have been degraded after Ca^{2+} addition by NADase. Since Ca^{2+} did not stimulate NADase activity, a direct effect of Ca^{2+} to promote hydrolysis of NAD is unlikely. In addition, the hydrolysis of exogeneously added NAD by the mitochondrial preparation suggests that the nucelosidase is external to the mitochondrial matrix and, therefore, would not be expected to hydrolyze the intramitochondrial NAD. A more likely explanation for the loss of NAD^+ can be postulated based on the known effect of Ca^{2+} to increase NAD permeability of liver mitochondria (Vinodrogradov *et al.*, 1972; Kaufman and Kaplan, 1960; Hunter and Ford, 1955). If lung mitochondria behave similarly, addition of Ca^{2+} may cause leakage of pyridine nucleotides from the mitochondria and lead to subsequent hydrolysis of extramitochondrial NAD by NAD-nucleosidase. But inhibition of NADase activity by nicotinamide did not prevent Ca^{2+} depression of respiration suggesting that leakage or binding of nucleotides rather than hydrolysis is the major factor in the respiratory effects of Ca^{2+}. Kaufman and Kaplan (1960) reached similar conclusions after study of liver mitochondria, although Ca^{2+} concentrations required for the effects with the latter preparation were approximately tenfold greater than required with lung mitochondria. But regardless of the mechanism, the relatively high concentration of Ca^{2+} required to induce effects on NAD-linked substrate oxidation suggests that this mechanism may not be important *in vivo*.

On the other hand, the concentration of Ca^{2+} required to activate the lung mitochondrial glycerophosphate dehydrogenase was in the range of 10^{-9}–10^{-7} M free Ca^{2+}, and Ca^{2+} activation of this enzyme may be physiologically important. As a possible physiologic role, Ca^{2+} may affect the rate of transfer of reducing equivalents from cytosolic to mitochondrial spaces *via* the α-glycerophosphate cycle (Borst, 1963). However, this cycle has not yet been demonstrated in lung cells and the low level of cytoplasmic glycerophosphate dehydrogenase (Lee and Lardy, 1965) suggests that the shuttle may not be normally operative. Alternatively, Ca^{2+} and α-glycerophosphate may be involved in phospholipid synthesis. α-Glycerophosphate is thought to be a key intermediate for the synthesis of dipalmitoyllecithin, a major component of the surface active alveolar lining layer (Lands, 1957; Felts, 1965) and intracellular Ca^{2+}, by determining α-GP availability, may affect the rate of lecithin synthesis by lung cells. However, the precise role of α-glycerophosphate and Ca^{2+} in the lung will require further studies.

Acknowledgments

The technical assistance provided by S. Mellen is greatly appreciated. M. Lagimoniere and J. D'Angelo, Philadelphia Veterans Administration Hospital, kindly provided technical support for electron microscopy and Dr. P. Skerrett loaned electron microscopy facilities.

References

Bertalanffy, F. D. (1964), *Int. Rev. Cytol. 16*, 233.
Borst, P. (1963), *in* Funktionelle und Morphologische Organisation Der Zelle, Karlson, P., Ed., Berlin, Springer-Verlag, p 137.
Carafoli, E., and Lehninger, A. L. (1971), *Biochem. J. 122*, 681.
Chance, B., and Schoener, B. (1966), *J. Biol. Chem. 241*, 4567.
Chance, B., and Williams, G. R. (1956), *Advan. Enzymol. 17*, 65.
Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.
Felts, J. M. (1965), *Med. Thorac. 22*, 89.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Hansford, R. G., and Chappell, J. B. (1967), *Biochem. Biophys. Res. Commun. 27*, 686.
Heinemann, H. O., and Fishman, A. P. (1969), *Physiol. Rev. 49*, 1.
Hunter, F. E., Jr., and Ford, L. (1955), *J. Biol. Chem. 216*, 357.
Kaufman, B. T., and Kaplan, N. O. (1960), *Biochim. Biophys. Acta 39*, 332.
Klingenberg, M., Slenczka, W., and Ritt, E. (1959), *Biochem. Z. 332*, 47.
Kyle, J. L., and Riesen, W. H. (1970), *Arch. Environ. Health 21*, 492.
Lands, W. E. M. (1957), *J. Biol. Chem. 231*, 883.
Lee, Y. P., and Lardy, H. A. (1965), *J. Biol. Chem. 240*, 1427.
Matsubara, T., and Tochino, Y. (1971), *J. Biochem. (Tokyo)*

70, 981.
Portzehl, H., Caldwell, P. C., and Rüegg, J. C. (1964), *Biochim. Biophys. Acta 79*, 581.
Reiss, O. K. (1965), *Med. Thorac. 22*, 100.
Reiss, O. K. (1966), *J. Cell Biol. 30*, 45.
Sayeed, M. M., and Baue, A. E. (1971), *Amer. J. Physiol. 220*, 1275.
Scarpa, A. (1972), *Methods Enzymol. 24*, 343.
Scarpa, A., and Azzi, A. (1968), *Biochim. Biophys. Acta 150*, 473.
Van Gelder, B. F. (1966), *Biochim. Biophys. Acta 118*, 36.
Vinodrogradov, A., Scarpa, A., and Chance, B. (1972), *Arch. Biochim. Biophys. 152*, 646.
Weber, K. C., and Visscher, M. B. (1969), *Amer. J. Physiol. 217*, 1044.
Williams, J. N., Jr. (1968), *Biochim. Biophys. Acta 162*, 175.
Williamson, J. R., and Corkey, B. E. (1969), *Methods Enzymol. 13*, 434.
Wilson, D. F., and Epel, D. (1968), *Arch. Biochim. Biophys. 126*, 83.
Yuan, J. H., and Anderson, B. M. (1971), *J. Biol. Chem. 246*, 2111.

Effect of Phenobarbital and Naphthalene on Some of the Components of the Electron Transport System and the Hydroxylating Activity of House Fly Microsomes†

Jorge Capdevila,‡ Antonio Morello,§ Albert S. Perry,¶ and Moises Agosin*,||

ABSTRACT: (1) A house fly microsomal enzyme system that hydroxylates several type I substrates is inducible by naphthalene and phenobarbital administered in the food. Both inducers increase the levels of cytochrome P-450, NADPH–cytochrome *c* reductase, and cytochrome b_5. (2) Untreated house flies show a peak absorbance at 450 nm in the CO-differential spectrum, whereas the naphthalene- or phenobarbital-treated insects show a maximum absorbance at 448 nm. (3) Cessation of phenobarbital or naphthalene treatment is promptly followed by a progressive reduction in the amounts of cytochromes P-450 and b_5 and in NADPH–cytochrome *c* reductase. (4) During the induction period, the relationship between the level of cytochrome P-450 and the hydroxylating activity of microsomal preparations depends on the substrate hydroxylated and the inducer employed. Good correlation is obtained with naphthalene hydroxylation and aminopyrine N-demethylation, but not with 2-isopropoxyphenyl-*N*-methylcarbamate (Baygon) hydroxylation because of competition with the substrate for cytochrome P-450 binding site I. This effect is not evident with naphthalene because the latter apparently is metabolized at a faster rate than phenobarbital, thus making the cytochrome P-450 binding site I available for other substrates. (5) The CO and ethyl isocyanide differential spectra, as well as the metabolic activities of induced cytochrome P-450, suggest that contrary to what happens in mammals, both phenobarbital and naphthalene stimulate the synthesis of a new species of hemoprotein, cytochrome P-448.

A moderate to substantial increase in the activity of insect microsomal mixed function oxidases can be produced by the administration of several insecticides as well as by phenobarbital and 3-methylcholanthrene (Agosin *et al.*, 1969; Gil *et al.*, 1968; Morello, 1964; Perry *et al.*, 1971; Plapp and Casida, 1970; Walker and Terriere, 1970). The increase in enzyme activity is preceded by an increase in the synthesis of messenger-like RNA (Balazs and Agosin, 1968; Litvak and Agosin, 1968) and of DNA-dependent RNA polymerase levels (Agosin, 1971). These effects can be prevented by the administration of RNA and protein synthesis inhibitors such as actinomycin D (Balazs and Agosin, 1968) and cycloheximide (Walker and Terriere, 1970). These observations indicate that house fly treatment with the above chemicals results in an increase in the quantity of microsomal enzymes. The latter, as in the case of mammalian microsomes (Hayaishi, 1969), correspond to a NADPH[1]-linked enzyme system which has as its terminal oxidase the CO-binding hemoprotein P-450 (Ray, 1967; Perry, 1970; Morello *et al.*, 1971). The administration of dieldrin (Matthews and Casida, 1970) or phenobarbital (Perry *et al.*, 1971) increases the levels of cytochrome P-450 in house flies, but a corresponding increase in microsomal activity is not always proportional. This may probably be due to the fact that the chemically induced changes in the kinetics of microsomal enzymes are complex functions of the inducing agent used and the substrate investigated (Gram *et al.*, 1968). Another possibility which might explain the lack of correlation between levels of cytochrome P-450 and hydroxylating activity may be the existence of more than one microsomal enzyme system involving cytochrome P-450

† From the Department of Zoology, University of Georgia, Athens, Georgia 30602, and the Center for Disease Control, U. S. Public Health Service, Savannah, Georgia 31402. *Received September 20, 1972.*

‡ University of Georgia; in partial fulfillment of the University of Georgia Graduate School requirements for a Ph.D. degree.

§ University of Georgia; present address: Department of Chemistry and Biochemistry University of Chile School of Medicine, Santiago, Chile.

¶ Center for Disease Control, U. S. Public Health Service.

|| Supported in part by U. S. Public Health Service Research Grant No. AI-09902-02.

[1] Abbreviations used are: NAD^+ and NADPH, oxidized and reduced nicotinamide adenine dinucleotides, respectively.