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THE POSSIBLE FUNCTION OF PYRUVATE IN THE STEROIDOGENIC RESPONSE OF RAT ADRENAL CELL SUSPENSIONS TO ACTH, CYCLIC-3′,5′-AMP AND DIBUTYRYL CYCLIC-3′,5′-AMP

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SUMMARY

Corticosterone synthesis and glycolysis were investigated in rat adrenal cell suspensions. Adrenocorticotropin (ACTH), adenosine-3′,5′-cyclic monophosphate and its dibutyryl derivative, N⁶-2¹-0-dibutyryl-adenosine-3′,5′-monophosphate were found to stimulate corticosterone synthesis as well as the formation of pyruvate and lactate. Arsenite prevented the utilization of pyruvate and also abolished the stimulation of corticosterone synthesis. Iodoacetate inhibited the formation of pyruvate with a concommitant inhibition of corticosterone synthesis which was partially overcome by the addition of pyruvate but not by citrate, isocitrate or malate. The results indicate that the formation and utilization of pyruvate are essential, perhaps obligatory, for ACTH to exert its steroidogenic effect in the rat adrenal and strengthen our earlier suggestion that pyruvate may be the physiological substrate utilized by the mitochondria of the rat adrenal cell to make available intramitochondrial NADPH during ACTH stimulated steroidogenesis.

INTRODUCTION

THE ROLE played by glycolysis in the steroidogenic action of ACTH in the rat adrenal gland has been indicated by Bell *et al.* [1]. After the *in vivo* administration of ACTH in hypophysectomized rats, crossovers in substrate concentrations at the level of phosphofructokinase (E.C. 2.7.1.11) and glyceraldehyde dehydrogenase (E.C. 1.2.1.12) enzymes were found. This not only showed that an activation of these enzymes occurred in the stimulated adrenals but suggests that end-products of glycolysis, pyruvic and lactic acids, might be produced in greater amounts than in the non-ACTH activated glands. In the studies carried out *in vitro* with rat adrenal sections [2] it was found that addition of cyclic-AMP, the mediator of ACTH action at the adrenal level [3–5], caused a large increase in the amount of lactic acid and corticosterone released into the incubation medium but led to little change in the amount of pyruvic acid when the incubations were carried out in the presence or absence of glucose. Nevertheless, the sum of the lactate and pyruvate produced by the sections was always 2–3 times greater when optimal levels of cyclic-AMP were present in the incubation medium than

Abbreviations and trivial names: ACTH, adrenocorticotropic hormone; CAMP, cyclic-AMP, adenosine-3′,5′-cyclic monophosphate; DBCAMP, N^6-2^1-0-dibutyryladenosine-3′,5′-cyclic monophosphate; TCA, trichloracetic acid; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; DOC, 21-hydroxy-4-pregnen-3-one.

when this cyclic nucleotide was absent. This showed that cyclic-AMP does indeed increase glycolysis and supports the conclusion derived from the *in vivo* studies of Bell *et al.* [1]. Other data obtained in our *in vitro* incubations carried out in the presence of the glycolytic inhibitors 2-deoxyglucose and iodoacetate [2] led us to conclude that perhaps the function of glycolysis in the rat adrenal gland was to provide lactate or more probably pyruvate as oxidizable substrate(s) for the mitochondria of the adrenal cell which must produce reducing equivalents (NADPH) for mitochondrial steroid hydroxylations [6–14]. This conclusion is not only supported by the fact that additions of pyruvate or that which can be derived from glucose-6-phosphate to cell-free preparations of rat adrenal tissue [15, 16] leads to efficient 11β-hydroxylation of 11-deoxycorticosterone (DOC) but by the finding that in the absence of a malate shuttle in this tissue [31] as contrasted to its presence in the bovine adrenal cortex [17–19], pyruvate might be the substrate more likely to be made available by a normal glycolyzing adrenal cell for intramitochondrial NADPH production. The fact that when pyruvate oxidation was inhibited by the presence of arsenite (inhibits mitochondrial pyruvate dehydrogenase E.C. 1.2.4.1), cyclic-AMP had no stimulatory effect on steroidogenesis [2] also lends support to the above thesis. Thus, even though cyclic AMP greatly stimulated glycolysis as reflected by the substantial accumulation of lactate and pyruvate in these experiments, steroidogenesis did not occur. These findings coupled to those reported in the same study that pyruvate addition in the absence of cyclic AMP does not lead to corticoid biosynthesis led us to suggest that pyruvate production and utilization might be an obligatory accompaniment for the manifestation of the steroidogenic action of cyclic-AMP. The function of lactate production in the steroidogenic action of cyclic-AMP (or ACTH), however, remained obscure although lack of O_2 diffusion and a predominant anaerobic state of some of the innermost cells of the incubated rat adrenal sections may have been responsible for the accumulation of lactate. If this was the case, one might expect when O_2 is in short supply, that the NADH formed during glycolysis (at the glyceraldehyde-3-phosphate dehydrogenase level) would be oxidized by lactic acid dehydrogenase of the adrenal and lead to lactic acid formation from pyruvate. We therefore sought an incubation system which would minimize as much as possible the problem of anaerobiosis and also allow us to carry out experiments with ACTH as well as cyclic-AMP where conditions for pyruvate formation and presumably its utilization would be optimal. We decided to conduct such experiments with rat adrenal cell suspensions prepared by means of proteolytic enzymes essentially as described by several investigators [20–24]. It was reasoned that when the dissociated adrenal cells were suspended and incubated in a buffer medium gassed with 95% O_2–5% CO_2 these would not be subjected to anaerobic conditions since O_2 would saturate the incubation system used. This would allow for an easy diffusion of O_2 inside the cell. Although pyruvate production might be expected to be decreased (because of the Pasteur effect) concommitantly with a decreased glycolysis, lactate production on the other hand should be reduced drastically. With the above incubation system we could reasonably expect therefore, a clearer picture of the function of pyruvate in ACTH and cyclic-AMP initiated steroidogenesis to be painted.

We wish to report results of such studies and bring attention to some of the difficulties which have been encountered in terms of evaluating the role played by pyruvate in the steroidogenic action of ACTH and cyclic-AMP.

EXPERIMENTAL

Chemicals. ACTH used in this study was the U.S.P. corticotropin reference standard distributed by U.S. Pharmacopeial Convention, Inc. Cyclic-3',5'-AMP (CAMP), dibutyryl cyclic-3',5'-AMP (DBCAMP), NAD^+, NADH, lactate dehydrogenase, were purchased from Sigma Chemical Co.; trypsin, collagenase and lima bean trypsin inhibitor from Worthington Biochemical Corp. and bovine serum albumin (BSA), fraction V powder, from Pentex, Incorp. All other chemicals were of reagent grade purity obtained from various sources.

Preparation of Cell Suspensions. The technique of preparing cell suspensions was essentially that of Sayers *et al.*[23] with some changes. The following solutions were prepared in Krebs–Ringer bicarbonate buffer, pH 7·2, containing 2 mg glucose per ml (KRBG).

Solution A – 150 units of collagenase per ml of KRBG.

Solution B – 2·5 mg of trypsin per ml of KRBG.

Solution C – 3 mg of trypsin inhibitor + 7·5 mg of BSA per ml of KRBG.

Adrenals from 16–20 male Sprague–Dawley rats (200–250 g) were collected immediately after decapitation in ice-cold KRBG. After removing the adhering fat, the glands were carefully cut into 8–12 approximately equal pieces and placed in a 25 ml Erlenmeyer flask containing 2 ml of Solution A. The flask was gently swirled by hand for a 1 min period, 18 ml of Solution B was added and two glass marbles 1 cm dia. were introduced into the flask. The latter was next incubated in a Dubnoff metabolic incubator for 20 min with constant agitation (100 shakes per min.) at 37°C in an atmosphere of 95% O_2 : 5% CO_2. After this period of incubation the flask was placed on crushed ice to allow intact tissues to settle. The supernatant containing isolated cells was transferred to another flask and the collagenase-trypsin treatment repeated 4–6 times more. The pooled supernatant from all treatments was centrifuged at 100 *g* for 40 min at 4°C. Final speed was attained gradually in the first 10 min. The resulting supernatant was discarded and the sedimented cells were suspended in a known volume of Solution C. Small aliquots of this suspension were mixed with Eosin Y, Neisser's A or Neisser's B stain [25] and the cells counted in a hemacytometer. Even though the above procedure for preparing the different batches of cells was followed rigorously on different days of experimentation the yield of cells varied from 100,000–250,000 per adrenal.

Incubations. All incubations were performed in duplicate in 10 ml beakers and the values reported in the tables represent the mean of the duplicates. In most if not all cases the individual values obtained were within 10% of one another. Each beaker contained 1·0 ml of cell suspension, test substance(s) dissolved in KRBG and an appropriate volume of KRBG to make the final volume 1·5 ml. All incubations were carried out for 2 h, unless stated otherwise, in a Dubnoff metabolic shaker (64 shakes per min.) at 37°C in an atmosphere of 95% O_2 : 5% CO_2.

All glassware that came in contact with the cells was siliconized by rinsing the articles used in Siliclad (Siliclad, Clay Adams).

At the end of the incubation period the beakers were immediately chilled in ice and appropriate aliquots of the cell suspensions immediately transferred to tubes containing aqueous ethanol (13% v/v) for fluorometric determination of corticosterone by the method of Silber *et al.*[26]. Following the removal of the above aliquots for corticosterone determination, 10% aqueous TCA was added to the remainder of the incubate (0·3 ml per one ml incubate) and the precipitated proteins removed by centrifugation. The supernatant from the hard packed pellet

was transferred to clean tubes and neutralized to a pH of 7·0 with 1N NaOH. Appropriate aliquots of the neutralized supernatant were taken for the measurement of pyruvate and lactate by the enzymatic method using lactate dehydrogenase as described before [16].

All values shown for corticosterone, pyruvate and lactate in the tables, are the net values obtained after substracting the zero time controls and are reported in mμmoles/beaker/2 h incubation except where designated.

RESULTS

Response of rat adrenal cell suspensions to ACTH, CAMP *and* DBCAMP

The first experiments were carried out to make certain that we could prepare viable cells which would respond to ACTH and CAMP. The intricacies of preparing cell suspensions had become evident to us when in reviewing the literature we found that several laboratories reported different degrees of steroidogenic response to ACTH in their cell suspensions [21–24]. Control experiments carried out in the absence of ACTH, CAMP and DBCAMP showed that even in a two hour incubation the synthesis of corticosterone from endogenous precursors was negligible; often zero and always less than 0·1 μg. The cells, however, responded to small amounts of the three stimulating agents with increased corticosterone synthesis (Table 1). The effect of ACTH was quite evident at 100 μU/ml, although not at 10 μU/ml, and levelled off at 1–2 mU/ml. Sayers *et al.* [23] and Kitabchi *et al.* [24] have reported maximal stimulation of steroidogenesis with smaller amounts of ACTH. Our data on the other hand is similar to that published by Haning *et al.* [22].

Table 1. The steroidogenic effect of ACTH, CAMP and DBCAMP in rat adrenal cell suspensions

Expt. 1		Expt. 2			
ACTH Added	Corticosterone formed	CAMP Added	Corticosterone formed	DBCAMP Added	Corticosterone formed
1 μU/ml	0	0·4 mM	0·32	0·04 mM	1·85
10 μU/ml	0	1·0 mM	0·66	0·1 mM	3·58
100 μU/ml	1·27	2·0 mM	2·77	0·2 mM	4·60
500 μU/ml	6·01	5·0 mM	4·45	0·5 mM	5·03
1 mU/ml	7·31	9·0 mM	5·03	1·0 mM	5·03
2 mU/ml	7·74				
5 mU/ml	8·18				

Each beaker contained 270,000 cells in Expt. 1 and 100,000 cells in Expt. 2.

Maximal stimulation of corticosterone synthesis with DBCAMP and CAMP was obtained around 0·5 and 10 mM concentrations of the two nucleotides respectively and agreed with the results of Kitabchi *et al.* [24]. Table 2 shows that ACTH, CAMP and DBCAMP also stimulated glycolysis. Increased amounts of both pyruvate and lactate were obtained after a 2 h incubation with all three agents. Having thus established that we could obtain cell suspensions which could be used in ACTH and CAMP studies we proceeded with the other experiments outlined below taking care to always use exactly the same procedure outlined in the Materials and Methods section for the preparation of the cell suspensions.

Table 2. The effect of ACTH, CAMP and DBCAMP on glycolysis in rat adrenal cell suspensions

Additions	Pyruvate	Lactate	Pyruvate + Lactate
None	8·3	1·7	10·0
ACTH 1 mU/ml	16·6	15·6	32·2
ACTH 5 mU/ml	18·7	32·8	51·5
ACTH 10 mU/ml	22·8	26·6	49·4
CAMP 2 mM	10·4	22·4	32·8
CAMP 5 mM	14·5	15·8	30·3
CAMP 10 mM	16·6	28·2	44·8
DBCAMP 0·2 mM	12·5	30·7	43·2
DBCAMP 0·5 mM	12·5	40·6	53·1
DBCAMP 1·0 mM	16·6	32·4	49·0

Each beaker contained 100,000 cells.

Effect of arsenite on corticosterone synthesis and on the accumulation of pyruvate and lactate

Arsenite is known to inhibit the utilization of pyruvate by the mitochondrial enzyme pyruvate dehydrogenase and leads to pyruvate and lactate accumulation in rat adrenal gland sections incubated *in vitro*[2]. If the same arsenite inhibition prevailed in the adrenal cell suspensions used in these experiments then pyruvate and perhaps lactate could reasonably be expected to accumulate when the cells were stimulated with ACTH, CAMP and DBCAMP.

Table 3, shows that in the control incubations pyruvate and lactate were present in almost equal amounts. With 10 mU ACTH there was an increased

Table 3. Effect of ACTH and arsenite on the accumulation of pyruvate and lactate and synthesis of corticosterone in rat adrenal cell suspensions

Time of incubation	30 min			60 min			120 min		
Additions	P	L	B	P	L	B	P	L	B
Control	2·1	3·5	0	8·3	12·5	0	20·8	18·0	0
ACTH 10 mU/ml	8·3	13·6	1·3	16·6	16·4	3·8	37·3	46·3	7·9
Arsenite 2 mM	12·5	15·9	0	27·0	35·0	0	62·2	77·4	0
ACTH 10 mU/ml + Arsenite 2 mM	16·6	29·0	0	29·3	51·2	0	68·4	85·1	0

Each beaker contained 250,000 cells. All values, are the net values after subtracting zero time controls and are reported in mμ moles/beaker for the incubation times designated. P = Pyruvate, L = Lactate and B = Corticosterone.

accumulation of these substances at the time periods studied. Arsenite, whether added with or without ACTH also caused an accumulation of pyruvate and lactate. However, when arsenite was present, corticosterone synthesis was abolished.

A similar effect of arsenite on steroidogenesis was obtained with CAMP and DBCAMP as the stimulating agents (Table 4).

Table 4. Effect of arsenite on corticosterone synthesis in rat adrenal cell suspensions incubated with ACTH, CAMP and DBCAMP

Additions	Corticosterone formed
None	0
Arsenite 2 mM	0
ACTH 10 mU/ml	7·37
ACTH 10 mU/ml + Arsenite 2 mM	0
CAMP 5 mM	6·04
CAMP 5 mM + Arsenite 2 mM	0
DBCAMP 0·5 mM	7·28
DBCAMP 0·5 mM + Arsenite 2 mM	0

Each beaker contained 180,000 cells.

Effect of iodoacetate on glycolysis and steroidogenesis

Iodoacetate which was shown to almost completely inhibit corticosterone, lactic and pyruvic acid production in incubated rat adrenal sections [2] was next tested with the cell suspensions. This inhibitor of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase level would be expected, if permeable to the cell membrane, to inhibit pyruvate and lactate production leading perhaps to an inhibition of the steroidogenic action of ACTH. Table 5 shows that iodoacetate did indeed inhibit glycolysis as evidenced by the decrease in the accumulation of pyruvate and lactate. Accompanying the inhibition of glycolysis was an inhibition of corticosterone synthesis which was almost complete at 600 μM levels of addition of iodoacetate. Whereas the presence of 5 mM pyruvate *alone* with the incubated cells had absolutely no effect on corticosterone production, its addition to those incubated with ACTH but inhibited by iodoacetate reversed, albeit only partially, the iodoacetate inhibitory effect at all concentrations of iodoacetate tested. Similar effects of iodoacetate were obtained when DBCAMP was substituted for ACTH (Table 6).

Table 5. Inhibition by iodoacetate of the glycolytic and steroidogenic response of rat adrenal cells to ACTH

Experiment	Additions	No pyruvate added	5 mM pyruvate added	No pyruvate added		
		Corticosterone		Pyruvate	Lactate	Pyruvate + Lactate
1	None	0	0	19·9	31·2	51·1
	ACTH 10 mU/ml	10·34	9·65	41·9	59·6	101·5
	ACTH 10 mU/ml + Iodoacetate 50 μM	5·40	6·33	17·7	34·2	51·9
2	None	0	0	31·6	45·6	77·2
	ACTH 10 mU/ml	12·66	11·50	53·1	103·6	156·7
	ACTH 10 mU/ml + Iodoacetate 100 μM	3·55	6·01	5·1	17·4	22·5
	ACTH 10 mU/ml + Iodoacetate 200 μM	1·65	4·39	1·7	12·4	14·1
	ACTH 10 mU/ml + Iodoacetate 400 μM	0·95	2·98	1·7	0	1·7
	ACTH 10 mU/ml + Iodoacetate 600 μM	0·38	1·79	0	0·8	0·8
	ACTH 10 mU/ml + Iodoacetate 800 μM	0·38	1·79	0	6·6	6·6

Each beaker contained 280,000 cells in Expt. 1 and 350,000 cells in Expt. 2.

Table 6. Inhibition by iodoacetate of the glycolytic and steroidogenic response of rat adrenal cells to DBCAMP

Additions	No pyruvate added	5 mM pyruvate added	No pyruvate added		
	Corticosterone		Pyruvate	Lactate	Pyruvate + Lactate
None	0	0	9·9	3·3	13·2
DBCAMP 0·5 mM	4·33	4·48	13·2	18·5	31·7
DBCAMP 0·5 mM + Iodoacetate 50 μm	2·66	3·64	9·9	4·9	14·8
DBCAMP 0·5 mM + Iodoacetate 100 μm	2·02	2·60	3·3	4·1	7·4
DBCAMP 0·5 mM + Iodoacetate 400 μm	0·98	2·05	0·0	0·6	0·6

Each beaker contained 180,000 cells.

Effect of Krebs cycle substrates on iodoacetate inhibition of corticosteroid synthesis

Because pyruvate was found to overcome the iodoacetate inhibition of steroid synthesis only partially, it was thought possible that this incomplete reversal of the iodoacetate block might be due to non-specific inhibition by iodoacetate of some other enzyme(s) involved in the complex machinery of corticosterone biosynthesis. One can reason that if iodoacetate could partially block enzymes like pyruvate dehydrogenase or one of the several enzymes involved in steroid hydroxylation, then addition of pyruvate to the iodacetate inhibited system might lead to only a partial restoration of corticosterone synthesis (see Table 5). Thus it was decided to test the effectiveness of some Krebs cycle intermediates in overcoming the inhibitory effects of iodoacetate in the ACTH stimulated cells. Since oxidation of these substrates occurs after the pyruvate dehydrogenase enzymatic step it was thought possible that their addition to the iodoacetate blocked system might provide optimal intramitochondrial NADPH levels necessary to establish maximal corticosterone synthesis. Tables 7 and 8 show representative results of several experiments. As found previously (Table 5), the addition of pyruvate removed the iodoacetate inhibition only partially. Addition of 10 or 50 μM oxaloacetate (which might have become rate limiting in the cells because of a possible inhibition of pyruvate carboxylase by iodoacetate) had no effect. Similar

Table 7. Effect of oxaloacetate + pyruvate on iodoacetate inhibition of corticosterone synthesis

Additions	Corticosterone formed
None	0
Oxaloacetate 10, 50 or 100 μM	0
ACTH 10 mU/ml	4·83
ACTH 10 mU/ml + Iodoacetate 200 μM	0·84
ACTH 10 mU/ml + Iodoacetate 200 μM + Pyruvate 5 mM	2·02
ACTH 10 mU/ml + Iodoacetate 200 μM + Pyruvate 5 mM + Oxaloacetate 10 μM	2·17
ACTH 10 mU/ml + Iodoacetate 200 μM + Pyruvate 5 mM + Oxaloacetate 50 μM	2·17

Each beaker contained 150,000 cells.

Table 8. Effect of some Krebs cycle acids on corticosterone synthesis in the presence of ACTH or ACTH + iodoacetate

Additions		ACTH 10 mU/ml	ACTH 10 mU/ml + Iodoacetate 200 μM
		Corticosterone formed	
None		3·49	0·95
Pyruvate	5 mM		2·14
	10 mM	3·93	2·28
Citrate	5 mM		0·61
	10 mM	3·47	0·0
Isocitrate	5 mM		0·87
	10 mM	3·70	0·72
Malate	5 mM		0·66
	10 mM	3·18	0·46

Each beaker contained 150,000 cells.

results were obtained with 10 or 50 μM citrate, isocitrate and malate (not shown in the table) which are known to prime pyruvate utilization and NADPH production at these levels of concentration in mitochondrial incubations[16]. Table 8 also shows that while 5 mM pyruvate as usual was effective in partially removing the iodoacetate inhibition, other Krebs cycle intermediates at concentrations as high as 10 mM were not.

To test the possibility that oxaloacetate accumulated rather than was limiting in the incubations where iodoacetate and pyruvate were present, and was thereby inhibiting the enzymes of the Krebs cycle[27], incubations with glutamate and cysteine sulfinate were carried out. Addition of these substances would be expected to reduce the concentration of oxaloacetate in the cells by virtue of transamination[28, 29] and thus lead to the reestablishment of NADPH production for corticosterone synthesis. The data in Table 9 shows that neither glutamate nor cysteine sulfinate had an additional effect on the pyruvate reversal of iodo-

Table 9. Effect of glutamate and cysteine sulfinate on corticosterone synthesis by rat adrenal cells incubated in the presence of ACTH and ACTH + iodoacetate

Additions	ACTH 10 mU/ml	ACTH 10 mU/ml + Iodoacetate 200 μM
	Corticosterone formed	
None	5·43	1·16
Pyruvate 5 mM	5·23	2·63
Glutamate 5 mM	5·23	1·16
Cysteine sulfinate 5 mM	4·57	1·30
Pyruvate 5 mM + glutamate 5 mM	—	2·14
Pyruvate 5 mM + cysteine sulfinate 5 mM	—	2·69

Each beaker contained 180,000 cells.

acetate inhibition whereas when 10 mM malate was used in rat adrenal mitochondria incubations which probably lead to oxaloacetate (OAA) accumulation [31], OAA inhibition was removed and effective mitochondrial 11β-hydroxylation took place.

The 11β-hydroxylation of DOC in adrenal cells

The failure to demonstrate a reestablishment of ACTH-initiated steroidogenesis by some Krebs cycle intermediates (Table 8) which could act as substrates in the iodoacetate inhibited cell suspensions needed clarification. As an initial attempt to see if this phenomenon might not be due to lack of substrate uptake by the incubated cells, these were incubated in the presence of the various Krebs cycle intermediates and pyruvate shown in Table 10. It could be assumed, if these were freely diffusible into the cells, that they might be present at the mitochondrial level at sufficiently high concentrations to support 11β-hydroxylation of DOC, a reaction well-known to occur in incubated rat adrenal mitochondria fortified with these substrates [30]. Although, as previously shown (Table 1), the cell suspensions were unable to synthesize corticosterone from endogenous substrates in the absence of ACTH or CAMP, they did convert exogenously added DOC to corticosterone. This conversion was enhanced several fold by 5 mM pyruvate additions. On the other hand, substrate supported 11β-hydroxylation of DOC by 5 mM citrate, isocitrate or malate was not observed. Thus, even though our results clearly indicated an availability of DOC and pyruvate to the mitochondria of the adrenal cells it would appear that the latter substrates are not permeable to the outer cell membrane of the cells in our preparation. Alternatively, these substrates may not have diffused within the cells in a manner making them available for oxidation and NADPH production at the mitochondrial level.

Table 10. Inability of some Krebs cycle intermediates to support 11β-hydroxylation of DOC in adrenal cell suspensions

Additions	DOC 230 μM	DOC 230 μM + Iodacetate 200 μM
	Corticosterone formed	
None	15·9	7·8
Pyruvate 5 mM	54·6	44·2
Isocitrate 5 mM	16·5	8·7
Citrate 5 mM	12·1	6·4
Malate 5 mM	17·1	7·5

Each beaker contained 180,000 cells.

DISCUSSION

Although it is difficult to make a quantitative comparison of results obtained in the present study using cell suspensions with those obtained with adrenal quarters [2], in both tissue preparations increase in glycolysis accompanied the stimulation of steroidogenesis by ACTH, CAMP or DBCAMP. This was reflected in the increased accumulation of both pyruvate and lactate in response to the above mentioned stimulating agents (Table 2). However, the interesting finding was made that the ratio of lactate to pyruvate in the experiments with adrenal sections was about 10 : 1 [2], whereas in the experiments reported here the two substances were found in almost equal amounts and the ratio never exceeded 2 : 1. Whether the increase in lactate is solely an accompaniment of increased pyruvate formation in our cell suspensions and has some function in the steroidogenic action of ACTH remains unknown. It is doubtful that lactate

formation might be due to anaerobic conditions prevailing in the buffer medium used to incubate the suspended cells since this was gassed with an atmosphere of 95% O_2:5% CO_2 throughout the incubation period. On the other hand, it would appear that pyruvate derived from glucose plays a supportive, perhaps obligatory, role in the steroidogenic action of ACTH. This conclusion is supported by the experiments where addition of arsenite led to the accumulation of pyruvate (and lactate) and almost completely abolished the ACTH stimulation of corticosterone synthesis. Probably pyruvate utilization was inhibited in the cell suspensions by arsenite resulting in a reduced flux in the Krebs cycle and decreased formation of intramitochondrial NADPH. It is well known that generation of intramitochondrial NADPH is essential for the conversion of cholesterol to pregnenolone and 11β-hydroxylation of DOC. In the presence of arsenite, ACTH action in terms of protein synthesis [31–34], activation of kinases [35, 36] etc. may have been manifested but due to the lack of sufficient intramitochondrial reducing pressure final expression of ACTH effect, i.e. stimulation of corticosterone synthesis was abolished.

That pyruvate production may be an obligatory accompaniment for the steroidogenic effect of ACTH is also supported by the results of iodoacetate experiments which show that inhibition of pyruvate formation is accompanied by a decrease in corticosterone synthesis. Even though addition of exogenous pyruvate reestablished corticosterone synthesis only partially it is clear that of all the substances tested, pyruvate was the only one utilized by the cells to overcome the iodoacetate inhibition. The reason why exogenous pyruvate does not overcome the iodoacetate inhibition completely in the cell suspensions or the adrenal quarters [2] remains obscure.

Attempts to reestablish corticosterone production in the iodoacetate blocked cells with several Krebs cycle intermediates (citrate, isocitrate and malate) were unsuccessful; little or no effect was observed even when these substances were added at concentrations which are known to support steroid hydroxylations in the mitochondrial preparations [30]. The lack of effect of these substrates may have been due to their inability to permeate the cells in our preparation as was indicated in incubation experiments with exogenously added DOC (Table 10). Tracer studies with radioactive Krebs cycle intermediates should clarify this point at a future date.

If the results of these experiments reflect physiological events within the cell, the data obtained in the experiments with iodoacetate and arsenite strengthen the conclusion that pyruvate might be the "physiological" substrate utilized by the rat adrenal mitochondria to make available intramitochondrial NADPH during ACTH stimulated steroidogenesis. This conclusion would also appear tenable in view of the fact that rat adrenal cells do not appear to possess an active malate shuttle [37].

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REFERENCES

1. Bell J., Brooker G. and Harding B. W.: *Biochem. biophys. Res. Comm.* **44** (1970) 938.
2. Tsang C. P. W. and Péron F. G.: *Steroids* **17** (1971) 453.

3. Haynes R. C. and Berthet L.: *J. biol. Chem.* **225** (1957) 115.
4. Sutherland E. W., Oye E. and Butcher R. W.: *Rec. Progr. Horm. Res.* **21** (1965) 623.
5. Robison, G. A., Butcher R. W. and Sutherland E. W.: *An. Rev. biochem.* **37** (1968) 149.
6. Sweat M. L. and Lipscomb M. D.: *J. Am. Chem. Soc.* **77** (1955) 845.
7. Cooper D. Y., Narasimhulu S., Rosenthal O. and Estabrook R. W.: In *Functions of the Adrenal Cortex* (Edited by K. W. McKerns). Appleton-Century-Crofts, New York, Vol. 2 (1966) p. 897.
8. Harding B. W., Bell J. J., Oldham S. B. and Wilson L. D.: Ibid p. 831.
9. Milton J. R. and Boyd G. S.: *Proc. biochem. Soc.* (*London*) **103** (1967) 17.
10. Simpson E. R. and Boyd G. S.: *Eur. J. Biochem.* **2** (1967) 275.
11. Simpson E. R. and Boyd G. S.: *Eur. J. Biochem.* **22** (1971) 489.
12. Koritz S. B. and Péron F. G.: *J. biol. Chem.* **230** (1958) 343.
13. Péron F. G. and McCarthy J. L.: In *Functions of the Adrenal Cortex*, (Edited by K. W. McKerns). Appleton-Century-Crofts, New York, Vol. 1 (1966) p. 261.
14. Péron F. G.: In *Protein and Polypeptide Hormones*, (Edited by M. Margoulies). *Exc. Med. Found.* Amsterdam, Vol. 3 (1969) p. 768.
15. Tsang C. P. W. and Péron F. G.: *Steroids* **16** (1970) 41.
16. Tsang C. P. W. and Péron F. G.: *Steroids* **15** (1970) 251.
17. Simpson E. R. and Estabrook R. W.: *Arch. biochem. Biophys.* **126** (1968) 977.
18. Simpson E. R., Cammer W. and Estabrook R. W.: *Biochem. biophys. Res. Comm.* **31** (1968) 113.
19. Simpson E. R. and Estabrook R. W.: *Arch. biochem. Biophys.* **129** (1969) 384.
20. Halkerston I. D. K. and Feinstein M.: *Fed. Proc.* **27** (1968) 27.
21. Kloppenberg P. W. C., Island D. P., Liddle G. W., Michelakis A. M. and Nicholson W. E.: *Endocrinology* **82** (1968) 1053.
22. Haning R., Tait S. A. S. and Tait J. F.: *Endocrinology* **87** (1970) 1147.
23. Sayers G., Swallow R. L. and Giordano N. D.: *Endocrinology* **88** (1971) 1063.
24. Kitabchi A. E. and Sharma R. K.: *Endocrinology* **88** (1971) 1109.
25. Gurr E.: In *Use of Dyes in Biology and General Staining Methods*. Williams and Wilkins, Baltimore (1965) p. 216.
26. Silber R. H., Busch R. D. and Oslapas R.: *Clin. Chem.* **4** (1958) 278.
27. Quastel J. H.: In *Metabolic Inhibitors*, (Edited by R. M. Hochster and J. H. Quastel). Academic Press, New York (1963) Vol. 2, p. 473.
28. Haslam J. M. and Krebs H.: *Biochem. J.* **107** (1968) 659.
29. Singer J. P. and Kearney E. B.: *Arch biochem. Biophys.* **61** (1956) 397.
30. Guerra F., Péron F. G. and McCarthy J. L.: *Biochim. biophys. Acta* **117** (1966) 433.
31. Péron F. G. and Tsang C. P. W.: *Biochim. biophys. Acta* **180** (1969) 445.
32. Bransome E. D., Jr. and Reddy W. J.: *Endocrinology* **69** (1963) 997.
33. Farese R. V. and Reddy W. J.: *Biochim. biophys. Acta* **76** (1963) 145.
34. Farese R. V.: *Endocrinology* **76** (1965) 795.
35. Ferguson J. J., Jr.: *J. biol. Chem.* **238** (1963) 2754.
36. Gill G. N. and Garren L. D.: *Proc. Natn. Acad. Sci.* (*U.S.*) **63** (1969) 512.
37. Gill G. N. and Garren L. D.: *Biochem. Biophys. Res. Comm.* **39** (1970) 335.