Catsimpoolas, N. (1969), *Biochim. Biophys. Acta 175*, 214.
Florini, J. R. (1964), *Biochemistry 3*, 209.
Florini, J. R. (1970), *Biochemistry 9*, 909.
Florini, J. R., and Breuer, C. B. (1965), *Biochemistry 4*, 253.
Florini, J. R., Brivio, R. P., and Battelle, B. (1971), *Anal. Biochem. 40*, 345.
Heywood, S. W., Dowben, R. M., and Rich, A. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1002.
Holliday, T. A., Julian, L. M., and Asmundson, V. S. (1968), *Anat. Rec. 160*, 207.
Ionasescu, V., Zellweger, H., and Conway, T. (1971), *Arch. Biochem. Biophys. 144*, 51.
Ionasescu, V., Zellweger, H., Filer, L. J., and Conway, T. (1970), *Arch. Neurol. 23*, 128.
Kabat, D. (1970), *Biochemistry 9*, 4160.
Leach, A. A. (1959), *J. Biochem. 74*, 70.
Loening, U. E. (1967), *Biochem. J. 102*, 251.
Monckton, G., and Nihei, T. (1971), *Int. Congr. Muscular Dis., 2nd, Abstr.* 16.
Nihei, T., and Tataryn, D. (1971), *Int. Congr. Muscular Dis., 2nd Abstr.* 18.
Seifter, S., and Gallop, P. M. (1962), *Methods Enzymol. 5*, 659.
Seifter, S., and Harper, E. (1970), *Methods Enzymol. 19*, 613.
Simpson, G. G., Roe, A., and Loewontin, R. C. (1960), Quantitative Zoology, New York, N. Y., Harcourt, Brace and World.
Snedocer, G. W., and Cochran, W. G. (1967), Statistical Methods, Ames, Iowa, Iowa State University Press.
Srivastava, U. (1972), *Can. J. Biochem. 50*, 409.
Tappel, A. L., Zalkin, H., Caldwell, K. A., Desai, I. D., and Shibko, S. (1962), *Arch. Biochem. Biophys. 96*, 304.
Traught, J. A., and Traut, R. R. (1972), *Biochemistry 11*, 2503.
Warburg, O., and Christian, J. (1942), *Biochem. Z. 310*, 384.
Watts, R. L., and Reid, J. D. (1969), *Biochem. J. 115*, 377.
Weinstock, I. M., Soh, T. S., Freedman, H. S., and Cutler, M. E. (1969), *Biochem. Med. 2*, 345.

A Stimulation by Phosphate of Malate Transport and Oxidation in Rat Adrenal Mitochondria†

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ABSTRACT: In isolated rat adrenal cortex mitochondria inorganic phosphate (P_i) stimulated the rates of the following malate-supported reactions: (1) large amplitude swelling (in isotonic ammonium malate); (2) intramitochondrial pyridine nucleotide reduction; (3) 11β steroid hydroxylation; and (4) pyruvate efflux into the medium. Mersalyl or *p*-iodobenzylmalonate, inhibitors of the P_i or malate exchange-diffusion carriers, respectively, either decreased or abolished the increments in these rates due to P_i. KCN inhibited the rate of P_i-stimulated 11β hydroxylation or pyruvate efflux by 60%. A portion of the rate of pyruvate formation which remained in the presence of KCN occurred as a result of a dismutation of malate to succinate and pyruvate. Rotenone inhibited this dismutation. The clear supernatant fluids obtained following centrifugation of sonicated mitochondrial suspensions contained $NADP^+$- and NAD^+-linked malic enzyme activities. It is concluded that rat adrenal cortex mitochondria contain P_i-stimulated malate carrier systems that increase the rate of malate entry to $NADP^+$- and NAD^+-linked malic enzyme(s) contained in the matrix space. The NADPH and NADH are available to steroid hydroxylation or oxidative phosphorylation, respectively, and the pyruvate leaves the mitochondria.

Several of the hydroxylations that occur during adrenal steroid hormone biosynthesis take place within the mitochondria. The NADPH required by the steroid hydroxylases can be generated inside the mitochondria either by the joint action of several NAD^+-linked dehydrogenases and a transhydrogenase or directly by $NADP^+$-linked dehydrogenases (Guerra *et al.*, 1966; Harding *et al.*, 1965; Cammer and Estabrook, 1967; Sauer and Mulrow, 1969). It is now known that specific carriers mediate the exchange of charged molecules, such as the substrate anions, across the inner mitochondrial membrane and it becomes of interest to determine: (1) which substrates provide the major source of the reducing equivalents for steroid hydroxylation; (2) how the substrates are generated outside of the mitochondria and enter the intramitochondrial space; and (3) how they are oxidized. In a previous report we demonstrated that inorganic phosphate (P_i) or arsenate stimulated the rate of malate-supported 11β hydroxylation in isolated rat adrenal cortex mitochondria (Sauer and Mulrow, 1969). P_i was also found to stimulate a malate-dependent formation of pyruvate. Since arsenate and P_i are known to stimulate malate entry *via* P_i–dicarboxylate anion-exchange-diffusion carriers (Chappell and Haarhof, 1967), it seemed likely that a study of the P_i effect would provide information on some of the points outlined above. We report here that P_i stimulates malate entry into rat adrenal cortex mitochondria *via* a P_i–malate exchange-diffusion carrier and that the malate is oxidized by malic enzyme (s) con-

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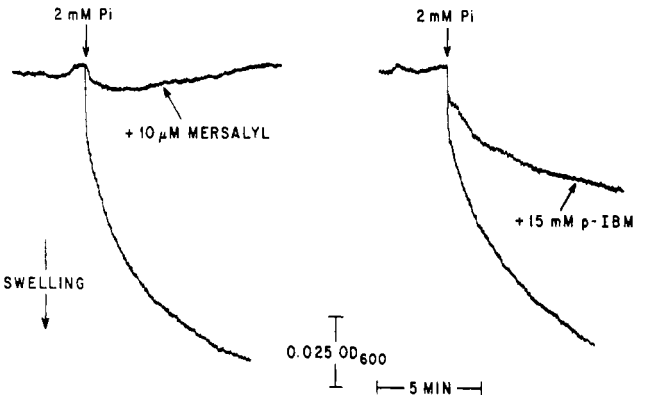

FIGURE 1: P_i-dependent swelling of rat adrenal cortex mitochondria suspended in ammonium malate. Swelling was started by the addition of 2 mM P_i to rat adrenal mitochondria (0.29 mg of protein) suspended in 1 ml of 90 mM ammonium malate (pH 7.4) containing 0.01 mM rotenone and 5 mM sucrose (from the mitochondrial suspension). In the two upper traces the medium also contained mersalyl or pIBzMal, as indicated.

tained in the matrix space. The reduced pyridine nucleotides formed are available to steroid hydroxylation and oxidative phosphorylation.

Materials and Methods

The enzymes and chemicals used in these studies were obtained from the following sources: lactic dehydrogenase, (−)-malic, pyruvic, and fumaric acids (Calbiochem Co., Los Angeles, Calif.); zone-refined succinic acid (Fisher Chemical Co., Pittsburgh, Pa.); sodium mersalyl and pyridine nucleotides (Sigma Chemical Co., St. Louis, Mo.); *p*-iodobenzylmalonic acid and rotenone (K & K Laboratories, Inc., Plainview, N. Y.); uniformly labeled [^{14}C]malic acid (40 Ci/mol) and other radioactive substrates (Amersham–Searle Corp., Arlington Heights, Ill.,); and crystallized bovine serum albumin (Miles Laboratories, Inc., Kankakee, Ill.). All other chemicals were reagent grade from commercial sources.

Mitochondria were prepared, by differential centrifugation (Sauer and Mulrow, 1969), from the adrenals of male Sprague-Dawley rats weighing 150–200 g. The homogenate was prepared in 0.25 M sucrose, 1 mM EDTA, 30 mM Tris-HCl, and 1% bovine serum albumin, all at pH 7.4. In a few experiments the medullary portions of the cleaned glands were removed under a dissecting microscope. The adrenal cortex fragments that remained yielded a mitochondrial preparation enriched in adrenal cortex mitochondria. Protein was determined by the biuret method (Szarkowska and Klingenberg, 1963). Mitochondria were disrupted by sonication (Sauer and Mulrow, 1969). Before sonication the mitochondria were washed twice and resuspended in 4 ml of the above homogenization medium containing no bovine serum albumin. The clear supernatant fluid containing the soluble mitochondrial enzymes was obtained by centrifugation (105,000*g* for 60 min).

The rate of pyruvate efflux from the intact mitochondria was determined by incubating the mitochondria at 37° in a medium containing 50 mM sucrose, 1 mM EDTA, 30 mM KCl, 30 mM Tris-HCl, and 1% bovine serum albumin (pH 7.4). Malate and other additions were as indicated in either the figure or Table legends. Samples (0.5 or 1 ml) were removed at various times and the mitochondria sedimented by centrifugation for 20 sec at 10,000*g* in a Fisher Model 59 centrifuge. The supernatants were decanted and stored in ice. Pyruvate was determined enzymatically (Estabrook and Maitra, 1962). There was no further pyruvate formation after the mitochondria were removed. The rate of pyruvate formation by the soluble enzymes in the sonicated mitochondrial supernatant was determined by similar incubations, except that the samples were quenched with 7% perchloric acid. Pyruvate was measured in the KOH-neutralized acid extracts. The rate of pyruvate formation in the sonicate is expressed per milligram of total mitochondrial protein so that it can be compared to the rate of pyruvate efflux from the intact mitochondria.

The radioactive products formed during the incubation of rat adrenal mitochondria with uniformly labeled [^{14}C]malate were separated by ion-exchange chromatography. The method of Busch *et al.* (1952), as modified by LaNoue *et al.* (1970), was used with slight further modification. The perchloric acid extract of the mitochondrial incubate was neutralized to pH 6.5 with KOH. A 0.5-ml aliquot was chromatographed on a 0.9 × 14 cm Dowex 1 (formate) column (Bio-Rad AG1-X10) equilibrated with 0.25 N formic acid. The elution gradient was formed by allowing 0.5 N formic acid to flow into a mixing reservoir containing 250 ml of 0.25 N formic acid. The column was eluted with this gradient until the malic acid peak was removed (275–280 ml). (In our experiments, the zero to 3.0 N formic acid gradient-forming system used by LaNoue *et al.* (1970) did not adequately separate succinate from malate.) The column was then eluted with 1.5 N formic acid until the pyruvic acid peak was removed (420-ml total effluent) and with 3 N formic acid until fumaric acid was removed (600-ml total effluent). Fractions of 2 ml were collected during the gradient elution and 5-ml fractions thereafter. Portions (1 ml) were counted in 10 ml of Bray's solution (Bray, 1960). The products were identified by comparison with elution profiles of ^{14}C-labeled standard acids chromatographed under identical conditions on the same Dowex 1(formate) columns or by thin-layer chromatography on cellulose (Myers and Huang, 1966) or silica gel G (Randerath, 1963) plates. Malate, fumarate, succinate, and pyruvate are well separated from aspartate during the ion-exchange (LaNoue *et al.*, 1970) and thin-layer (Myers and Huang, 1966) chromatographic separations. In addition, no glutamate or other nitrogen source was present in the incubates. No evidence for aspartate formation was obtained.

The P_i-induced swelling of mitochondria in isotonic ammonium malate was measured as described by Chappell and Haarhof (1967). Intramitochondrial pyridine nucleotide reduction was measured in stirred solutions in an Aminco-Bowman spectrofluorometer. The malic enzyme activity of the sonicated mitochondrial supernates was assayed in a Gilford Model 2000 spectrophotometer by the method of Ochoa (1955) with $NADP^+$ or NAD^+ as hydrogen acceptor except that 0.1 mM dithiothreitol was added. Incubations for the measurement of 11β steroid hydroxylation were performed as previously described (Sauer, 1970). Corticosterone was assayed by acid fluorescence (Silber *et al.*, 1958).

Results

Intact Mitochondria. MALATE ENTRY. Chappell and Haarhof (1967) have described a large amplitude P_i- or arsenate-dependent swelling of mitochondria suspended in isotonic ammonium malate. This finding is interpreted as evidence for P_i–malate exchange reaction. Figure 1 illustrates that rat adrenal cortex mitochondria show similar P_i-induced swelling phenomena. The swelling was completely inhibited by 10

TABLE I: Effect of Mersalyl and Oligomycin on P_i-Stimulated 11β Hydroxylation in Rat Adrenal Mitochondria.[a]

Additions	Corticosterone Production[d]
Complete system[b]	1.5
+P_i	4.4
+Mersalyl	0.7
+P_i + mersalyl	0.6
Complete system[c]	9.9
+P_i	15.6
+P_i + oligomycin	13.1
Complete system[c]	9.4
+KCN	3.4
+KCN + P_i	6.6
+KCN + P_i + oligomycin	5.9

[a] Incubation conditions and corticosterone measurements were as described under Methods. The values are means of duplicate or triplicate determinations. [b] The complete system contained: 0.2 M sucrose; 20 mM Tris-HCl; 40 μM dinitrophenol, 150 μM deoxycorticosterone; 10 mM malate and 0.49 mg of mitochondrial protein. P_i and mersalyl were 2 mM and 30 μM, respectively. Total volume was 1 ml. [c] The complete system contained 0.25 or 0.37 (experiment with KCN) mg of mitochondrial protein suspended in 1 ml of the medium described in Methods, containing 150 μM deoxycorticosterone and 10 mM malate. P_i was 2 mM. KCN was 2 mM. Oligomycin (in ethanol) was 2 μg/ml. The control flasks received an equivalent amount of ethanol. [d] nmol/min per mg of mitochondrial protein.

μM mersalyl and was partially inhibited by 15 mM *p*-iodobenzylmalonate (pIBzMal),[1] inhibitors, respectively, of the P_i (Tyler, 1969) and the malate (Robinson and Williams, 1969) exchange-diffusion carriers. Higher concentrations of pIBzMal gave even greater inhibitions of the rate of swelling.

MALATE ENTRY AND OXIDATION. P_i increased the rate of reduction of the intramitochondrial pyridine nucleotides by malate (Figure 2). The rate of reduction, taken as a per cent of the total reduction, was 61 and 23% reduced/30 sec in the presence and absence of P_i, respectively. The addition of mersalyl plus P_i abolished the stimulation by P_i. Deoxycorticosterone addition caused an immediate oxidation of a portion of the reduced pyridine nucleotides (presumably mostly NADPH). This occurred even in the presence of mersalyl indicating that 11β hydroxylation was not inhibited by the mercurial.

The P_i stimulation of malate-supported 11β steroid hydroxylation in rat adrenal mitochondria was inhibited by mersalyl (Table I). In this experiment dinitrophenol (N_2ph) was added to increase the magnitude of the P_i stimulation (Sauer and Mulrow, 1969). Table I also shows that the effect of P_i on corticosterone production was only slightly affected by oligomycin. Therefore, in contrast to the P_i stimulation with α-ketoglutarate described earlier (Sauer and Mulrow, 1969), ATP formation does not play a role in the malate-supported reaction. Since arsenate, an uncoupler of oxidative phosphorylation, also stimulated 11β steroid hydroxylation (or pyruvate efflux) under these conditions, it is very unlikely that energy coupling is involved in either the P_i or arsenate effects.

[1] Abbreviation used is: pIBzMal, *p*-iodobenzylmalonate.

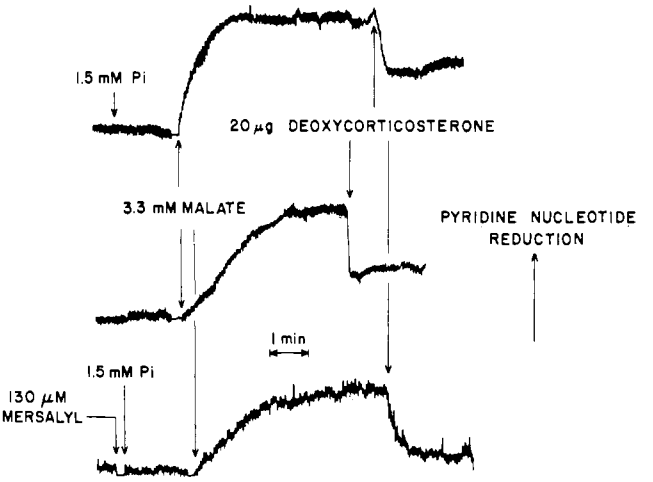

FIGURE 2: Effect of P_i on the rate of malate-dependent intramitochondrial pyridine nucleotide reduction. Rat adrenal mitochondria (1.51 mg of protein) were suspended in 1.5 ml of the incubation medium described under Methods. Rotenone was 3 μM. Other additions were as indicated. Pyridine nucleotide fluorescence was activated at 360 nm and measured at 465 nm.

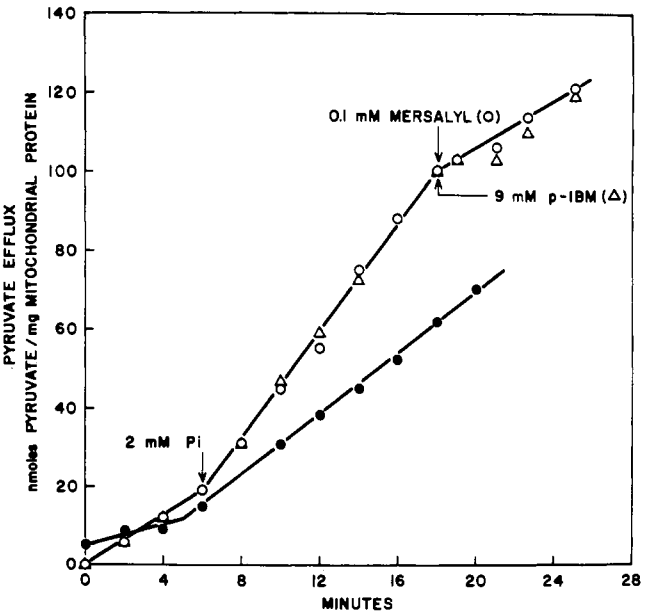

FIGURE 3: Pyruvate efflux from rat adrenal mitochondria. The reactons were started at zero time by the addition of either 2 mM (Δ) or 5 mM (●) malate or 10 mM succinate (○). The incubates with malate also contained 1 mM arsenite and were made 2 mM in P_i at 5 min and 0.1 mM in mersalyl (○) or 9 mM in pIBzMal (Δ) at 18 min. The incubation with succinate contained 3 mM P_i, 5 mM $MgCl_2$, 10 mM glucose, 0.6 mM ADP, and 0.36 mg of yeast hexokinase. No arsenite was present. The mitochondrial protein concentrations were 0.55 (●), 0.67 (○), and 0.74 (Δ) mg/ml and represent three different mitochondrial preparations.

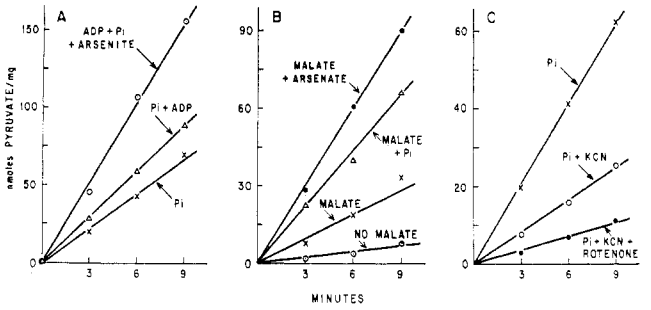

FIGURE 4: Properties of the malate-dependent pyruvate efflux from rat adrenal mitochondria. The incubation and sampling procedures were as described for intact mitochondria under Methods. The reactions were started by the addition of 10 mM malate at zero time. The other additions were: arsenite, 1 mM; P_i or arsenate, 2 mM; ADP, 0.6 mM; KCN, 1 mM; rotenone, 0.01 mM. Arsenite was absent in part A, except as indicated, and present in both parts B and C.

The effect of P_i on the rate of pyruvate efflux from rat adrenal mitochondria incubated at 37° with malate is shown in Figure 3. Pyruvate efflux began immediately on addition of malate and was stimulated more than twofold by 2 mM P_i. Both mersalyl and pIBzMal inhibited the rate of pyruvate efflux. The P_i-stimulated portion was inhibited by mersalyl. The inhibition by pIBzMal depended on the concentration added. At 37° a complete block of pyruvate efflux occurred only at low malate and high pIBzMal concentrations. This is similar to the findings of Robinson and Williams (1970) in rat liver mitochondria. Figure 3 also shows that pyruvate efflux occurred during state 3 respiration in the presence of succinate. After a lag period of from 4 to 5 min, the rate of pyruvate efflux was constant. Arsenite need not be present for pyruvate efflux in the presence of succinate (Figure 3) or malate (see below). Also, neither Mg nor Mn ion was required for pyruvate efflux from intact mitochondria with either substrate.

Some further properties of the P_i-stimulated pyruvate efflux are shown in Figure 4. Pyruvate efflux was stimulated by conditions that increased the rate of malate oxidation, *e.g.*, oxidative phosphorylation (Figure 4A) or uncoupling with arsenate (Figure 4B). Likewise, inhibition of respiration with KCN or KCN plus rotenone inhibited pyruvate efflux (Figure 4C). Arsenite enhanced the rate of pyruvate efflux (Figure 4A), suggesting that a portion of the pyruvate must be oxidized in the absence of arsenite. Also, the rate of pyruvate efflux was low in the absence of malate.

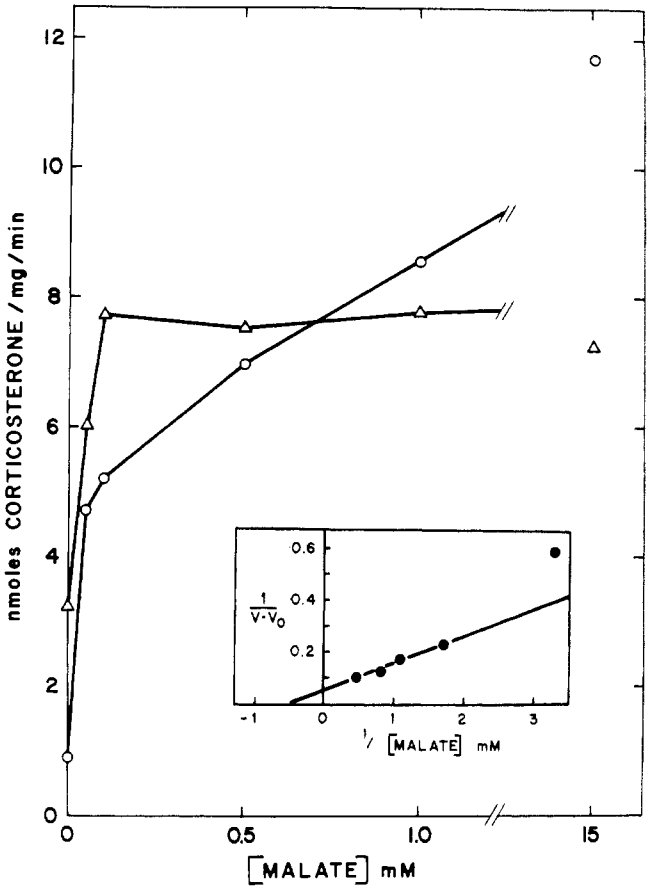

FIGURE 6: Inhibition by P_i of the rate of corticosterone formation at low malate concentrations. Incubation conditions for the determination of the rate of corticosterone production were as described under methods. The additions were as follows: deoxycorticosterone, 150 μM P_i, 2 mM (○) or absent (△); and malate, as indicated. The inset is a double-reciprocal plot of the rate of pyruvate efflux against the malate concentration. The P_i concentration was 2 mM. Arsenite was 1 mM. V is the rate of pyruvate efflux (nmol/mg of mitochondrial protein per min) at the malate concentrations indicated. V_0 is the rate in the absence of malate.

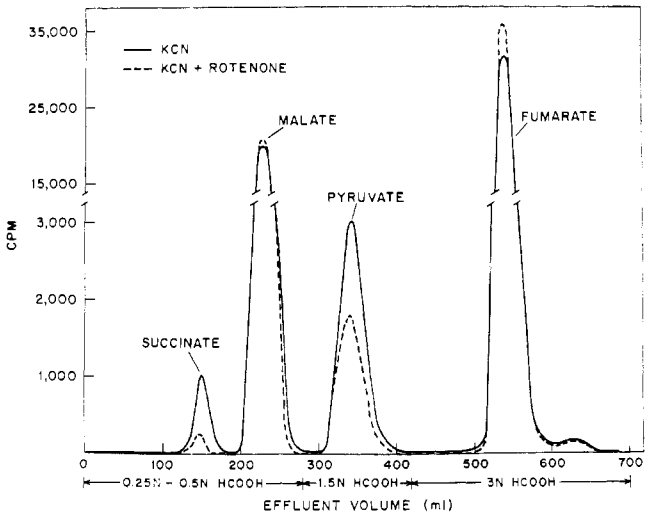

FIGURE 5: Separation by chromatography on Dowex 1 (formate) of the products of [^{14}C]malate oxidation formed in the presence of KCN or KCN plus rotenone. The procedures of incubation, product separation, counting of radioactivity, and product identification were as described under Methods.

Rotenone is a potent inhibitor of NADH oxidase and of reversed electron transport over site I in these mitochondria (Sauer, 1972). It was of interest, therefore, to determine why KCN plus rotenone inhibited pyruvate efflux more than KCN alone (Figure 4C). Figure 5 shows that in rat adrenal mitochondria, as in rat heart mitochondria (LaNoue *et al.*, 1970), fumarate is produced from [^{14}C]malate in the presence of KCN or KCN plus rotenone. Succinate was also generated in the presence of KCN but the addition of rotenone markedly decreased the amount that accumulated. Rotenone also decreased the accumulation of pyruvate and increased the concentrations of malate and fumarate slightly. Therefore, the portion of the rate of KCN-insensitive pyruvate efflux (Figure 4C) which is sensitive to rotenone is due to a dismutation of malate to pyruvate and succinate. The succinate was formed *via* the rotenone-sensitive oxidation of NADH and the con-

TABLE II: NAD+- and NADP+-Linked Malic Enzyme Activities in Soluble Fractions of Sonicated Rat Adrenal Mitochondria.[a]

Additions	nmol formed/min per mg of Mitochondrial Protein	
	NAD+ and/or NADP+ Reduction	Pyruvate
Complete system[b] + NADP+	13.3	12.9
Minus NADP+	0.2	1.1
Minus Mn ion	0.0	0.5
Minus malate	1.7	0.9
Complete system[b] + NAD+	10.4	12.7
Minus NAD+	0.4	
Minus Mn ion	0.0	
Minus malate	1.3	
Complete system[b] + NAD+ and NADP+	15.5	12.7

[a] Pyruvate formation and NAD+ or NADP+ reduction were measured as described under Methods. [b] The complete system contained: 10 mM malate; 1 mM arsenite; 0.1 mM dithiothreitol; 1 mM KCN; 0.01 mM rotenone; 5 mM $MnCl_2$; 0.5 mM NAD and/or NADP; and 30 mM Tris-HCl (pH 7.4). Rotenone, KCN, and arsenite were omitted during the spectrophotometric measurement of NADP+ and/or NAD+ reduction. The reactions were started by the addition of malate or pyridine nucleotide in the absence of malate.

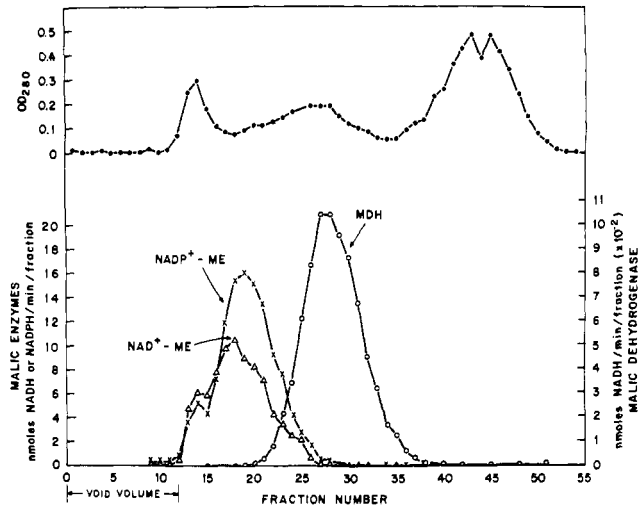

FIGURE 7: The distribution of malic dehydrogenase and the NAD+- and NADP+-linked malic enzyme activities following gel filtration of a rat adrenal mitochondrial sonicate. 1.5 ml of the clear supernatant (prepared as described under Methods) containing 5.1 mg of protein was passed through a 2 × 21.5 cm column of Sephadex G-200 equilibrated with 30 mM Tris-HCl, 0.5 mM EDTA, and 0.1 mM dithiothreitol (pH 7.4). The flow rate was 11 ml/hr. 1.8-ml fractions were collected into tubes containing 0.5 μmol of dithiothreitol. The malic enzyme activities were measured as described under Methods. Malic dehydrogenase was assayed by NADH disappearance at 340 nm in solutions containing 0.1 mM NADH, 50 mM Tris-HCl (pH 7.4), 0.1 mM dithiothreitol, 2 mM oxaloacetate, and an appropriate aliquot of sample. The reaction was started with oxaloacetate.

sequent reduction of fumarate by succinic dehydrogenase. Malonate also inhibited KCN-insensitive pyruvate efflux (not shown), but the mechanism is less clear since malonate inhibits malic enzyme (Ochoa, 1955), and dicarboxylate anion transport (Van Dam and Tsou, 1968) as well as succinic acid dehydrogenase.

Quagliariello and Palmieri (1968) have shown that P_i may compete with malate for entry into rat liver mitochondria. We have observed similar P_i–malate relationships in our rat adrenal cortex mitochondrial preparations. For example, at low malate concentrations P_i inhibited the rate of corticosterone formation (Figure 6). The crossover point between the inhibition and stimulation of 11β hydroxylation occurred at about 0.5 mM malate. At 5–15 mM malate, 2 mM P_i consistently stimulated both the rate of pyruvate efflux and 11β hydroxylation (Sauer and Mulrow, 1969). The inset in Figure 6 shows that the apparent K_m for malate of the P_i-stimulated pyruvate efflux is 2.2 mM. An inhibitory effect of P_i on pyruvate efflux at low malate concentrations caused these points to fall above the linear plot on a Lineweaver–Burk presentation.

Sonicated Mitochondria. Sonication of rat adrenal mitochondria released latent malic enzyme activities that reduced either NADP+ or NAD+. The malic enzyme activities produced constant rates of pyridine nucleotide reduction and pyruvate and CO_2 formation (not shown), and were mainly associated with the soluble mitochondrial contents. The activities in the soluble fraction and in the submitochondrial particles were additive. As shown in Table II, pyridine nucleotide reduction and pyruvate formation required malate, Mn ion and either NAD+ or NADP+. P_i had no effect on the rate of pyruvate formation catalyzed by the malic enzymes in the soluble fraction. This is in contrast with pyruvate formation in intact mitochondria which required only malate and which was stimulated by P_i (Figures 3 and 4). Mg ion could replace Mn ion for pyruvate formation and pyridine nucleotide reduction by the soluble enzymes. When both NAD+ and NADP+ were present the rates of product formation were slightly enhanced but were never exactly additive.

Because only a small amount of mitochondria can be obtained from the pooled adrenals of even several rats and the activity of the mitochondrial malic enzymes is quite low (relative, for example, to malic dehydrogenase), multistep purification procedures (Simpson and Estabrook, 1969; Frenkel, 1971) with their inherent losses proved unsatisfactory. However, gel filtration on Sephadex G-200 (Figure 7) did allow the partial separation of the NADP+- and NAD+-linked malic enzyme activities from the very active malic dehydrogenase. The two malic enzyme activities eluted before the malic dehydrogenase were metal dependent and were easily distinguishable from the nonmetal requiring malic dehydrogenase. Even wider separations were obtained following gel filtration on Sepharose columns (not shown). No oxaloacetic acid decarboxylase activity could be detected in the sonicates.

Discussion

In this report we have presented the results of several different types of experiments, all of which support the concept that P_i controls the rate of malate entry and oxidation in rat adrenal cortex mitochondria. On the basis of these findings we propose that the mechanism of P_i stimulation of malate oxidation is an increased rate of malate entry. A scheme for the proposed mechanism is summarized in Figure 8. This scheme incorporates our findings within the framework established by others in rat liver mitochondria (*cf.* Slater, 1969).

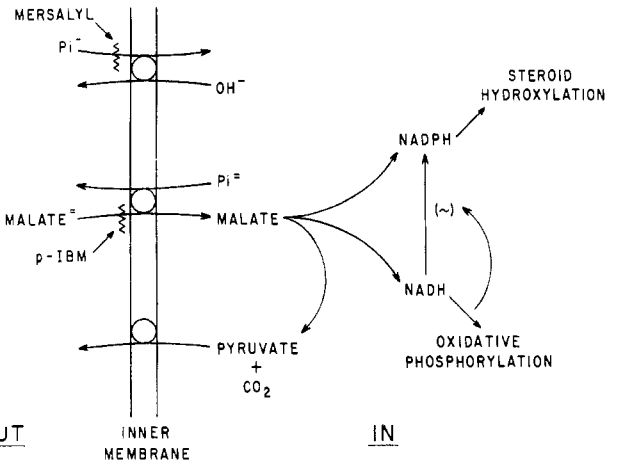

FIGURE 8: Proposed mechanism for the P_i-stimulation of malate-supported reactions in rat adrenal cortex mitochondria.

Although the transport of pyruvate across the inner membrane of rat liver mitochondria is mediated by a specific translocator (Papa *et al.*, 1971), we have not determined if the pyruvate leaves rat adrenal mitochondria in exchange for another anion or as the undissociated acid. Despite many attempts we were unable to demonstrate consistently either a P_i-dependent accumulation of [^{14}C]malate or an increase in the rate of [^{14}C]malate penetration into rat adrenal mitochondria. These phenomena, which are direct evidence for a P_i stimulation of malate entry, have been demonstrated by several investigators in rat liver mitochondria. The reason for our inconsistent success seems to be that adrenal cortex mitochondria contain active malic enzymes. The oxidation of malate by these enzymes occurs even in the presence of inhibitors of respiration (Figures 4C and 5). Therefore, once the mitochondria are separated from the medium and further malate entry ceases, the remaining intramitochondrial malate is converted to pyruvate and CO_2. Both of these compounds leave the mitochondria. In rat liver mitochondria, which contain only low amounts of malic enzyme (Brdiczka and Pette, 1971) and have no rotenone-insensitive pathway for reduced pyridine nucleotide oxidation (Ernster *et al.*, 1963), intramitochondrial malate would be more stable. In rat adrenal cortex mitochondria, the KCN- and rotenone-insensitive oxidation of reduced pyridine nucleotides (Sauer, 1971) is most reasonably explained by an energy-independent hydroxylation of endogenous steroids, presumably mostly cholesterol.

It was of interest to find that pyruvate formation in the presence of KCN occurred *via* a dismutation of malate. It had been considered that KCN-insensitive malate-supported steroid hydroxylation was non-energy-linked (Guerra *et al.*, 1966; Sauer *et al.*, 1969). The effect of the rotenone addition (Figures 4C and 5) indicated that energy coupling at site I could support KCN-insensitive energy-dependent steroid hydroxylation. This reaction, proceeding *via* the NAD-dependent malic enzyme activity and the energy-linked transhydrogenase, would be inhibited by rotenone.

In our earlier studies we were able to demonstrate pyruvate formation in our intact rat adrenal mitochondrial preparations, but were unable to provide spectrophotometric evidence for malic enzyme activity by $NADP^+$ reduction in sonicates (Sauer and Mulrow, 1969). The reason for this is probably because the malic enzyme activity is low (relative to malic dehydrogenase and the $NADP^+$-linked isocitric acid dehydrogenase), the enzyme(s) has allosteric properties (see below) of which we were unaware at that time, and the sonicates contain small amounts of endogenous substrates which made $NADP^+$ reduction not dependent on malate. We now routinely find the NAD^+- and $NADP^+$-linked malic enzymes to be present in constant activities (R. D. Mandella and L. A. Sauer, in preparation).

Simpson and Estabrook (1969) and Simpson and Frenkel (1969) have demonstrated that beef adrenal cortex mitochondria contain an $NADP^+$-linked malic enzyme and that most of the pyruvate formed leaves the mitochondria. Their studies established that a "malate shuttle" could exist in the adrenal cortex between the extramitochondrial soluble fraction and the intramitochondrial matrix space. Our findings indicate that the P_i–malate exchange is important for malate entry in adrenal cortex mitochondria and that this exchange must be considered in any "malate shuttle" experimental model.

The intra- and extramitochondrial malic enzymes have recently been studied by several investigators (Simpson and Estabrook, 1969; Frenkel, 1971; Brdiczka and Pette, 1971). Their presence in the adrenal cortex, especially in the mitochondria, is of particular importance as a possible source for non-energy-dependent NADPH formation during steroid hormone biosynthesis. We were surprised to find NAD^+-linked malic enzyme activity in our mitochondrial preparations. This enzyme is known to occur in some streptococci (London and Meyer, 1969a) and in mitochondria from higher plants (Macrae and Moorhouse, 1970) and from Ascaris (Papa *et al.*, 1970) but, to our knowledge, had not been described in higher animals. However, in addition to our finding there have been other recent reports of mitochondrial NAD^+-linked malic enzymes in rabbit and cod ovary (Mounib and Eisan, 1971) and in beef heart (Lin and Davis, 1972). The enzyme may prove to be more widespread than previously suspected.

Because only small amounts of mitochondria can be obtained from the rat adrenal cortex, purification of the malic enzymes is difficult. However, both activities can be separated from malic dehydrogenase by gel filtration. Therefore, the NAD^+-linked activity is not due to the combined effects of the $NADP^+$-linked malic enzyme and malic dehydrogenase. Also, the extramitochondrial cell sap contains both $NADP^+$-linked malic enzyme and malic dehydrogenase but no NAD^+-linked malic enzyme. During gel filtration the two malic enzyme activities are closely associated. For the following reasons we feel the two activities may reside in the same enzyme: (1) the activities with NAD^+ and $NADP^+$ are not additive; (2) both activities show sigmoid kinetics in plots of activity *vs.* the malate concentration; (3) as recently reported by Frenkel (1972), positive cooperativity was found with succinate for the $NADP^+$-linked enzyme. We find similar cooperative effects for the NAD^+-linked enzyme; and (4) as in *Streptococcus faecalis* (London and Meyer, 1969b), the NAD^+-linked malic enzyme was inhibited by ATP. We find a similar inhibition of the $NADP^+$-linked enzyme by ATP. Final proof, however, of whether or not these activities are the result of one or two enzymes must await further purification. In a subsequent report, we will provide more detailed information on the kinetic properties of these interesting malic enzymes.

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References

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Brdiczka, D., and Pette, D. (1971), *Eur. J. Biochem. 19*, 546.
Busch, H., Hurlbert, R. B., and Potter, V. R. (1952), *J. Biol. Chem. 196*, 717.
Cammer, W., and Estabrook, R. W. (1967), *Arch. Biochem. Biophys. 122*, 721.
Chappell, J. B., and Haarhof, K. N. (1967), *in* Biochemistry of Mitochondria, Slater, E. C., Kaniuga, Z., and Wojtczak, L., Ed., London and Warsaw, Academic Press and Polish Scientific Publishers, p 75.
Ernster, L., Dallner, G., and Azzone, G. F. (1963), *Biochem. Biophys. Res. Commun. 10*, 23.
Estabrook, R. W., and Maitra, P. K. (1962), *Anal. Biochem. 3*, 369.
Frenkel, R. (1971), *J. Biol. Chem. 246*, 3069.
Frenkel, R. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 858 Abstr.
Guerra, F., Peron, F. G., and McCarthy, J. L. (1966), *Biochim. Biophys. Acta 117*, 433.
Harding, B. W., Wilson, L. D., Wong, S. H., and Nelson, D. H. (1965), *Steroids Suppl. II*, 51.
LaNoue, K., Nicklas, W. J., and Williamson, J. R. (1970), *J. Biol. Chem. 245*, 102.
Lin, R. C., and Davis, E. J. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 831 Abs.
London, J., and Meyer, E. Y. (1969a), *J. Bacteriol. 98*, 705.
London, J., and Meyer, E. Y. (1969b), *Biochim. Biophys. Acta 178*, 205.
Macrae, A. R., and Moorhouse, R. (1970), *Eur. J. Biochem. 16*, 96.
Mounib, M. S., and Eisan, J. S. (1971), *Endocrinology 88, Suppl.*, 237.
Myers, W. F., and Huang, K. Y. (1966), *Anal. Biochem. 17*, 210.
Ochoa, S. (1955), *Methods Enzymol. 2*, 739.
Papa, S., Cheah, K. S., Rasmussen, H. N., Lee, I. Y., and Chance, B. (1970), *Eur. J. Biochem. 12*, 540.
Papa, S., Francavilla, A., Paradies, G., and Meduri, B. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12*, 285.
Quagliariello, E., and Palmieri, F. (1968), *Eur. J. Biochem. 4*, 20.
Randerath, K. (1963), Thin Layer Chromatography, New York, N. Y., Academic Press, p 218.
Robinson, B. H., and Williams, G. R. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 5*, 301.
Robinson, B. H., and Williams, G. R. (1970), *Biochim. Biophys. Acta 216*, 63.
Sauer, L. A. (1970), *Arch. Biochem. Biophys. 139*, 340.
Sauer, L. A. (1971), *Biochim. Biophys. Acta 234*, 287.
Sauer, L. A. (1972), *Arch. Biochem. Biophys. 149*, 42.
Sauer, L. A., and Mulrow, P. J. (1969), *Arch. Biochem. Biophys. 134*, 486.
Silber, R. H., Busch, R. D., and Oslapas, R. (1958), *Clin. Chem. 4*, 278.
Simpson, E. R., and Estabrook, R. W. (1969), *Arch. Biochem. Biophys. 129*, 384.
Simpson, E. R., and Frenkel, R. (1969), *Biochem. Biophys. Res. Commun. 35*, 765.
Slater, E. C. (1969), *in* The Energy Level and Metabolic Control in Mitochondria, Papa, S., Tager, J. M., Quagliariello, E., and Slater, E. C., Ed., Bari, Adriatics Editrice, p 15.
Szarkowska, L., and Klingenberg, M. (1963), *Biochem. Z. 338*, 674.
Tyler, D. D. (1969), *Biochem. J. 111*, 665.
Van Dam, K., and Tsou, C. S. (1968), *Biochim. Biophys. Acta 162*, 301.