The Activation of Adrenal Cortex Mitochondrial Malic Enzyme by Ca^{2+} and Mg^{2+} †

D. R. Pfeiffer‡ and T. T. Tchen*

ABSTRACT: Adrenal cortex mitochondria prepared by a standard method do not exhibit malic enzyme activity. Addition of physiological concentrations of Ca^{2+} and Mg^{2+} enables these mitochondria to reduce added $NADP^+$ by malate to form free NADPH. Half-maximum activation of the mitochondrial malic enzyme requires 0.3 mM Ca^{2+} and 1 mM Mg^{2+}. Solubilized mitochondrial malic enzyme is independent of Ca^{2+} and has a K_M of 0.2 mM for Mg^{2+}. The Ca^{2+} effect is dependent on an initial period of active Ca^{2+} uptake which also causes other changes in respiratory properties similar to those observed with mitochondria from other tissues. After Ca^{2+} accumulation has taken place, free Ca^{2+}, but not additional accumulation, is still required for malic enzyme activity. The requirement for Mg^{2+} can be met by Mn^{2+} (1 mM). This concentration of Mn^{2+} alone yielded only a slight activation of mitochondrial malic enzyme while higher concentrations of Mn^{2+} alone gave good activation of the mitochondrial malic enzyme. The NADPH generated by the Ca^{2+}-Mg^{2+} activated malic enzyme effectively supports the 11β-hydroxylation of deoxycorticosterone, whereas in the presence of malate, or malate plus Mg^{2+} but absence of Ca^{2+}, the energy linked transhydrogenase supplies all the required NADPH. The activated malic enzyme appears to be more efficient than transhydrogenase in generating NADPH to support 11β-hydroxylation. Cyanide and azide have been found to inhibit solubilized mitochondrial malic enzyme.

Bovine adrenal cortex mitochondria contain an $NADP^+$ dependent malic enzyme (Kimura, 1966; Simpson *et al.*, 1968, Simpson and Estabrook, 1968; Pfeiffer *et al.*, 1972), the function of which is not known. In previous communications (Pfeiffer, 1973; Pfeiffer and Tchen, 1973), we reported that these mitochondria will reduce extramitochondrial $NADP^+$ by intramitochondrial malic enzyme if exposed to physiological levels of Ca^{2+} and Mg^{2+} under conditions where energy dependent Ca^{2+} accumulation may occur. We now present more fully this effect of Ca^{2+} and other ions on the activity of malic enzyme and demonstrate the ability of this system to support 11β-hydroxylation of deoxycorticosterone. Some respiratory properties of these mitochondria under conditions which lead to malic enzyme activation are also presented. The possible physiological significance of Ca^{2+} and its effects on adrenal cortex mitochondria in the control of steroidogenesis are discussed.

Experimental Procedure

Bovine adrenal cortex mitochondria were prepared as described previously (Pfeiffer *et al.*, 1972; Pfeiffer and Tchen, 1973). Solubilized malic enzyme was obtained by sonicating the mitochondria (Pfeiffer *et al.*, 1972) and assaying either the whole sonicate or the supernatant obtained by centrifuging at 109,000*g* for 1 hr, 0°. The supernatant contained approximately 80% of the malic enzyme activity of the whole sonicate.

Malic enzyme was assayed by monitoring OD_{340} or by the fluorescence method. The respiratory studies (including 11β-hydroxylation of deoxycorticosterone) were performed on a Gilson Oxygraph with Clark type electrode. All data were obtained at 25°. The standard medium for determining malic enzyme activity of "intact mitochondria" contained in a volume of 3.0 ml, 0.32 M sucrose, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and an appropriate amount of mitochondrial protein. Solubilized malic enzyme was assayed under the same conditions except that calcium was normally omitted. Other conditions used are detailed in the appropriate figure legends.

$NADP^+$, NAD^+, NADH, ATP (all sodium salts), deoxycorticosterone, glutathione reductase, oxidized glutathione, Tris, bovine serum albumin (low fatty acid type), EGTA,[1] ruthenium red, antimycin A, and oligomycin were purchased from Sigma. All other reagents and substrates were commercially available reagent grade.

All sucrose solutions used in these studies were prepared from a solution which had been treated with a mixed bed ion exchange resin as described by Allmann *et al.* (1970a).

Antimycin A, oligomycin, and deoxycorticosterone were utilized as ethanolic solutions.

Protein was determined by the biuret reaction in the presence of deoxycholate (1% final concentration).

Results

Intact mitochondria[2] incapable of reducing added $NADP^+$ by malate can be induced to do so by the addition of Mg^{2+} and Ca^{2+}. Figure 1 shows that for this activity, Mg^{2+}, Ca^{2+}, malate, and $NADP^+$ are all required. $NADP^+$ reduction began immediately following the addition of the last component (curves B–D) except in the case of Ca^{2+} (curve A). In this latter case there is a lag period, the length of which varied between 2 and 10 min with dif-

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received February 15, 1974.* Taken from a dissertation submitted by D.R.P. to Wayne State University in partial fulfillment of the Ph.D. degree. This work was supported by U. S. Public Health Service Grants AM 05384 and AM 13724.

‡ Current address: The Enzyme Institute, University of Wisconsin, Madison, Wisconsin 53706.

[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

[2] Unless otherwise specified, mitochondria refers to bovine adrenal cortex mitochondria, obtained predominantly from the fasciculata portion of the gland.

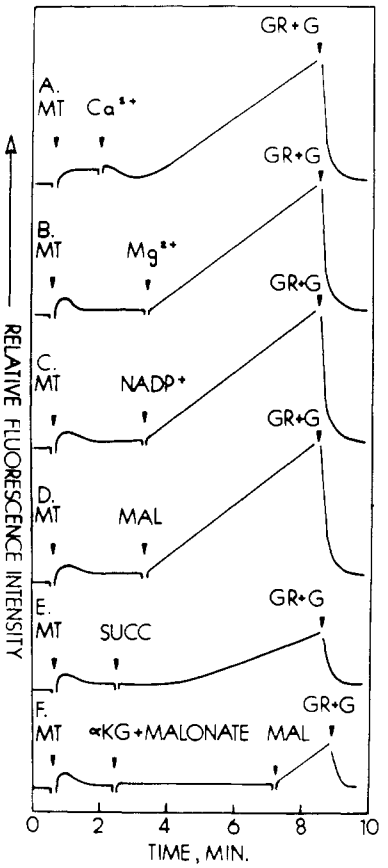

FIGURE 1: The requirements for the reduction of extramitochondrial $NADP^+$ by intramitochondrial malic enzyme. NADPH formation was monitored fluorometrically. In curves A–D, the complete reaction mixture was the standard mixture described in Experimental Procedure with 0.2 mg of mitochondrial protein/ml. Reaction mixtures containing all but one component (Ca^{2+}, Mg^{2+}, $NADP^+$, or malate) were incubated until the fluorescence became constant. Malic enzyme reaction was then initiated by addition of the missing component as indicated. Malate was omitted for curve E and reduction initiated by the addition of 7 mM sodium succinate. In curve F, α-ketoglutarate (αKG) (7 mM) and malonate (5 mM) were substituted for malate and failed to support reduction. The further addition of malate produced the normal rate of reduction. NADPH was identified by reoxidation with added glutathione reductase (G.R.) (activity 1 μmol/min) and oxidized glutathione (G) (1 μmol). These results were obtained not only with freshly prepared mitochondria, but also with mitochondria which had been frozen and thawed. For the subsequent experiments, however, only freshly prepared mitochondria were used. In these and later experiments, the concentrations of malate and NADP used were 7 and 1.6 mM, respectively. In this and later figures, MT represents mitochondria.

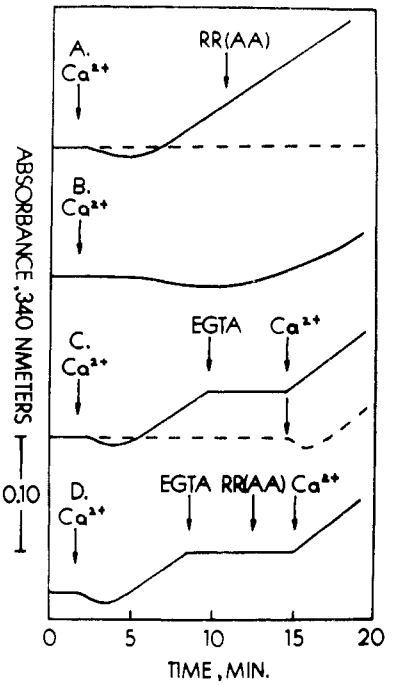

FIGURE 2: The effects of ruthenium red (RR), antimycin A (AA), and EGTA on the Ca^{2+} dependent "unveiling" of malic enzyme activity. All assays were performed in the standard medium with a total of 0.3 mg of mitochondrial protein. The original additions of Ca^{2+} were 0.5 mM. The second additions of Ca^{2+} were twice the amount of the first addition. (A) RR (10 nmol) or AA (5 μg) was added either before Ca^{2+} (- - -) or after Ca^{2+} (· · ·). (B) 0.50 nmol of RR was added before Ca^{2+}. (C) 0.6 mM EGTA was added before Ca^{2+} (- - -) or after Ca^{2+} (—). The addition of a second aliquot of Ca^{2+} as indicated overcame the EGTA inhibition. (D) This experiment is similar to (C) except for the addition of 10 nmol of RR or 5 μg of AA as shown.

ferent mitochondrial preparations. Substitution of succinate for malate (curve E) led to a longer lag period, probably reflecting the formation of malate from succinate. α-Ketoglutarate, in the presence of malonate to inhibit succinate dehydrogenase, cannot reduce $NADP^+$ (curve F). A subsequent addition of malate led to $NADP^+$ reduction at the normal rate. In all cases, the addition of glutathione reductase and oxidized glutathione rapidly reoxidized the NADPH formed. These results show that the observed increase in fluorescence is due to $NADP^+$ linked malic enzyme activity and not the result of combined NAD^+-linked dehydrogenase and a NADH–$NADP^+$ transhydrogenase.

To explore the mechanism of action of Ca^{2+}, we studied the effects of ruthenium red (an inhibitor of energy-dependent Ca^{2+} transport by mitochondria) (Moore, 1971, Vasington *et al.*, 1972), antimycin A (a commonly used inhibitor of electron transport and thereby energy production), and EGTA (a chelator specific for Ca^{2+} in the presence of Mg^{2+}). The results are shown in Figure 2. Curve A shows that when ruthenium red or antimycin A was added at sufficiently high concentration before the addition of Ca^{2+}, little or no malic enzyme activity was obtained. However, if these inhibitors were added after Ca^{2+} and the appearance of malic enzyme activity no inhibition of $NADP^+$ reduction was observed. When lower levels of ruthenium red were added before Ca^{2+} (curve B), the reduction of $NADP^+$ showed a lag period followed by gradual increase in rate. EGTA, in molar excess of Ca^{2+}, inhibits the Ca^{2+} induced malic enzyme activity whether added before or after Ca^{2+}. This inhibition can be overcome by addition of excess Ca^{2+} (curve C). Finally, curve D shows that when ruthenium red or antimycin A was added after initial Ca^{2+} activation and EGTA inhibition of malic enzyme, the enzyme could be reactivated by the addition of excess Ca^{2+}.

Since the results shown in Figure 2 imply the involvement of an energy dependent Ca^{2+} pump, the respiratory properties of these mitochondria in the presence or absence of Ca^{2+} and various inhibitors were examined. Most of the results are in agreement with well-known effects of Ca^{2+} on liver mitochondria, or previous reports on adrenal cortex mitochondria (Cammer and Estabrook, 1967; Harding *et al.*, 1968) and are summarized as follows. (1) In the presence of phosphate, these mitochondria show respiratory control for succinate and malate oxidation upon the addition of ADP. The control ratio and P/O ratio determined by oxygen consumption showed some variability between preparations. Control ratios of 3–4 were commonly ob-

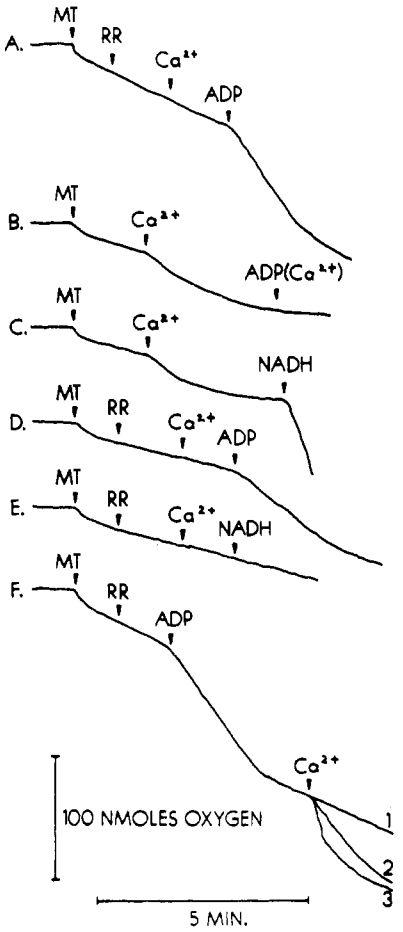

FIGURE 3: Some effects of Ca^{2+} on the respiratory properties of bovine adrenal cortex mitochondria. All assays were performed using 1.0 mg of mitochondrial protein in a final volume of 1.6 ml. The media contained sucrose, Tris, Mg^{2+} at the levels employed for malic enzyme assay, and 5 mM sodium phosphate (pH 7.4). The substrate was 7 mM succinate for curves A and F and 7 mM malate for curves B–E. Further additions as indicated were 0.30 μmol of ADP, 1.6 μmol of NADH, 1.6 μmol of Ca^{2+}, and 10 nmol of ruthenium red except in curve F where Ca^{2+} was added at 0.40 μmol. In this curve, Ruthenium Red was present at 10 nmol (line 1), 0.40 nmol (line 2), or absent (line 3).

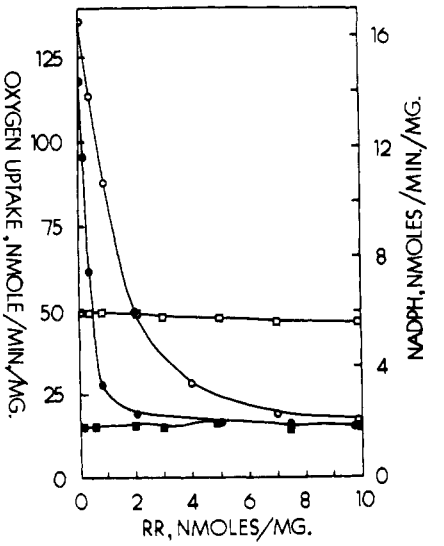

FIGURE 4: Comparison of the effect of ruthenium red (RR) on some respiratory properties and on the Ca^{2+} stimulated malic enzyme activity of bovine adrenal cortex mitochondria. The experiments were performed as described in the legend to Figure 3, curve F, using several concentrations of RR. The rates of state 4 (■), state 3 (□), and the Ca^{2+} stimulated (●) respiration were obtained from the appropriate portions of the curves. The malic enzyme data (○) were obtained from experiments analogous to Figure 2, curve B where the NADPH accumulated during the first 12 min after Ca^{2+} addition was used to determine the specific activity.

served and P/O ratios as high as 2 and 2.9, for succinate and malate, respectively, were seen with many preparations. (2) When oxidizing succinate, an accumulation of limited amounts of Ca^{2+} (approximately 50 nmol of Ca^{2+}/mg of protein) with concomitant increase in oxygen consumption at a ratio of $Ca^{2+}/O = 4$ was obtained in the absence of phosphate. Higher levels of Ca^{2+} (1 μmol/mg of protein) in the presence of phosphate produced a continuing elevated rate of oxidation, unless ATP or oligomycin was present. In these cases, a state 4 rate was regained and Ca^{2+}/O ratio of approximately 4 was again observed.[3] (3) With malate as substrate, the respiratory responses to small amounts of Ca^{2+} were similar to those observed with succinate except that Ca^{2+}/O increased to nearly 6 in the presence of ATP and phosphate. In the absence of ATP, higher levels of Ca^{2+} (~1 μmol/mg of protein) produced an initial stimulation followed by inhibition of respiration. This inhibition could not be overcome by further addition of Ca^{2+} or ADP. However, addition of NADH led to a very high rate of oxygen consumption. (4) The rapid oxidation of either succinate or NADH produced by high Ca^{2+} and phosphate returned to an apparent state 4 rate by the addition of EGTA in slight excess of Ca^{2+}. Such mitochondria did not respond well to the addition of ADP but still responded to a second aliquot of Ca^{2+} in excess of EGTA, returning to the initial Ca^{2+} stimulated rate of oxygen consumption.

In Figure 3, curves A–E show that ruthenium red at 10 nmol/mg of protein prevented the changes caused by high levels of Ca^{2+}, namely, rapid oxidation, impaired ATP synthesis with succinate or malate as substrate, and the ability to oxidize added NADH at high rate. Lower concentrations of ruthenium red gave partial inhibition of these Ca^{2+} effects.

In order to compare and correlate the effects of ruthenium red on Ca^{2+}-induced malic enzyme activity and on energy dependent Ca^{2+} uptake, respiratory experiments were conducted at different concentrations of ruthenium red. This is illustrated in Curve F of Figure 3. The complete results of several similar experiments on the effects of ruthenium red are shown in Figure 4. This figure shows that ruthenium red at these levels had no effect on the rates of state 3 and state 4 respiration but strongly inhibited both Ca^{2+}-induced malic enzymes and Ca^{2+}-stimulated succinate-supported respiration. Slightly higher levels of ruthenium red were required to inhibit malic enzyme to a degree comparable to the inhibition of Ca^{2+}-stimulated respiration. However, since the malic enzyme activity in the presence of low concentrations of ruthenium red increased with time (Figure 2, curve B) and since the rates plotted here are not initial rates but the amounts of NADPH formed in 12 min, the actual inhibitory effect of ruthenium red on Ca^{2+} induction of malic enzyme is probably more pronounced and may be comparable to the inhibition of oxygen consumption.

Some factors which affect the specific activity of malic

[3] For the calculation of Ca^{2+}/O ratios, it was assumed that under these conditions where the rate of respiration returned to the initial rate, the added Ca^{2+} was completely transported into and retained by the mitochondria.

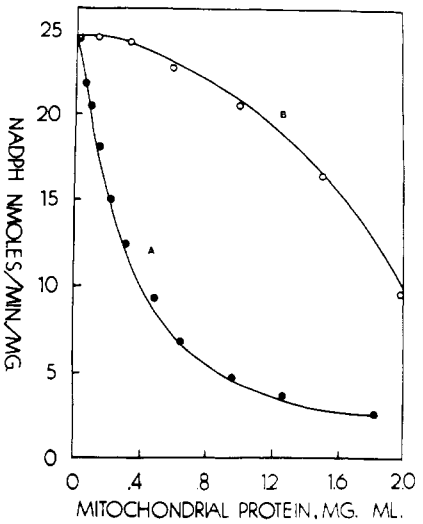

FIGURE 5: The effect of protein concentration on malic enzyme activity for "intact" and sonicated mitochondria. Malic enzyme activity was determined in the standard assay medium with reaction initiated by the addition of Ca^{2+} for intact mitochondria (●) or by $NADP^+$ for the solubilized enzyme (○). The protein concentration axis for the solubilized enzyme (109,000*g*, 1-hr supernatant) refers to the concentration of mitochondria from which the supernatant was obtained rather than the actual protein concentration of the supernatant fraction. NADPH formation was monitored by increase in OD_{340}.

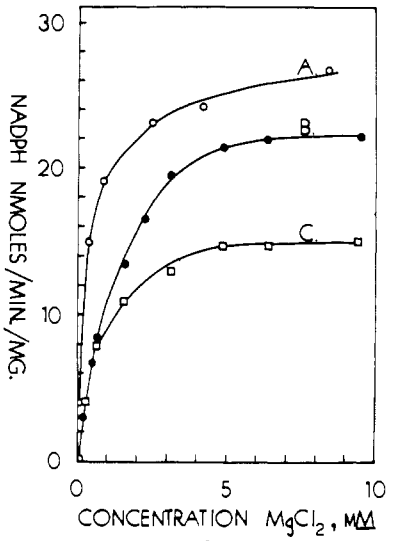

FIGURE 7: The effect of varying Mg^{2+} concentration on the activity of malic enzyme of "intact" or sonicated mitochondria. The media contained sucrose, Tris, and malate at the standard levels plus various concentrations of Mg^{2+} as shown. (A) Solubilized malic enzyme (supernatant equivalent to 0.21 mg of mitochondrial protein/ml fraction) with (1 mM) or without Ca^{2+}. (B and C) Intact mitochondria (0.10 mg/ml) with 1 mM and 0.3 mM Ca^{2+}, respectively. Reaction was initiated by the addition of Mg^{2+} for curve A or Ca^{2+} for curves B and C.

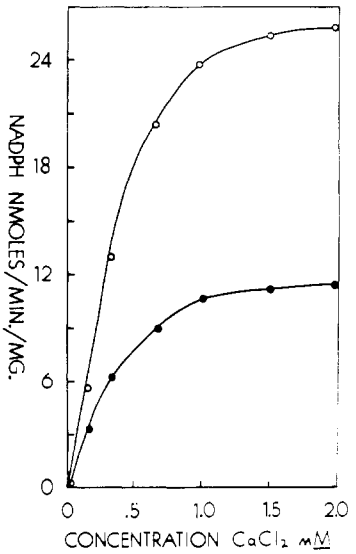

FIGURE 6: The effect of varying Ca^{2+} concentration on malic enzyme activity of "intact" mitochondria. Malic enzyme assays were conducted in the standard medium using intact mitochondria at 0.12 mg/ml (○) and 0.36 mg/ml (●) as enzyme source. Reaction was initiated by the addition of Ca^{2+} and NADPH formation monitored by increase in OD_{340}.

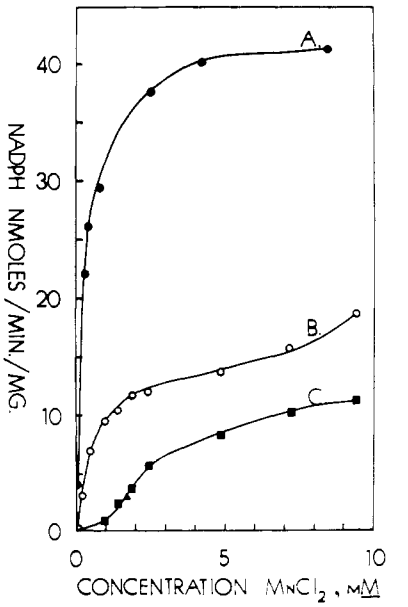

FIGURE 8: The effect of Mn^{2+} concentration on the activity of malic enzyme from intact or sonicated mitochondria. The media contained sucrose, Tris, malate, and $NADP^+$ at the standard levels. Various concentrations of Mn^{2+} were substituted for Mg^{2+} as shown. Curve A utilized solubilized malic enzyme equivalent to 0.21 mg of mitochondrial protein/ml. Curves B and C were obtained with "intact" mitochondria at 0.36 mg of protein/ml in the presence (B) or absence (C) of 1 mM Ca^{2+}. The triangle in curve C contained 5 mM Mg^{2+} in addition to Mn^{2+}. Reduction was initiated by the addition of Mn^{2+} and monitored by increase in OD_{340}. The relatively high protein concentration used in curve B is most likely responsible for the somewhat low specific activity when compared to Ca^{2+} and Mg^{2+} (Figure 7, curve B).

enzyme in intact mitochondria or after solubilization by sonication are presented in Figures 5–8. Figure 5 demonstrates that at constant Ca^{2+} and Mg^{2+} concentrations, the specific activity of malic enzyme is strongly dependent on protein concentration (curve A). The effect is much more pronounced with intact mitochondria than when the high speed supernatant from a sonicate was used as enzyme source (curve B). Figure 6 demonstrates that the observed inhibition with higher concentrations of intact mitochondria is not due to limiting Ca^{2+}. The apparent K_M for Ca^{2+} is not significantly different at low or high concentrations of mitochondria but the maximal specific activity obtainable is clearly decreased at high concentration of mitochondria. It is also important to note that at 1 mM Ca^{2+}, the *in vivo* Ca^{2+} concentration in bovine adrenals as reported by Allmann *et al.* (1970b), malic enzyme activity in the mitochondrial system has reached a rate nearly as high as sonicated mitochondria. With the solubilized enzyme, the pres-

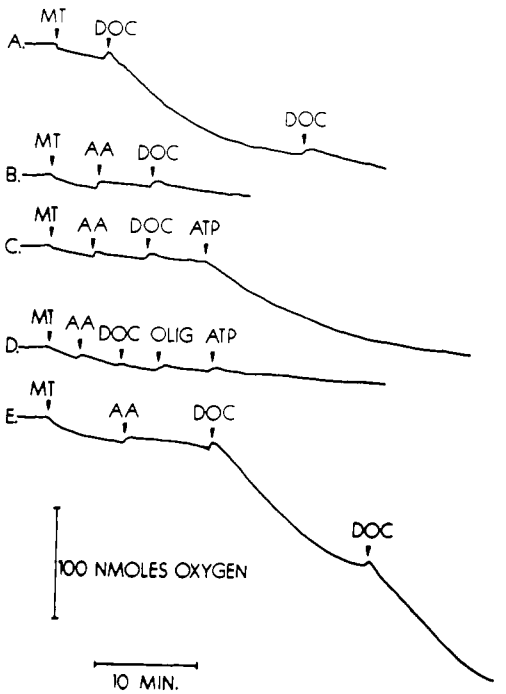

FIGURE 9: Malate supported 11β-hydroxylation *via* energy-dependent transhydrogenase or malic enzyme generated NADPH. In curves A–D, the media contained sucrose, Tris, and 7 mM malate. For curve E, Mg^{2+}, Ca^{2+}, and $NADP^+$ were present at the standard levels for malic enzyme activation. All assays were performed with 1 mg of mitochondrial protein in a total volume of 1.6 ml. Additions shown were deoxycorticosterone (DOC), 100 nmol, antimycin A (AA), 6 μg, 1 mM ATP, and 6 μg of oligomycin (olig.). The presence of 5 mM $MgCl_2$ for curves A–D did not influence the requirements for hydroxylation.

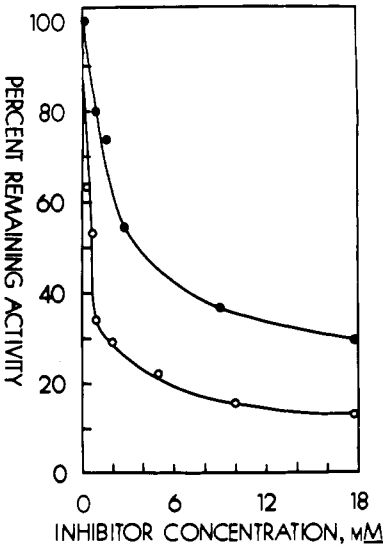

FIGURE 10: The effects of cyanide and azide on the activity of solubilized malic enzyme. Malic enzyme activity was assayed fluorometrically using the entire mitochondrial sonicate (equivalent to 0.50 mg of mitochondria) as the enzyme source. The reaction mixture contained sucrose, Tris, Mg^{2+}, and $NADP^+$ as described in Experimental Procedure, plus sodium cyanide (○) or azide (●) as shown. Reaction was initiated by the addition of $NADP^+$.

ence or absence of Ca^{2+} had no effect on enzyme activity. Figure 7 demonstrates that both the solubilized enzyme (curve A) and the mitochondrial system at low or high protein concentrations (curves B and C, respectively) are dependent on the Mg^{2+} concentration. Comparison of the solubilized and mitochondrial enzyme activities shows a lower K_M with the solubilized enzyme, suggesting that the Mg^{2+} requirement with mitochondria is not simply to serve as a cofactor for the enzyme.

The well-known involvement of Mn^{2+} in malic enzyme and the ability of Mn^{2+} to be actively accumulated by other mitochondria prompted us to investigate its effects on malic enzyme. Initial experiments, analogous to Figure 3, curve F, where Ca^{2+} was replaced by Mn^{2+}, showed that Mn^{2+} is also accumulated by these mitochondria and that this activity is sensitive to ruthenium red (data not shown). Figure 8, curve A, shows that the solubilized enzyme is activated more effectively by Mn^{2+} than by Mg^{2+}. In the case of intact mitochondria in the presence of 1 mM Ca^{2+}, Mn^{2+} can also be substituted for Mg^{2+} effectively. Since Mn^{2+} at a concentration of 1 mM is actively accumulated and can effectively substitute (1) for Ca^{2+} in inducing light scattering changes of mitochondria (Pfeiffer 1973b), (2) for Mg^{2+} with solubilized enzyme, and (3) for Mg^{2+} in activating intact mitochondrial enzyme in the presence of Ca^{2+}, it might be expected that this concentration of Mn^{2+} alone would be sufficient to induce intact mitochondrial malic enzyme activity. Experimentally, however, it was observed that much higher concentration of Mn^{2+} was required (curve C, Figure 8). Also, 1 mM Mn^{2+} together with 5 mM Mg^{2+} gave negligible malic enzyme activity with intact mitochondria (triangle in Figure 8). The activity observed with Mn^{2+} alone showed the expected sensitivity to antimycin A or ruthenium red (data not shown).

To investigate the possible physiological significance of the Ca^{2+} activated reduction of extramitochondrial $NADP^+$ by mitochondrial malic enzyme, the ability of these mitochondria to perform 11β-hydroxylation of deoxycorticosterone was examined. The results are shown in Figure 9. Curve A shows that malate, in the absence of the other components required for malic enzyme activity, supports 11β-hydroxylation. This activity is inhibited by antimycin A (curve B) suggesting that the energy dependent transhydrogenase and not malic enzyme is responsible for generating the required NADPH. Antimycin A was found to have no effect on solubilized malic enzyme. The ability of ATP to overcome antimycin A inhibition (curve C) and the sensitivity of this effect of ATP to oligomycin (curve D) provide further evidence that the transhydrogenase is generating the required NADPH under these conditions. Curve A also shows a gradual decrease in the rate of hydroxylation, less than theoretical oxygen consumption (1 mol of oxygen/mol of deoxycorticosterone), and failure to respond to a second aliquot of deoxycorticosterone. In contrast to the data of curves A–D, curve E shows that when mitochondria were preincubated with all the components required for malic enzyme activity the subsequent addition of antimycin A (analogous to Figure 2 curve A) no longer inhibited 11β-hydroxylation. The deoxycorticosterone to oxygen ratio was 1.01 for two aliquots of deoxycorticosterone. It is, therefore, clear that the Ca^{2+}-activated malic enzyme activity can effectively support 11β-hydroxylation.

Finally, during the course of these studies we have observed that cyanide, and to a lesser extent azide, are rather effective inhibitors of the mitochondrial malic enzyme. Typical inhibition curves for solubilized malic enzyme obtained by sonication are shown in Figure 10.

Discussion

The results presented here demonstrate that Ca^{2+} and Mg^{2+} can unveil the malic enzyme activity of adrenal cor-

tex mitochondria to reduce added $NADP^+$ by malate. With low concentrations of mitochondria and optimal concentrations of Ca^{2+} and Mg^{2+} which are within their physiological range in the adrenal cortex, the specific activity of malic enzyme is comparable to that obtained with sonicated mitochondria. Higher concentrations of intact mitochondria and, to a lesser extent, sonicated mitochondria cause partial inhibition by an unknown mechanism. Results shown in Figure 6 demonstrate that the lower specific activity obtained with higher protein concentrations is not due to an insufficiency in Ca^{2+}. Sauer (1973a,b) has recently reported the existence of multiple malic enzymes in rat adrenal mitochondria. His work and work by Frenkel (1971, 1972) with heart mitochondria malic enzyme have identified a number of mitochondrial activators and inhibitors of this activity. These results, plus preliminary results in our laboratory, suggest that an inhibitor present in these mitochondria is responsible for the effect of high mitochondrial concentration on the specific activity of Ca^{2+} activated malic enzyme. The variation in specific activity from different mitochondrial preparations reported earlier (Pfeiffer and Tchen, 1973) is eliminated when the assays are performed at the same concentration of mitochondrial protein.

The finding that Ca^{2+} and Mg^{2+} can activate mitochondrial malic enzyme raises two major questions: (1) what are the modes of action of Ca^{2+} and Mg^{2+}; (2) does this phenomenon play a physiological role in the control of steroidogenesis?

With regard to the role(s) of the metal ions, it is clear that both Ca^{2+} and Mg^{2+} are required. Since other malic enzymes are known to require Mg^{2+} or Mn^{2+} (Hsu and Lardy, 1967; Mowneb and Elsan, 1972), it seemed possible that the requirement for Mg^{2+} was simply to meet this requirement. However, the experimental results showed that the malic enzyme activity of intact mitochondria requires considerably higher concentrations of Mg^{2+} than the solubilized malic enzyme. This would suggest that Mg^{2+} may serve another unknown function.

In the case of Ca^{2+}, no requirement for this ion was observed with solubilized malic enzyme. Its effects on mitochondria are numerous. Aside from the activation of malic enzyme, Ca^{2+} causes apparent "leakiness" to pyridine nucleotides (as shown by the rapid oxidation of extramitochondrial NADH) and a lowered efficiency of ATP synthesis. All of these effects require active Ca^{2+} accumulation. Reports from Green's laboratory (Allmann *et al.*, 1970b) have demonstrated that ultrastructural transformation from the aggregated to the orthodox form is accompanied by a decrease in 90° light scattering and is probably involved in the other Ca^{2+} induced alterations in the properties of these mitochondria. Work from our laboratory have confirmed and extended these results (Pfeiffer, 1973b). It was found that the time required to complete the Ca^{2+} induced light scattering decrease and ultrastructure changes are very similar to the length of the lag period for malic enzyme activation seen when Ca^{2+} is added last. The initial period of energy dependent Ca^{2+} accumulation in unveiling malic enzyme activity represents a requirement to transform the mitochondria from the aggregated to the orthodox form.

In addition to the above described ultrastructural changes, there is apparently another role for Ca^{2+} in the activation of malic enzyme. This is shown by the experiment in Figure 2D. Malic enzyme was first activated by the addition of Ca^{2+} and then inhibited by the addition of EGTA. Ruthenium red or antimycin A was then added to inhibit any subsequent active Ca^{2+} uptake and a second dose of Ca^{2+} in excess of the amount of EGTA was added. Malic enzyme was reactivated. These results demonstrate that even after the initial ultrastructural changes have been induced (EGTA did not reverse light scattering changes or ultrastructure induced by Ca^{2+} (Pfeiffer, 1973a)) free Ca^{2+} is still required to obtain malic enzyme activity. The reason for this second Ca^{2+} requirement, as well as the requirement for higher concentration of Mg^{2+} by intact mitochondria than by the solubilized enzyme, is unknown. Since these ions bind phospholipids, it is possible that these requirements may involve their binding to membrane phospholipids with alteration of membrane structure or permeability (Scarpa and Azzi, 1968; Papahadjopoulos, 1972).

The dual requirements for Ca^{2+} are further demonstrated by the results presented on the effects of Mn^{2+} in activating malic enzyme. Like Ca^{2+}, Mn^{2+} at 1 mM was actively accumulated by the mitochondria producing a decrease in 90° light scattering and ultrastructural transformation to the orthodox from (Pfeiffer, 1973a). In addition, this concentration of Mn^{2+} effectively replaced Mg^{2+} in both the activation of solubilized malic enzyme, or with mitochondria which had previously been treated with 1 mM Ca^{2+}. If producing the ultrastructural transformation, with any attendant leakiness to pyridine nucleotides, were the only effects of Ca^{2+} relevant to malic enzyme activation, it would be expected that Mn^{2+} alone at 1 mM would produce substantial activation. However, Figure 8 shows that essentially no activity is present at this Mn^{2+} concentration with or without the presence of 5 mM Mg^{2+}. Higher levels of Mn^{2+} (5 mM) did yield good activity indicating that at these concentrations Mn^{2+} can fulfill both roles of Ca^{2+}. However, it should be pointed out that this level of Mn^{2+} is in all probability unphysiological.

The physiological function of the Ca^{2+} effects reported here is not clear. First, it may be questioned whether these reflect properties of mitochondria *in vivo*. Although no definitive answer can be given in the case of adrenal cortex, it has been shown that active accumulation of Ca^{2+} not only occurs *in vitro* by mitochondria from various tissues, but also by mitochondria in intact Ehrlic ascites tumor cells (Cittadini *et al.*, 1973). In the latter case, all the Ca^{2+} accumulation by these cells is due to Ca^{2+} accumulation by mitochondria. The consequence of Ca^{2+} uptake by these mitochondria *in vivo* appeared very similar to effects observed under *in vitro* conditions using mitochondria isolated from these cells.

If one were to assume that the adrenal cortex mitochondria can also actively accumulate Ca^{2+} *in vivo*, one would expect to find activation of the mitochondrial malic enzyme which may play a key role in the regulation of steroidogenesis. In recent years, there has been a great deal of speculation concerning the role of Ca^{2+} in the medium (Birmingham *et al.*, 1953; Peron and Kortiz, 1958). Stimulation by dibutyryl-cAMP is also substantially (~50%) reduced by the omission of Ca^{2+} (Farese, 1967; Sayers *et al.*, 1972). If the effects of Ca^{2+} reported here were to be involved in hormonal stimulation of glucocorticoid synthesis and secretion, one must demonstrate that (1) intracellular free Ca^{2+} is increased after stimulation, causing activation of mitochondrial malic enzyme, and (2) the NADPH produced supports the various sterol oxygenases. The determination of intracellular free (unchelated) Ca^{2+} is extremely difficult and data on this are not available. With respect to the generation of NADPH for sterol oxygenases, the results pre-

sented in this paper show that although intact mitochondria do not utilize malic enzyme to support 11β-hydroxylation in the absence of Ca^{2+}, they can utilize malic enzyme efficiently for this process when Ca^{2+} and Mg^{2+} are present.

The conversion of cholesterol to corticosterone (or cortisol) requires 5 (or 6) equiv of NADPH, 4 of which are used by mitochondrial oxygenases (3 for side-chain cleavage, 1 for 11β-hydroxylation). During hormone-stimulated steroidogenesis, the extra demand for NADPH may come from (1) the mitochondrial transhydrogenase, (2) the mitochondrial malic enzyme and/or isocitric dehydrogenase, or (3) cytosolic sources.

Among the cytosolic sources, the pentose-phosphate shunt activity was reported to be enhanced following hormone stimulation (McKerns, 1964; Weaver and Landau, 1963). More recent work, however, has indicated that this is probably not true (Kowal *et al.*, 1972). Whether changes in the cytosolic malic enzyme activity occur after hormone stimulation is not known. It has, however, been proposed that it may serve as part of a shuttle mechanism for NADPH transport into the mitochondria (Simpson and Estabrook, 1968, 1969). According to this scheme, the cytosolic malic enzyme would generate malate from NADPH and pyruvate, the malate would enter the mitochondria where it is used by the mitochondrial malic enzyme to regenerate NADPH. No net NADPH is generated but cytosolic NADPH generally considered to be not available to mitochondrial oxygenases is thus "transported" into the mitochondria. The mitochondrial permeability barrier is, however not inviolate as the results presented here and earlier *in vitro* reports show that mitochondria in the presence of Ca^{2+} can utilize external NADPH (Horshfield and Kortiz, 1964; Peron *et al.*, 1965). On the whole, available evidence seems to indicate that there is no increase in cytosolic NADPH generating activity after hormonal stimulation, although cytosolic NADPH might become available to mitochondrial oxygenases.

The mitochondrial sources of NADPH, transhydrogenase and malic enzyme, have both been shown to be capable of supporting 11β-hydroxylation *in vitro*. Several lines of reasoning have led us to favor the malic enzyme as the *in vivo* source of additional NADPH required for stimulated steroidogenesis. (1) Under the *in vitro* conditions reported here, malic enzyme supported 11β-hydroxylation (Figure 9). (2) In a previous communication, we have reported that superfused adrenals, whose mitochondria show extensive structural fragmentation, respond well to ACTH stimulation (Kuo and Tchen, 1973). Under *in vitro* conditions, such damaged mitochondria do not normally retain a good capacity for coupled functions such as transhydrogenation, suggesting that with these cells, well-coupled mitochondria are not essential for the stimulation of steroidogenesism. (3) Allmann *et al.* (1970b,c) have previously presented evidence that Ca^{2+} induces transformation of mitochondria from aggregate to orthodox structure and suggested that this may represent a switch from coupled functions (including transhydrogenase) to specialized function. (4) Others have shown that the mitochondrial malic enzyme(s) is an allosteric enzyme and suggested that it may play an important regulatory function (Sauer, 1973a,b; Frenkel, 1972). (5) If the mitochondrial transhydrogenase were the source of NADPH for sterol oxygenases, one should find during stimulated steroidogenesis an increase in tricarboxylic acid cycle activity. Experimental results with an ACTH-sensitive cell line, however, showed that there is no increase in

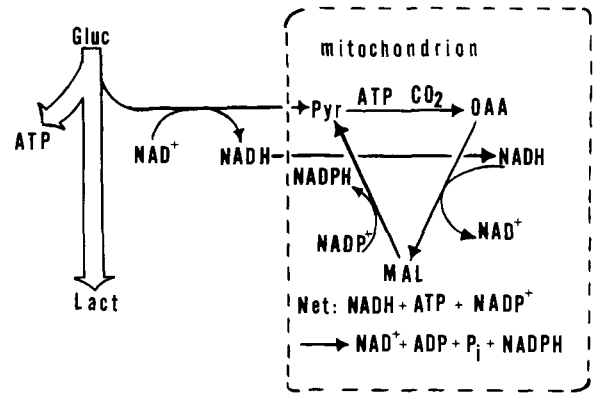

FIGURE 11: Hypothetical scheme of relationship between glycolysis and mitochondrial NADPH generation. The symbols used represent the following: Gluc, glucose; Lact, lactate; PYR, pyruvate; OAA, oxalacetate; and MAL, malate. The heavy arrows indicate that quantitatively most of the glucose is converted to lactate with concomitant ATP generation.

pyruvate oxidation (Kowal *et al.*, 1972). Instead, the rate of glycolysis is greatly stimulated leading to accumulation of lactate, and presumably also some pyruvate. Similar increase in lactate was reported for an *in vivo* system (Bartova *et al.*, 1973). (6) With intact cells, stimulated steroidogenesis is dependent on external supply of glucose, indicating that oxidation of fatty acid does not meet the demand for energy and/or NADPH.

The above mentioned results can be fit into a common scheme as illustrated in Figure 11. During hormonal stimulation, mitochondria are exposed to increased levels of free Ca^{2+} and become less coupled for ATP generation but activated in terms of malic enzyme. Glycolysis is stimulated to generate both ATP and lactate. The increased lactate pool increases potential amounts of NADH and increases the steady-state pyruvate concentration. A pyruvate-oxalacetate-malate cycle as illustrated in the brackets would function as a transhydrogenase to convert NADH to NADPH. Previous workers have shown that pyruvate can indeed support 11β-hydroxylation although the mitochondrial pyruvic carboxykinase activity was rather low (1.6 nmol per min per mg of mitochondrial protein) (Simpson and Boyd, 1971). However, we have found that our mitochondria preparations can carry out pyruvate carboxylation at a rate at least comparable to NADPH formation by malic enzyme (17 nmol per min per mg of mitochondrial protein).

Two other schemes can be readily constructed by minor variations of the scheme shown above. First, it is possible to form oxalacetate from phosphoenolpyruvate instead of from pyruvate. Second, based on the recent report by Sauer (1973a,b) that adrenal cortex mitochondria contain both NAD^+- and $NADP^+$-linked malic enzymes, one can eliminate oxalacetate from the scheme and use the coupling of the two malic enzymes to convert NADH to NADPH.

These schemes provide an explanation for the functions of hormone-stimulated glycolysis, namely, to generate ATP, NADH, and pyruvate. The Ca^{2+} activated malic enzyme plays a key role in the generation of additional NADPH and the Ca^{2+} induced permeability changes of the mitochondria allows the required transfer of small molecules across the mitochondrial membrane. This explains the dependence of steroidogenesis on external supply of glucose. However, it should be stressed that the proposed schemes and concepts are not proven, but only working hypotheses.

They concern *how* extra NADPH may be generated after hormonal stimulation, but do not answer the question of whether this is the cause of enhanced steroidogenesis. They also leave many other observations unanswered. First, there is no apparent link between the schemes discussed and the labile steroidogenic protein which is synthesized after hormonal stimulation and required for enhanced steroidogenesis (see Garren *et al.*, 1971) but whose identity and function(s) are unknown. Second, our results showed that malate supported 11β-hydroxylation in the absence of Ca^{2+} was completely inhibited by antimycin A while earlier reports of similar experiments did not show antimycin A inhibition (Cammer and Estabrook, 1967; Harding *et al.*, 1968). This apparent discrepancy may relate to effects of phosphate which have been reported to influence mitochondrial malic enzyme activity by increasing malate entry (Sauer and Park, 1973). Third, one should also raise the question whether isocitric dehydrogenase is involved in NADPH generation. Fourth, if the mitochondria are indeed "uncoupled," why does this have no effect on the rate of pyruvate oxidation? Finally, the schemes are dependent on an increase in intracellular free Ca^{2+} which remains to be proven.

It is clear from the above discussion that much remains to be done to clarify the source of NADPH for mitochondrial sterol oxygenases, to determine whether it is generated by the mitochondrial transhydrogenase, shuttle mechanisms involving malate, or Ca^{2+} activated malic enzyme.

Finally, the inhibition of solubilized mitochondrial malic enzyme by cyanide and azide is unexpected. This observation may be of some practical importance as cyanide is often used as a specific inhibitor of cytochrome oxidase, in distinction to oxygenases which are much less sensitive to these agents. The inhibition of malic enzyme by cyanide and azide indicates that these agents may indirectly affect oxygenases by reducing NADPH supply under certain conditions.

Acknowledgment

The authors thank Dr. T. Kimura for valuable contributions and Miss Kathleen Trivich for expert technical assistance, during the course of this work.

References

Allmann, D. W., Wakabuyashi, T., Korman, E. F., and Green, D. E. (1970a), *J. Bioenerg. 1,* 73–86.

Allmann, D. W., Munroe, J., Wakabayashi, T., and Green, D. E., (1970b), *J. Bioenerg. 1,* 87–107.

Allmann, D. W., Munroe, J., Wakabayashi, T., and Green, D. W., (1970c), *J. Bioenerg. 1,* 331–353.

Bartova, A., Tibagong, M., Holzbauer, M., and Birmingham, M. K., (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 1513.

Birmingham, M. K., Elliot, F. H., and Valere, P. H. L. (1953), *Endocrinology 53,* 687–689.

Cammer, W., and Estabrook, R. W., (1967), *Arch. Biochem. Biophys. 122,* 721–734.

Cittadini, A., Scarpa, A., and Chance, B. (1973), *Biochim. Biophys. Acta 291,* 246–259.

Frenkel, R. (1972), *Biochem. Biophys. Res. Commun. 47,* 931–937.

Garren, L. D., Gill, G. N., Masui, H., and Walton, G. M. (1971), *Recent Progr. Horm. Res. 27,* 433–487.

Harding, B. W., Bell, J. J., Oldham, S. B., and Wilson, L. D. (1968), *in* Function of the Adrenal Cortex, Vol. II, McKerns, K. W., Ed., New York, N.Y., Appleton, p 831.

Horshfield, I. N., and Kortiz, S. B. (1964), *Biochemistry 3,* 1994–1998.

Hsu, R. Y., and Lardy, H. A. (1967), *J. Biol. Chem. 242,* 520–526.

Kimura, T. (1966), *J. Jap. Biochem. Soc. 38,* 209–223.

Kowal, J., Frenkel, R., and Angee, I. (1972), *Endocrinology 91,* 1219–1226.

Kuo, T., and Tchen, T. T. (1973), *J. Biol. Chem. 248,* 6670.

McKerns, K. W. (1964), *Biochim. Biophys. Acta 90,* 357–371.

Moore, C. L. (1971), *Biochem. Biophys. Res. Commun. 42,* 298–305.

Mowneb, M. S., and Elsan, J. S. (1972), *Endocrinology 91,* 1375–1379.

Papahadjopoulos, D. (1972), *Biochim. Biophys. Acta 265,* 169–186.

Peron, F. G., Guerra, F., and McCarthy, J. L. (1965), *Biochim. Biophys. Acta 110,* 277–289.

Peron, F. G., and Kortiz, S. B. (1958), *J. Biol. Chem. 233,* 256–259.

Pfeiffer, D. R. (1973a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 176.

Pfeiffer, D. R. (1973b), Dissertation, Wayne State University.

Pfeiffer, D. R., Kimura, T., and Tchen, T. T. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22,* 165–168.

Pfeiffer, D. R., and Tchen, T. T. (1973), *Biochem. Biophys. Res. Commun. 50,* 807–813.

Rasmussen, H. (1970), *Science 170,* 404–412.

Rasmussen, H., and Tenenhouse, H. (1968), *Proc. Nat. Acad. Sci. U.S. 59,* 1364–1370.

Sauer, L. A. (1973a), *Biochem. Biophys. Res. Commun. 50,* 524–531.

Sauer, L. A. (1973b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* abs. no. 2025.

Sauer, L. A., and Park, R. (1973), *Biochemistry 12,* 643–649.

Sayers, G., Bell, R. J., and Seelig, S. (1972), *Science 175,* 1131–1133.

Scarpa, A., and Azzi, A. (1968), *Biochim. Biophys. Acta 150,* 473–481.

Simpson, E. R., and Boyd, G. S. (1971), *Eur. J. Biochem. 22,* 489–499.

Simpson, E. R., Cammer, W., and Estabrook, R. W. (1968), *Biochem. Biophys. Res. Commun. 31,* 113–118.

Simpson, E. R., and Estabrook, R. W. (1968), *Arch. Biochem. Biophys. 126,* 997–978.

Simpson, E. R., and Estabrook, R. W. (1969), *Arch. Biochem. Biophys. 129,* 385–395.

Vasington, F. D., Gazzotti, P., Tiozzo, R., and Carafoli, E. (1972), *Biochim. Biophys. Acta 256,* 43–54.

Weaver, G., and Landau, B. R. (1963), *Endocrinology 73,* 640–646.