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FURTHER STUDIES ON CORTICOSTEROIDOGENESIS

VI. PYRUVATE AND MALATE SUPPORTED STEROID 11β-HYDROXYLATION IN RAT ADRENAL GLAND MITOCHONDRIA

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SUMMARY

Pyruvate and pyruvate *plus* ATP have been shown to support 11β-hydroxylation of 11-deoxycorticosterone into corticosterone in incubated rat adrenal gland mitochondria. Corticosterone production with pyruvate *plus* ATP was not as great as with malate *plus* P_i, malate *plus* ATP or malate *plus* pyruvate. Respiratory chain inhibitors, *trans*-aconitate, oxaloacetate, arsenite and the uncoupler 2,4-dinitrophenol, inhibited corticosterone formation. On the other hand, cysteine sulfinate and pyruvate, which led to the removal of excess metabolic oxaloacetate formed from malate oxidation, increased rat adrenal mitochondrial O_2 consumption as well as corticosterone production from 11-deoxycorticosterone. P_i and ATP also appeared to act in the same way in that these agents brought about a greater conversion rate of oxaloacetate into pyruvate. Pyruvate, resulting from the oxidation of malate, accumulated in the incubation system only when arsenite was added. Arsenite additions to malate and isocitrate inhibited the conversion of 11-deoxycorticosterone into corticosterone except when the 11β-hydroxylation of 11-deoxycorticosterone was supported with exogenous NADPH in Ca^{2+}-swollen mitochondria. These results as well as the observations that NAD-linked malate dehydrogenase (L-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) is at least 10 times as active as the NADP-linked enzyme (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.39) in sonicated rat adrenal gland mitochondria, led to the conclusion that under our incubation conditions malate was mainly oxidized *via* the NAD-linked malate dehydrogenase. The fact that in malate incubations pyruvate did not accumulate because of its further metabolism in rat adrenal gland mitochondria, does not support the possibility that these mitochondria are the source of pyruvate for a "malate shuttle" originally thought to occur in rat adrenal gland[7]. This shuttle would have depended on the formation of pyruvate from malate in rat adrenal gland mitochondria followed by extrusion of the pyruvate formed intramitochondrially into the cytoplasm of the cell.

The systematic chemical names of substances for which trivial names are used on this report are: 11-deoxycorticosterone, 21-hydroxy-4-pregnen-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnen-3,20-dione; pyruvate, sodium salt of pyruvic acid.

INTRODUCTION

In a recent paper[1] an attempt was made to deduce what possible oxidizable substrate(s) may be present in the cytosol of the cell (103000 × *g* supernatant fraction obtained from whole adrenal gland homogenates from rats, hereafter referred to as Sup. fraction), which provides rat adrenal gland mitochondria with a source of reducing pressure in the form of intramitochondrial NADPH necessary for the conversion of 11-deoxycorticosterone into corticosterone. Because corticosterone production from 11-deoxycorticosterone by these mitochondria was increased by adding Sup. fraction or Sup. fraction in combination with substances such as NADPH, pyruvate and CO_2, we deduced that the Sup. fraction contained small molecular weight substances such as pyruvate which could be converted into malate by NADP-linked malate dehydrogenase (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) in the Sup. fraction. Since malate stimulates mitochondrial steroid 11β-hydroxylation[2,5,9] and is known to be oxidized to pyruvate in bovine adrenal cortex mitochondria *via* the NADP-linked malate dehydrogenase[2,6], a scheme was suggested[7] to explain the roles of malate and of pyruvate in the hydroxylation reaction. This scheme suggested that malate was oxidized to pyruvate in rat adrenal gland mitochondria with concurrent formation of intramitochondrial NADPH necessary for steroid 11β-hydroxylation. Pyruvate, leaking out of the mitochondria, could then be recarboxylated in the presence of NADPH *plus* CO_2 (ref. 1) into malate by the malate dehydrogenase in the rat Sup. fraction. Thus, this sequence of events suggested a "malate shuttle"[7], a machanism for the transfer of reducing equivalents (NADPH) from the cytosol into the mitochondrion.

The work published by SIMPSON AND ESTABROOK[8,21] clearly shows that such a mechanism is possible in bovine adrenal cortex mitochondria. As proposed by these workers, who have isolated both the cytosol and mitochondrial NADP-linked malate dehydrogenase[6], the "malate shuttle" provides a mechanism whereby "extramitochondrially generated NADPH can be utilized for the function of the key mitochondrial mixed function oxidases"[8]. In spite of the above rationale, which would make the "malate shuttle" scheme for the provenance of intramitochondrial NADPH for steroid hydroxylation easily acceptable, we have been disturbed by the fact that inhibitors of the respiratory chain have led to a decreased respiration as well as to a concomitant inhibition of corticosterone production from 11-deoxycorticosterone in rat adrenal mitochondria incubated in the presence of malate[1]. This suggested that the oxidation of malate in rat adrenal mitochondria may be carried out by the NAD-linked malate dehydrogenase (L-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) and that the activity of the respiratory chain is intimately linked with the production of NADPH *via* the energy-dependent transhydrogenase (NADPH:NAD^+ oxidoreductase, EC 1.6.1.1) reaction[17]. This mechanism could account for the availability of NADPH to the P450 chain subsequent to the removal of oxaloacetate, the metabolic product of malate oxidation. It can be assumed that if oxaloacetate accumulation occurs, this would lead to some inhibition of malate dehydrogenase. This would be reflected in the generation of lower levels of intramitochondrial NADH and NADPH and in turn would be reflected in a lowering of corticosterone production from 11-deoxycorticosterone. Conversely, if a mechanism for removal of oxaloacetate exists, corticosterone production would be greater.

The present studies were initiated to investigate some of these possibilities and to continue our investigations on corticosteroidogenesis in rat adrenal mitochondria. We hoped to provide further information on the mechanism of malate utilization in rat adrenal mitochondria and to see whether the "malate shuttle"[7] is operative in this cellular entity. Resolution of some of these problems would provide a basis for future studies to test whether adrenocorticotropin (ACTH) or cyclic 3',5'-adenosine monophosphate (3',5'-AMP) has any action on the utilization of malate itself or on other biochemical parameters of the "malate shuttle"[8].

MATERIALS AND METHODS

The materials and methods used in these studies were essentially those reported previously[9]. Preparation of the mitochondrial pellet was the same as in our former publication[1] except that the mitochondrial pellet was washed twice to eliminate microsomal and soluble enzyme contaminants. Approx. 1 mg of mitochondrial pellet protein was used in each incubation. All incubations were carried out in 3.0-ml glass-stoppered tubes for 10 min at 37° in air. Essentially the same results as those to be described in the present studies were obtained if the incubations were carried out in 100 % O_2 or in O_2–CO_2 (95:5, v/v). The final incubation volume was 1 ml containing 15 mM Tris buffer (pH 7.4), 50 mM nicotinamide, 10 mM NaCl, 10 mM KCl, 140 mM sucrose, 120 μg 11-deoxycorticosterone (dissolved in 0.02 ml of a mixture of equal volumes of propylene glycol and abs. ethanol) and 1 mg of fatty acid-free bovine serum albumin (Pentex, Kankakee, Ill.). The concentration of other added substances shown in the tables, Oxygraph and Eppendorf fluorimeter readings, was 2 mM except where designated.

Corticosterone was quantitated by the H_2SO_4-induced fluorescence method of SILBER *et al.*[10]. Production of corticosterone from 11-deoxycorticosterone is reported in the tables in nmoles corticosterone produced per 10 min per mg of mitochondrial protein. In the Oxygraph and Eppendorf experiments (see below), corticosterone production is reported as nmoles corticosterone produced per min per mg of mitochondrial protein (corticosterone rate). Protein determinations were carried out by the method of LOWRY *et al.*[20].

Mitochondrial O_2 uptake was measured polarographically at 37° as described previously[1]. Qualitative changes in levels of nicotinamide–adenine nucleotides were continuously recorded in an Eppendorf fluorimeter attached to a Minneapolis–Honeywell recorder equipped with zero suppression features[4,11].

The activities of the malate and isocitrate dehydrogenases were measured in the supernatant fraction obtained by centrifuging sonicated mitochondria as follows. Approx. 1.0 ml of a mitochondrial suspension (equivalent to 7.0 mg mitochondrial protein) prepared in the same manner as for the incubation studies (see above) was subjected to ultrasonic vibrations in a small plastic tube for 5 min using the probe of the 10-kcycles Bronwill Biosonik ultrasonicator (Will Scientific Division, Rochester, N.Y., U.S.A.) having a 125-W maximal output. The tube was immersed in ice to avoid heat denaturation of the proteins. The sonicated mitochondrial suspension was then centrifuged in the cold for 60 min at 105000 × *g*, and the resultant clear supernatant fraction was used in the enzyme assays. Malate and isocitrate dehydrogenase activities were then determined in the clear supernatant aliquots (equivalent to 0.33 mg of

mitochondrial protein) by directly assaying the NADH and NADPH formed at 340 nm, respectively. No enzymatic activity with respect to the previously mentioned enzymes was found in the sedimented fraction of the sonicated mitochondrial suspension which contained more than half of the original mitochondrial protein.

For determining pyruvate in the arsenite incubations (see RESULTS), the incubation mixture, after the 10-min incubation at 37°, was first deproteinized with trichloroacetic acid. After removing the precipitated proteins by centrifugation, the pyruvate in the neutralized supernatant fraction was analyzed directly by assaying the added NADH which was oxidized by commercially available lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27).

RESULTS

In the initial experiments, rat adrenal mitochondria were incubated with the substances shown in Table I. Arsenite was added "to inhibit pyruvate oxidase"[6].

TABLE I

THE EFFECT OF ARSENITE ON 11β-HYDROXYLATION OF 11-DEOXYCORTICOSTERONE INTO CORTICOSTERONE BY RAT ADRENAL MITOCHONDRIA INCUBATED IN THE PRESENCE OF MALATE, ISOCITRATE, NADPH + Ca^{2+} AND PYRUVATE

Malate, isocitrate, pyruvate: 2 mM final concentration, and Ca^{2+}: 15 mM, NADPH: 1.0 mM. No corticosterone production was found without malate, isocitrate, pyruvate or in the absence of Ca^{2+} in the NADPH experiment. Results are expressed in nmoles corticosterone produced per 10 min per mg mitochondrial protein.

Arsenite added (mM)	*Expt. 1*		*Expt. 2*	*Expt. 3*
	Malate	*Isocitrate*	*NADPH + Ca^{2+}*	*Pyruvate + ATP*
—	—	—	—	25.2*
—	83.4	146.8	128.4	85.8
0.05	41.7	148.8	—	—
0.10	37.8	134.9	—	—
0.20	27.0	135.7	—	—
0.50	19.1	96.1	137.6	—
1.00	—	—	138.4	—
2.00	19.1	46.1	147.1	7.7

* No ATP added to system.

Corticosterone production from 11-deoxycorticosterone by intact rat adrenal mitochondria supported by malate, isocitrate or pyruvate *plus* ATP was inhibited by arsenite. On the other hand, corticosterone production by Ca^{2+}-swollen rat adrenal mitochondria[9,12] supplemented with exogenous NADPH was not inhibited. Thus, the steroid hydroxylating P450 chain was not inhibited by arsenite provided that sufficient reducing pressure in the form of NADPH was readily available. Therefore, arsenite appeared to inhibit the utilization of isocitrate for NADPH production *via* isocitrate

dehydrogenase (isocitrate:NADP+ oxidoreductase (decarboxylating), EC 1.1.1.42)* as well as the utilization of malate and pyruvate *plus* ATP.

Studies carried out with the Eppendorf fluorimeter using malate as a substrate showed (Fig. 1) that the addition of arsenite to rat adrenal mitochondria in which the pyridine nucleotides had reached a steady state level of reduction led to a rapid oxidation of the pyridine nucleotides. When isocitrate replaced malate (not

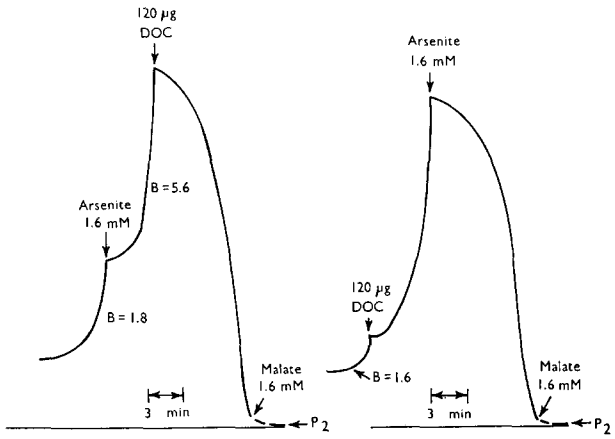

Fig. 1. Reduction of pyridine nucleotides in rat adrenal gland mitochondria incubated with malate. The traces are to be read from right to left. An upward deflection of the tracing indicates reduction of the pyridine nucleotides while a downward deflection an oxidation of the reduced pyridine nucleotides. Additions are as indicated on the outside of the trace while B = is the corticosterone rate, expressing the amount of corticosterone formed after 11-deoxycorticosterone (DOC) additions in terms of nmoles corticosterone produced per min per mg mitochondrial (P_2) protein. Initial reaction volume was 2.5 ml with 0.50 mg mitochondrial protein; reaction carried out at 24° in the same buffer (see MATERIALS AND METHODS) as for the 37° incubations.

shown in Fig. 1), oxidation of the pyridine nucleotides occurred to a much lesser extent. As shown in Table I, corticosterone production supported by isocitrate was also less affected by arsenite as compared to that supported by malate, particularly at low concentrations of the inhibitor.

Incubating malate with rat adrenal mitochondria and arsenite resulted in the accumulation of pyruvate (Table II).

The malate dehydrogenase assay described by OCHOA *et al.*[15] was carried out with sonicated rat adrenal mitochondria. The results showed that malate dehydrogenase activity was only slight, even in the presence of Mn^{2+}. On the other hand, over 10 times more NAD-linked malate dehydrogenase activity was detected in terms of

* In bovine adrenal cortex mitochondria, malate and succinate appear to be the substrates best utilized for steroid 11β-hydroxylation[2,5], whereas in rat adrenal gland mitochondria, isocitrate is the subtrate leading to the greatest corticosterone production from 11-deoxycorticosterone[19]. This latter observation as well as others[4,9] implied that isocitrate was utilized *via* the NADP-linked isocitrate dehydrogenase. With the enzyme preparation obtained from sonicated mitochondria used in this study (see MATERIALS AND METHODS), we have not been able to present evidence that isocitrate is metabolized *via* the NAD-linked isocitrate dehydrogenase since NAD^+ was not reduced when it replaced $NADP^+$ in the enzyme system. The apparent K_m for isocitrate obtained with the enzyme assay in the presence of $NADP^+$ was found to be 1.6 mM. It is to be noted here that the addition of Mn^{2+} (300 μM final concentration) thrice increased the initial rate (between zero time and 1 min after the addition of $NADP^+$) of the $NADP^+$ reaction. Addition of Mn^{2+} had no effect on the enzyme system when NAD^+ replaced $NADP^+$.

the amount of NAD^+ reduced in the presence of acetyl-CoA, cysteine sulfinate or glutamate. The rate of NAD^+ reduction was found to be Mn^{2+} independent. The apparent K_m for malate under these conditions was found to be 1.8 mM (Fig. 2). Acetyl-CoA in this system is more than likely required for the removal of oxaloacetate (*via* citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7)) which is known to inhibit the NAD-linked malate dehydrogenase. The function of glutamate and cysteine sulfinate is presumably one involved in the removal of oxaloacetate by transamination. These results strongly indicated, therefore, that the accumulation of pyruvate in the malate *plus* arsenite incubations carried out in the absence of Mn^{2+} is brought about mainly as a result of the malate dehydrogenase (NAD-linked) activity.

TABLE II

CORTICOSTERONE FORMATION AND PYRUVATE ACCUMULATION IN INCUBATIONS CARRIED OUT WITH MALATE + 11-DEOXYCORTICOSTERONE AND MALATE + ARSENITE + 11-DEOXYCORTICOSTERONE IN THE PRESENCE OR ABSENCE OF Mn^{2+}

nmoles: nmoles pyruvate accumulated or corticosterone formed per 10 min per 1 mg mitochondrial protein. Malate and arsenite: 2 mM final concentration. Mn^{2+}: 300 μM. Pyruvate was estimated enzymatically using lactate dehydrogenase (see MATERIALS AND METHODS). Pyruvate concentration was related to the amount of NADH oxidized, assuming complete utilization of pyruvate to form lactate. Boiled mitochondria were carried through exactly the same incubation procedures and served as blanks in the enzyme assay determinations.

Addition	*Pyruvate accumulated (nmoles)*	*Corticosterone formed (nmoles)*
Malate alone	4.8	43.7
Malate alone + Mn^{2+}	3.8	40.6
Malate + arsenite	65.8	10.8
Malate + arsenite + Mn^{2+}	83.0	10.3

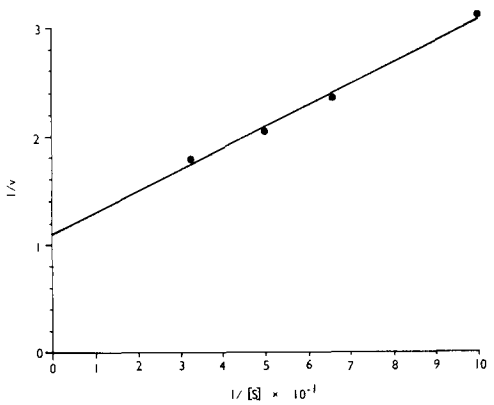

Fig. 2. Double reciprocal plot of malate concentration *versus* change in absorbance at 340 nm. The enzyme assay was carried out as described in MATERIALS AND METHODS with different concentrations of malate. Addition of P_i, ATP or Mn^{2+} to the enzyme system had little or no effect on the rate of NAD^+ reduction. NAD concentration used was 0.5 μmole/ml; acetyl-CoA, 0.5 μmole. v, change in absorbance/min; [S], concentration of malate (mM). Apparent K_m for malate, 1.8 mM. Under identical conditions the apparent K_m for malate in the NADP-linked malate dehydrogenase reactions, 27 mM. For optimal activity of the NADP-linked enzyme, Mn^{2+} was required.

TABLE III

THE EFFECT OF P_i, ATP AND OTHER PHOSPHORYLATED COMPOUNDS ON 11β-HYDROXYLATION OF 11-DEOXYCORTICOSTERONE BY RAT ADRENAL MITOCHONDRIA INCUBATED WITH MALATE AND PYRUVATE

Final concentration of substances was 2 mM except where designated. In other experiments, not shown, corticosterone production in presence of 2 mM malate was maximally stimulated by 1 mM P_i and 2 mM ATP, respectively, whereas with 2 mM pyruvate, the addition of 2 mM ATP led to maximal corticosterone production.

Addition	*Expt. 1*	*Addition*	*Expt. 1*	*Addition*	*Expt. 2*	*Addition*	*Expt. 3*
Malate	47.6	Pyruvate	17.7	No substrate + ATP	2.2	Malate	59.4
Malate + P_i	78.5	Pyruvate + P_i	20.7	ATP + pyruvate (10 μM)	5.2	Pyruvate	26.9
Malate + ATP	81.2	Pyruvate + ATP*	81.2	ATP + pyruvate (20 μM)	9.5	Malate + pyruvate	134.5
Malate + ADP	96.7	Pyruvate + ADP	23.3	ATP + pyruvate (50 μM)	22.8		
Malate + AMP	58.8	Pyruvate + AMP	14.8	ATP + pyruvate (100 μM)	48.4		*Expt. 4*
Malate + GTP	75.8	Pyruvate + GTP	13.6	ATP + pyruvate (500 μM)	63.3	Malate	52.1
Malate + 3′,5′-AMP	49.1	Pyruvate + 3′,5′-AMP	14.8	ATP + pyruvate (1 mM)	72.1	Malate + pyruvate (40μM)	70.4
Malate + cysteine sulfinate	92.6	Phosphoenolpyruvate	4.6	ATP + pyruvate (5 mM)	62.0	Malate + pyruvate (120μM)	83.2
						Malate + pyruvate (200μM)	99.1
						Malate + pyruvate (1 mM)	87.0

* Final concn., 1 mM.

Further support of this idea was the observation that oxaloacetate incubated either with the buffer (pH 7.4) or with rat adrenal mitochondria *plus* buffer also gave rise to pyruvate at 37°, as determined by the enzyme assay and by the isolation of pyruvate using the thin-layer system of MYERS AND HUANG[13]. If the amount of pyruvate (65 nmoles), accumulated in the presence of arsenite (Table II), was derived *via* the NADP-linked malate dehydrogenase, the amount of NADPH formed would have been ample for 11β-hydroxylation.

From these results, we deduced that if the pyruvate or oxaloacetate metabolism could be inhibited in rat adrenal mitochondria, this probably would lead to an inhibition of the corticosterone production from 11-deoxycorticosterone. Conversely, stimulating or increasing the removal of oxaloacetate and pyruvate metabolism would lead to an increase in corticosterone production. The following experiments (Tables III–VI) were designed to test this deduction and are representative of many other results obtained.

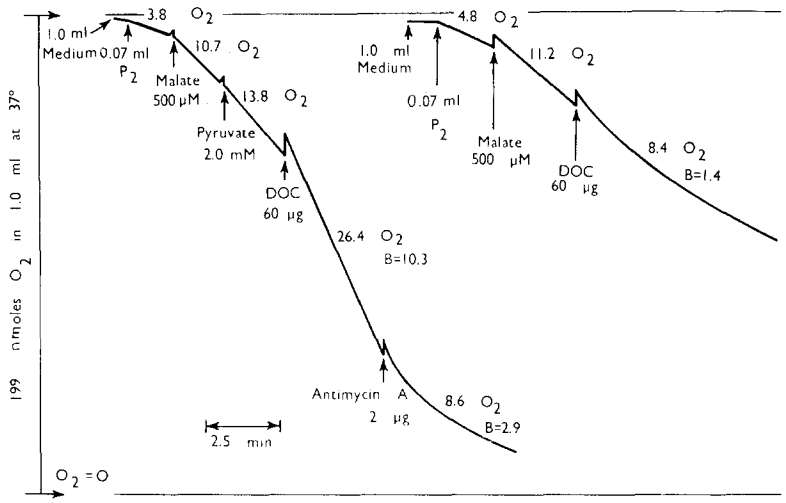

Fig. 3. Trace of O_2 uptake measured polarographically (see MATERIALS AND METHODS) at 37°. Incubation medium used was the same as that described in MATERIALS AND METHODS. Notations below the traces indicate sequential additions (from top to bottom) to the system. Notations above the traces report O_2 uptake prior to and after the additions at arrows, as well as the corticosterone rate (B), *i.e.* corticosterone production after addition of 11-deoxycorticostesone (DOC) to the system. Corticosterone concentration was determined in suitable aliquots withdrawn from the incubation system just before the designated additions shown below the traces. P_2, mitochondria.

As can be seen, the addition of malate to rat mitochondria resulted in a good production of corticosterone from 11-deoxycorticosterone (Table III). Addition of ATP or other phosphorylated compounds which might be expected to increase the uptake of malate or the removal of oxaloacetate[16] from rat adrenal mitochondria almost doubled corticosterone production. However, these compounds could be replaced by orthophosphate (P_i). On the other hand, a specific requirement of ATP for the efficient utilization of pyruvate was observed. The addition of pyruvate to malate also led to a greater utilization of malate for corticosterone production (Table III) and to a slight increase in the O_2 uptake by mitochondria and to a good corticosterone rate (Fig. 3). Both of these parameters of activity were inhibited by antimycin A.

As expected from the work of HASLAM AND KREBS[16], carried out with liver and heart mitochondria, additions of oxaloacetate to malate led to an oxidation of pyridine nucleotides (Fig. 4A), an inhibition of O_2 uptake (Figs. 4B, 4C) and an inhibition of the 11β-hydroxylation of 11-deoxycorticosterone (Table IV). Addition of cysteine sulfinate which removes oxaloacetate by transamination in heart mitochondria[16] also led to an increased utilization of malate as reflected in increased amounts of corticosterone

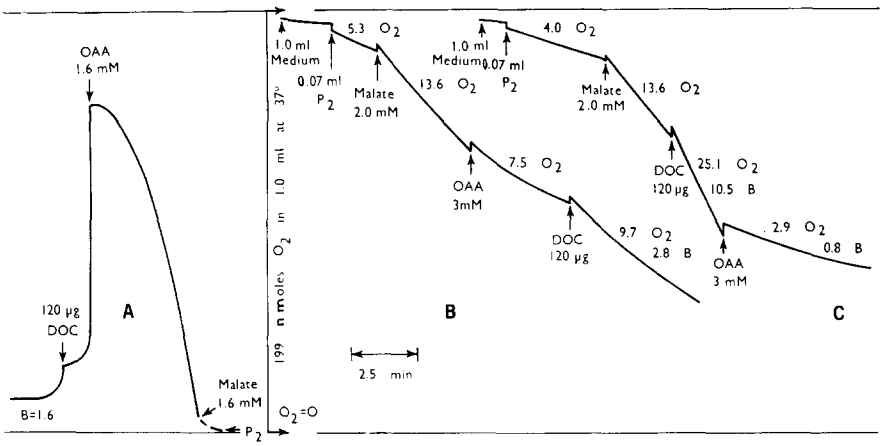

Fig. 4. (A) Effect of the addition of oxaloacetate on the steady state of pyridine nucleotide reduction brought about by malate in rat adrenal mitochondria incubated at 24°. Incubation conditions are those described in Fig. 1. (B and C) Inhibition of O_2 uptake and corticosterone production by oxaloacetate in rat adrenal mitochondria incubated with malate + 11-deoxycorticosterone at 37°. Incubation conditions are those mentioned in Fig. 3. Abbreviations: DOC, 11-deoxycorticosterone; OAA, oxaloacetate; B, corticosterone; P_2, rat adrenal mitochondria.

TABLE IV

THE EFFECT OF OXALOACETATE ON CORTICOSTERONE PRODUCTION BY RAT ADRENAL MITOCHONDRIA INCUBATED IN THE PRESENCE OF 11-DEOXYCORTICOSTERONE + MALATE, 11-DEOXYCORTICOSTERONE + PYRUVATE AND MALATE + PYRUVATE

Final concentrations of substances: 2 mM except values in parentheses designate incubations carried out with 500 µM pyruvate.

Additions	*Malate*		*Malate + pyruvate*	*Pyruvate + ATP*
Expt. 1				
—	75.7	17.8	139.7	
ATP	143.3	99.5	167.1	
Oxaloacetate	31.2	2.1	44.9	
Oxaloacetate + ATP	143.3	3.7	171.5	
Expt. 2				
—	44.8	26.7 (29.7)	—	
ATP	102.9	102.9 (99.7)	—	
Oligomycin (10 µg)	36.5	—	—	
P_i	101.5	—		
ATP + oligomycin (10 µg)	53.8	16.6		
Oxaloacetate (10 µM)	—	(45.5)		(125.3)
Oxaloacetate (100 µM)	—	(7.2)		(128.2)
Oxaloacetate (200 µM)	—	(5.1)		(122.1)
Oxaloacetate (3 mM)	29.9	—	—	

produced (Tables III and VI). *trans*-Aconitate which inhibits *cis*-aconitate decarboxylase (*cis*-aconitate carboxy-lyase, EC 4.1.1.6) was inhibitory at 10 mM, whereas inhibitors of the classical respiratory chain proved to be inhibitory at the concentrations designated in Table V. It is to be noted that oligomycin inhibited the ATP effect on pyruvate and on malate, whereas it has less effect on the 11β-hydroxylation of 11-deoxycorticosterone supported by P_i *plus* malate. The uncoupling effect of 2,4-dinitrophenol with malate is clearly seen in the Oxygraph experiments (Fig. 5) and led to an increased O_2 uptake as well as to a decreased corticosterone production (see Table V also). Addition of isocitrate to the uncoupled mitochondria led to a higher rate in O_2 uptake and reestablished a good rate of corticosterone production.

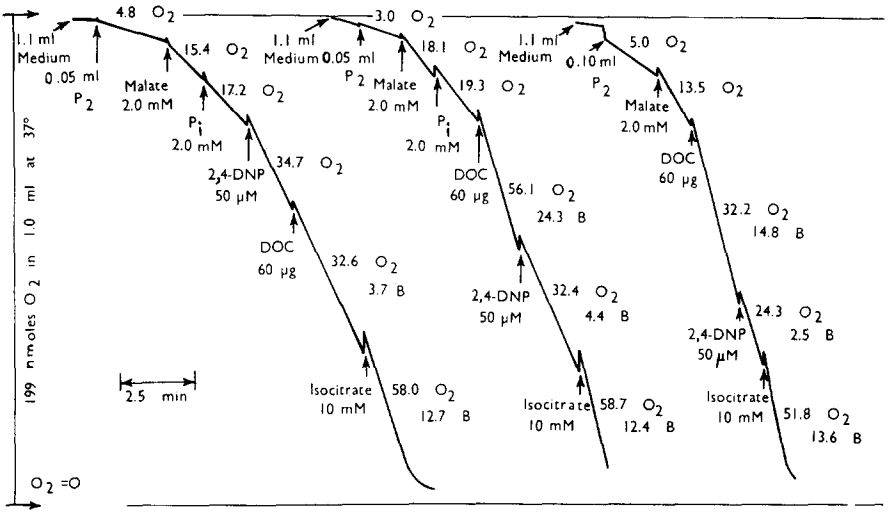

Fig. 5. The uncoupling effect brought about by a 50 μM concentration of 2,4-dinitrophenol in rat adrenal mitochondria incubated at 37° with malate and reflected in corticosterone production and O_2 consumption. Incubation conditions are those mentioned in Fig. 3. Abbreviations: 2,4-DNP, 2,4-dinitrophenol; DOC, 11-deoxycorticosterone; B, corticosterone; P_2, rat adrenal mitochondria.

The inhibitory effect on malate of the addition of oxaloacetate could be overcome by adding ATP to the system (Table IV) but not in the presence of oligomycin. Addition of small amounts of oxaloacetate (50 μM) to pyruvate (500 μM) or to pyruvate *plus* ATP led to an increase in pyruvate utilization as reflected by the increased amount of corticosterone produced (Table IV).

The last experiments (Table VI) were carried out to reaffirm our findings that oxaloacetate can give rise to pyruvate when incubated with buffer alone or when incubated in the presence of buffer *plus* mitochondria. Preincubation of oxaloacetate for 15 min in the presence or absence of the enzyme system (P_2 = rat adrenal mitochondria) led to some 11β-hydroxylation of 11-deoxycorticosterone in the final 10-min incubation period carried out in the presence of ATP (Conditions 1, 3, 4). When pyruvate was added to rat adrenal mitochondria prior to the 10-min final incubation, corticosterone production was small in the final incubation with ATP (Condition 7). On the other hand, if pyruvate was added to rat adrenal mitochondria and was kept on ice for 15 min (Condition 8) or if pyruvate was added just before the 10 min final

TABLE V

CORTICOSTERONE PRODUCTION FROM 11-DEOXYCORTICOSTERONE IN RAT ADRENAL MITOCHONDRIA INCUBATED IN THE PRESENCE OF RESPIRATORY CHAIN INHIBITORS, *trans*-ACONITATE, OLIGOMYCIN AND THE UNCOUPLER 2,4-DINITROPHENOL

Malate, ATP, P_i,pyruvate, all 2 mM final concentration.

Additions	*Malate*	*Malate* + ATP*	*Malate + P_i*	*Pyruvate + ATP*
—	—	53.5**	—	—
—	52.3	172.5	99.0	53.3
KCN (3 mM)	11.8	97.5	14.9	12.9
Amytal (5 mM)	6.6	18.1	8.9	2.4
Antimycin A (3 μg)	13.4	34.7	23.9	2.4
Oligomycin (10 μg)	38.9	92.3	60.2	8.9
2,4-Dinitrophenol (50 μM)	13.4	22.9	34.4	3.4
Arsenite (2 mM)	8.9	54.3	16.6	3.7
trans-Aconitate (10 mM)	3.2	5.5	3.9	1.8

* Different experiment.
** Value obtained with malate alone.

incubation (Condition 9) with ATP, a good corticosterone production was detected. Less corticosterone was produced in the experiment in which oxaloacetate was pre-incubated with rat adrenal mitochondria (Condition 1) compared to the experiments carried out in the absence of rat adrenal mitochondria (Conditions 3 and 4).

Whereas pyruvate was well utilized when ATP was added (Condition 10), the prensence of cysteine sulfinate lowered corticosterone production (Condition 12). In the presence of malate, cysteine sulfinate had the same effect as the addition of ATP to the incubation system (Conditions 13, 14, Table III also).

DISCUSSION

The inhibitory effect found with arsenite on the 11β-hydroxylation of 11-deoxycorticosterone by rat adrenal mitochondria incubated with malate was in contrast to that reported in bovine adrenal cortex mitochondria[3,6]. In bovine adrenal mitochondrial oxidation of malate occurs *via* the NADP-linked malate dehydrogenase[21] which is not inhibited by arsenite.

The data obtained in this paper as presented in the arsenite experiments, as well as those carried out with sonicated preparations, strongly indicate that in rat adrenal mitochondria malate is mainly oxidized *via* the NAD-linked malate dehydrogenase. Other data support this idea and some of the reasons for supporting this conclusion are enumerated as follows: (1) Malate utilization in rat adrenal mitochondria in the presence of arsenite resulted in the accumulation of pyruvate probably *via* its inhibitory action on pyruvate dehydrogenase (pyruvate:lipoate oxidoreductase (acceptor-acetylating), EC 1.2.4.1). This not only led to the accumulation of pyruvate but also of oxaloacetate, the latter in sufficient amounts to account for the oxidation of the pyridine nucleotide (NADH) as observed in the Eppendorf recording (Fig. 1). (2) Oxidation of malate by sonicated rat adrenal mitochondria *via* the NAD-linked malate dehydrogenase required the presence of acetyl-CoA (which removes oxaloacetate to form citrate) or substances (cysteine sulfinate, glutamate) which can remove oxaloacetate by transamination[16,22]. (3) While the removal of oxaloacetate by cysteine

TABLE VI

THE GENERATION OF PYRUVATE FROM OXALOACETATE IN RAT ADRENAL MITOCHONDRIA PREINCUBATED 15 min AT 37°

Final concentration of oxaloacetate and pyruvate: 100 μM except where designated; ATP, malate, cysteine sulfinate: 2 mM. The preincubation was carried out as usual at 37° except where (on ice) designate tubes were kept in ice for 15 min during the preincubation period. The final incubation period was carried out at 37°. P_2 = mitochondria.

Condition	*Substance present in 15-min preincubation period*	*Additions just prior to 10-min final incubation period*	*Corticosterone produced (nmoles) in 10 min final incubation*
Expt. 1			
1	P_2 + 11-deoxycorticosterone + oxaloacetate	ATP	9.5
2	P_2 + 11-deoxycorticosterone	Oxaloacetate + ATP	2.0
3	11-Deoxycorticosterone + oxaloacetate (50 μM)	P_2 + ATP	7.0
4	11-Deoxycorticosterone + oxaloacetate	P_2 + ATP	14.8
5	P_2 + 11-deoxycorticosterone + oxaloacetate (on ice)	ATP	2.0
6	P_2 + 11-deoxycorticosterone (on ice)	Oxaloacetate + ATP	1.5
7	P_2 + 11-deoxycorticosterone + pyruvate	ATP	2.9
8	P_2 + 11-deoxycorticosterone + pyruvate (on ice)	ATP	32.3
9	P_2 + 11-deoxycorticosterone (on ice)	Pyruvate + ATP	30.6
Expt. 2			
10	No preincubation	P_2 + 11-deoxycorticosterone + pyruvate (2 mM) + ATP	43.3
11	No preincubation	P_2 + 11-deoxycorticosterone + malate	42.6
12	No preincubation	P_2 + 11-deoxycorticosterone + pyruvate (2 mM) + ATP + cysteine sulfinate	28.5
13	No preincubation	P_2 + 11-deoxycorticosterone + malate + cysteine sulfinate	98.9
14	No preincubation	P_2 + 11-deoxycorticosterone + malate + ATP	125.7

sulfinate or pyruvate (Table III) led to an increased utilization of malate and corticosterone production, the addition of oxaloacetate brought about a decreased utilization of malate (by inhibiting malate dehydrogenase) and a corticosterone production. These observations, therefore, lead us to the conclusion that under physiological conditions, oxidation of malate occurs *via* oxaloacetate and pyruvate and that both of these latter substances are important in a feedback control on malate oxidation in rat adrenal mitochondria.

Enzyme assays on sonicated rat adrenal mitochondria showed that arsenite had no inhibitory effect on the NADP-linked isocitrate dehydrogenase. On the other hand, it was found that the addition of α-ketoglutarate reduced the rate of $NADP^+$ reduction by isocitrate. Since arsenite is known to inhibit α-ketoglutarate dehydrogenase (oxoglutarate dehydrogenase, 2- oxoglutarate:lipoate oxidoreductase (acceptor-acylating), EC 1.2.4.2), the action of arsenite on the utilization of isocitrate is probably due to the accumulation of α-ketoglutarate. This is another example of a feedback control mechanism which can occur in rat adrenal mitochondria.

For the first time pyruvate was shown to be utilized efficiently in rat adrenal mitochondria for corticosterone production from 11-deoxycorticosterone. More than likely, ATP was required for some oxaloacetate production which in turn was required for pyruvate metabolism *via* the citrate synthase. Small amounts of oxaloacetate added to pyruvate (no ATP present) led to some corticosterone production (Table IV); thus the above conclusion is supported. Thus, pyruvate metabolism in the presence of ATP yielded sufficient isocitrate to supply reducing equivalents (NADPH) for the 11β-hydroxylation of 11-deoxycorticosterone. As demonstrated in the Oxygraph studies (Fig. 3), the addition of pyruvate to malate led to an increased O_2 consumption and corticosterone production, also indicating an increased removal of oxaloacetate by pyruvate.

The P_i effect on the utilization of malate and reversal of oxaloacetate inhibition by ATP (Table IV) cannot be explained at the moment. On the other hand, the ATP effect (as well as that by GTP, ADP, Table III) is probably due to P_i liberated from the hydrolysis of ATP by ATPase (ATP phosphohydrolase, EC 3.6.1.3, EC 3.6.1.4) because oligomycin (Table VI) inhibited the ATP effect. It must be mentioned that L. A. SAUER, B. E. DREYER AND P. J. MULROW (personal communication) have also found a similar effect of P_i in rat adrenal mitochondria incubated with malate.

Addition of respiratory chain inhibitors as well as oxaloacetate to malate, malate *plus* pyruvate, malate *plus* P_i (or ATP) or pyruvate *plus* ATP decreased O_2 consumption and inhibited corticosterone production. Thus the production of NADPH by these substrates is related to an uninhibited respiratory chain. The amount of reducing equivalents (NADPH) needed for 11β-hydroxylation and arising from either the isocitrate metabolism or *via* the energy-linked transhydrogenase reaction[17,18] is unknown. Nevertheless, since 2,4-dinitrophenol uncoupled rat adrenal mitochondria as well as inhibited corticosterone production, it is indicated that not all NADPH production was the result of the isocitrate oxidation. It would appear, therefore, that some NADH arising from the oxidation of malate and from Krebs cycle intermediates gave rise to NADPH *via* the transhydrogenase reaction. The energy required for this reaction would be derived from respiratory chain activity and from the formation of high energy substances.

The experiments in Table VI serve to emphasize that the accumulation of

pyruvate in rat adrenal mitochondrial incubations with malate cannot be used as the sole index for the NADP-linked malate dehydrogenase activity. As was observed by HSU AND LARDY[14], oxaloacetate is an unstable compound and was decarboxylated to pyruvate with or without rat adrenal mitochondria. Whether oxaloacetate is decarboxylated spontaneously or enzymatically in rat adrenal mitochondria is a matter of conjecture at the present.

The data presented in this paper indicate, therefore, that the "malate shuttle" proposed in our former studies[7] and in those of other workers using bovine adrenal mitochondria[8] is not operative in rat adrenal cortex. Since pyruvate does not accumulate and is further metabolized by rat adrenal mitochondria when maximal corticosterone production and O_2 consumption occur, the provenance of pyruvate for the cytoplasmic malate dehydrogenase is not mitochondrial. Finally, NADPH required for the steroid hydroxylating chain in rat adrenal mitochondria is more than likely made available by the energy-linked transhydrogenase reaction as well as by the isocitrate oxidation which is known to give rise to NADPH production in rat adrenal mitochondria[4,9,12].

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