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Is the Adenine Nucleotide Translocator Rate-Limiting for Oxidative Phosphorylation?

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1. The effects of atractyloside and carboxyatractyloside (between 5 and 40μM) on O_2 uptake, glucose synthesis, urea synthesis, the adenine nucleotide content and the intracellular K^+ concentration were measured in isolated hepatocytes. 2. Urea synthesis was much less inhibited than glucose synthesis by both atractylosides. Measurements of intermediary metabolites of carbohydrate metabolism in freeze-clamped liver after injection of atractyloside into rats indicate that inhibition of gluconeogenesis is due to interference at the cytosolic reactions requiring ATP (phosphoenolpyruvate carboxykinase and 3-phosphoglycerate kinase). 3. The decrease in [ATP]/[ADP]$\times$[P_i] after addition of atractyloside or carboxyatractyloside was restricted to the cytosol. 4. Dihydroxyacetone can be converted either into glucose with the consumption of 2mol of ATP (per mol of glucose) or into lactate with the production of 2mol of ATP. In the presence of high concentrations of atractyloside and carboxyatractyloside more ATP was produced than was used for the synthesis of glucose from dihydroxyacetone, probably for the maintenance of intracellular [K^+]. 5. When the rates of respiration were altered by changing substrates, the degrees of inhibition of respiration and translocation by a given concentration of the atractylosides were the same, whereas at a given concentration of HCN the degree of inhibition was high at higher initial rates, and low at lower initial rates. 6. Inhibition of a complex series of reactions by atractyloside does not necessarily indicate that the translocator is a rate-limiting step in that sequence as Th. P. M. Akerboom, H. Bookelman & J. M. Tager [(1977) *FEBS. Lett.* **74**, 50–54] assume. This point is discussed.

This paper is concerned with the systematic investigation of the effect of atractyloside and carboxyatractyloside, inhibitors of adenine nucleotide translocation, on the metabolism of rat hepatocytes. According to Tager *et al.* (1973) and Akerboom *et al.* (1977) atractyloside in the presence of alanine inhibits glucose synthesis to a greater extent than urea synthesis. This is somewhat unexpected, because both processes require cytosolic ATP, and at maximum rates urea synthesis requires more ATP than glucose synthesis. Thus both processes depend on the activity of the translocator. One of the objectives of the present work was to explore the reasons for the differential inhibition of urea and glucose synthesis by atractyloside. Another objective concerned the question to what extent is translocation a rate-limiting factor in cellular energy metabolism (Heldt, 1967; Heldt & Pfaff, 1969; Kemp *et al.*, 1969; Klingenberg, 1970; Vignais, 1976; Davis & Lumeng, 1975).

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Materials and Methods

Rats

Female Wistar rats were obtained from Charles River (U.K.) Ltd., Margate, Kent, U.K. They were starved for 48h.

Preparation and treatment of isolated hepatocytes

Hepatocytes were prepared by the method of Berry & Friend (1969) as modified by Krebs *et al.* (1974). Incubations were either in Erlenmeyer flasks or, when O_2 uptake was to be measured, in Warburg manometer vessels (Krebs *et al.*, 1974). Usually the wet wt. of cells was between 40 and 80mg per flask in a final volume of 4ml containing 2.5% (w/v) dialysed bovine serum albumin. Incubations were for 1h at 37°C except where stated otherwise. At the end of the incubations the cell suspensions were either deproteinized with $HClO_4$ (final concentration 2%, v/v), or separated into cells and medium by rapid centrifugation through the separating device of Hems *et al.* (1975), or treated briefly with digitonin (Zuurendonk & Tager, 1974) before centrifugal

separation to obtain cytosolic and mitochondrial fractions. The technique of Zuurendonk & Tager (1974) was modified as follows: the cell suspensions (4ml, containing 150–180mg wet wt.) were rapidly cooled in an NaCl/ice mixture to 5°C, treated with digitonin (0.11–0.13g/g wet wt.) for 40s and poured into the large separating device of Hems *et al.* (1975). On centrifugation the mitochondrial fraction rapidly entered the bulb containing 4% (v/v) $HClO_4$. A sample of the cytosolic fraction from the upper chamber was removed and deproteinized with $HClO_4$.

Cyanide, where added, was in the form of a solution containing stoicheiometric proportions (see legends to Tables) of KCN and HCN.

Reagents

Atractyloside was obtained from Calbiochem, Hereford, U.K. Carboxyatractyloside, enzymes and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

Determination of metabolites

The metabolites were determined by the following standard enzymic methods on neutralized $HClO_4$ extracts; glucose (Slein, 1963), lactate and pyruvate (Hohorst *et al.*, 1959), glutamate and alanine successively in the same cuvette (Bernt & Bergmeyer, 1963) as modified by Cornell *et al.* (1974), ammonia and urea successively in the same cuvette (Bernt & Bergmeyer, 1970), 3-hydroxybutyrate and acetoacetate (Williamson *et al.*, 1962), ATP (Lamprecht & Trautschold, 1963), ADP and AMP (Adam, 1963), P_i (Wahler & Wollenberg, 1958), and K^+ by an atomic absorption spectrophotometer (Perkin–Elmer model 103). Unless stated otherwise, metabolites were measured in the cell suspensions rather than the separated fractions.

Calculations

A factor of 3.7 was used for conversion of dry wt. into wet wt. (Krebs *et al.*, 1974). The concentrations of adenine nucleotides and P_i in the mitochondrial matrix were based on the assumption that the volume of the matrix was 60μl/g wet wt. This value was derived from the finding that the volume of the matrix was 1.0μl/mg of mitochondrial protein (Pfaff *et al.*, 1968) and that there is 60mg of mitochondrial protein/g wet wt. (Scholz & Bücher, 1965). The $[P_i]$ in the cytosol was calculated on the assumption that 1g wet wt. of fresh liver corresponds to 400μl of cytosol (Bolender & Weibel, 1973).

Expression of results

Although in the presence of the inhibitors the rates of glucose and urea production were not linear with time (see Table 4), the results have been expressed as μmol/min per g wet wt. These are thus mean rates during 1 h so that they are comparable with published rates.

Results

Effects of atractyloside and carboxyatractyloside on glucose synthesis from lactate and on urea synthesis from NH_4Cl

Glucose synthesis from lactate and urea synthesis from NH_4Cl were measured simultaneously under conditions favourable for both processes (as described in Table 1 and Krebs *et al.*, 1976). At all inhibitor concentrations tested (5–40μM) urea synthesis was less inhibited than glucose synthesis. Thus at 5μM atractyloside inhibited glucose synthesis by 69% and urea synthesis by only 26%. The inhibitory effect of the two substances on urea synthesis was about the same, but carboxyatractyloside was

Table 1. *Effects of atractyloside and carboxyatractyloside on O_2 uptake and glucose and urea production in hepatocytes*
The experimental details are as described in the text. The incubations were made in the presence of lactate and NH_4Cl (10mM), ornithine, oleate and pyruvate (1mM). The results are expressed as μmol/min per g wet wt. and are means± S.E.M. with the numbers of observations in parentheses.

Inhibitor	O_2 uptake	Inhibition (%)	Glucose formed	Inhibition (%)	Urea formed	Inhibition (%)
None	9.65±0.52 (7)		1.72±0.10 (7)		4.93±0.18 (7)	
Atractyloside						
5μM	6.50±0.61 (4)	33	0.54±0.06 (4)	69	3.66±0.07 (4)	26
10μM	5.53±0.51 (4)	43	0.38±0.04 (4)	78	3.46±0.44 (4)	31
20μM	4.65±0.64 (3)	52	0.19±0.01 (4)	89	2.17±0.13 (4)	56
40μM	2.10 (1)	78	0.09 (1)	95	1.21 (1)	75
Carboxyatractyloside						
10μM	5.51±0.95 (4)	43	0.90±015 (4)	48	3.33±0.17 (4)	32
20μM	3.13±0.76 (4)	68	0.49±0.10 (4)	72	2.03±0.22 (4)	59
40μM	2.33±0.38 (4)	76	0.28±0.05 (4)	84	1.52±0.17 (4)	69

less effective than atractyloside in inhibiting glucose synthesis. The O_2 consumption, as expected from earlier work on mitochondria (Bruni *et al.*, 1962; Vignais *et al.*, 1962), was also inhibited, and the degree of the inhibition by the two substances was about the same.

When alanine was the precursor of glucose and urea (Table 2) the rate of alanine removal was affected by the two inhibitors to the same extent (35%) and urea formation was also equally inhibited (about 57%). Glucose synthesis was inhibited to a greater extent by atractyloside (81%) than by carboxyatractyloside (63%). Both inhibitors caused increases in the formation of lactate plus pyruvate and of aspartate, and increased the [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios, indicating a more reduced state of the cytosolic and mitochondrial NAD couples.

The large increase in lactate and aspartate formation from alanine may be related to the fact that these processes require no cytosolic ATP and therefore are expected to be preferred processes when cytosolic ATP is in short supply as a consequence of the inhibition of the translocator.

The rates of requirement of cytosolic ATP under the test conditions, as calculated from the rates of urea and glucose synthesis given in Table 1, were $1.72 \times 4 = 6.88\,\mu$mol/min per g for glucose synthesis and $4.93 \times 2 = 9.86\,\mu$mol/min per g for urea synthesis. Thus, although more ATP had to be translocated for urea synthesis than for glucose synthesis, urea synthesis was less inhibited. This raises the question of whether the inhibitors have effects other than those on the translocator. This was tested on the rate of lactate formation by erythrocytes, i.e. in cells that do not have mitochondria. Even at 80 μM inhibitor there was no effect.

Effects of injection of atractyloside on intermediates of carbohydrate metabolism

The problem of the site of action of an inhibitor may be approached by measuring the concentrations of the intermediary metabolites in the presence and absence of the inhibitor. In a metabolic sequence the concentrations of those metabolites arising beyond the point of inhibition decrease, whereas those arising before this point increase (Chance & Willams, 1956). Intermediates of carbohydrate metabolism were therefore measured in freeze-clamped livers. This material was used in preference to hepatocytes because the hepatocyte suspensions are too dilute for accurate measurements of those intermediates present at very low concentrations. As the rats had been starved the carbohydrate metabolism operated in the direction of gluconeogenesis.

Two different amounts of atractyloside were tested (Table 3). At the lower dose, which caused a fall in [ATP] from 1.78 to 1.10 mM, the concentrations of

Table 2. *Effects of atractyloside and carboxyatractyloside on the fate of alanine and oleate in hepatocytes*

Experimental details are as described in text. The results are expressed as μmol/g found at the end of a 60 min incubation and are means of duplicate determinations. The carbon balance is based on the assumption that 2 mol of alanine are needed for the synthesis of 1 mol each of glucose and glutamate, and 1 mol of alanine for the synthesis of 1 mol of aspartate and lactate or pyruvate. The initial concentration of alanine was 5 mM and oleate 1 mM. A minus sign designates disappearance of a metabolite.

	Metabolites formed or removed (μmol/g)										Metabolite ratios	
Inhibitor	Glucose	Lactate plus pyruvate	Urea	NH_4^+	Glutamate	Aspartate	Total ketone bodies	Alanine	Carbon found as C_3 units	Nitrogen found as NH_3	Lactate / Pyruvate	3-Hydroxybutyrate / Acetoacetate
None	54.6	8.1	62.5	8.4	12.6	1.9	59.0	−123	144	148	5.6	0.2
Atractyloside (20 μM)	10.5	34.2	27.5	3.1	12.7	7.7	58.5	−80.0	88.3	78.5	10.8	1.2
Carboxyatractyloside	20.2	21.2	26.4	5.5	9.0	10.3	47.2	−79.5	89.9	77.6	9.1	0.7

Table 3. *Effects of atractyloside in vivo on metabolite concentrations in rat liver*

Livers from 36h-starved rats were freeze-clamped and metabolites assayed as described by Williamson *et al.* (1967) and Hawkins *et al.* (1973) 4h after intraperitoneal injection of atractyloside. Metabolite concentrations are means ± S.E.M. for the numbers of observations given in parentheses.

Metabolite	Metabolite concentration (μmol/g wet wt.) (mean ± S.E.M.)		
	Control (8)	Treated with 170mg of atractyloside/kg (6)	Treated with 250mg of atractyloside/kg (3)
Lactate	2.310 ± 0.280	4.000 ± 0.380	7.190 ± 0.580
Pyruvate	0.026 ± 0.003	0.030 ± 0.002	0.027 ± 0.003
Malate	0.800 ± 0.060	1.230 ± 0.087	1.470 ± 0.030
Aspartate	0.960 ± 0.060	1.230 ± 0.065	2.130 ± 0.290
Phosphoenolpyruvate	0.028 ± 0.003	0.057 ± 0.006	0.012 ± 0.002
2-Phosphoglycerate	0.011 ± 0.002	0.025 ± 0.001	0.011 ± 0.002
3-Phosphoglycerate	0.090 ± 0.012	0.200 ± 0.018	0.069 ± 0.001
Glyceraldehyde 3-phosphate	0.005 ± 0.003	0.003 ± 0.0005	0.003 ± 0.0006
Dihydroxyacetone phosphate	0.021 ± 0.002	0.018 ± 0.001	0.010 ± 0.0006
Fructose 1,6-bisphosphate	0.020 ± 0.002	0.011 ± 0.001	0.008 ± 0.001
Glucose 6-phosphate	0.200 ± 0.028	0.048 ± 0.008	0.014 ± 0.0006
Glucose	5.300 ± 0.290	3.410 ± 0.260	0.660 ± 0.023
ATP	1.780 ± 0.096	1.110 ± 0.125	0.150 ± 0.012
ADP	1.000 ± 0.030	0.900 ± 0.040	0.290 ± 0.012
AMP	0.650 ± 0.060	0.560 ± 0.050	0.350 ± 0.035

Table 4. *Time course of the effects of atractyloside and carboxyatractyloside on glucose synthesis and the ATP content of hepatocytes*

Experimental details are as described in the text. Results (from a representative experiment) are expressed as μmol/g of metabolite found. Atractyloside and carboxyatractyloside were 20μM.

Time (min)	Addition ...	Glucose found			ATP found		
		None	Atractyloside	Carboxyatractyloside	None	Atractyloside	Carboxyatractyloside
5		7.0	6.3	8.3	2.37	1.76	2.16
10		13.5	7.4	12.1	2.30	1.78	1.70
15		19.3	7.9	17.1	2.30	1.51	1.59
30		38.2	10.1	24.7	2.05	1.22	0.68
60		72.2	11.5	29.5	2.00	0.80	0.40

lactate, malate, aspartate, phosphoenolpyruvate and 2- and 3-phosphoglycerate were raised above the control values, whereas those of glyceraldehyde 3-phosphate and of the subsequent intermediates fell below the control values. This indicates a block by atractyloside (170mg/kg) at the 3-phosphoglycerate kinase step. At the higher dose (250mg/kg), when ATP had fallen to the 0.1mM range and the glucose content from 5.3 to 0.66mM, the concentrations of lactate, malate and aspartate were raised and those of phosphoenolpyruvate and subsequent stages decreased. Thus under these conditions the block was at the phosphoenolpyruvate carboxykinase step.

The 3-phosphoglycerate kinase and phosphoenolpyruvate carboxykinase steps are the only ones in glucose synthesis that require nucleoside triphosphates (GTP and ATP) and depend on translocation. Thus inhibitions at these steps would be expected. The inhibitory effect at the higher dose of atractyloside at phosphoenolpyruvate carboxykinase masks the inhibition of 3-phosphoglycerate kinase observed at the lower dose of atractyloside. Carboxyatractyloside (50mg/kg) gave a similar metabolite profile to that seen with the higher concentration of atractyloside (not shown in the Table).

Relationships between the rate of glucose synthesis from lactate and ATP concentration in the presence of atractyloside and carboxyatractyloside

The time course of action of the inhibitors on the ATP concentration (Table 4) shows that carboxyatractyloside does not act significantly within the first 5min, whereas atractyloside causes early inhibition. This suggests that the penetration of carboxy-

atractyloside into the cell is slower than that of atractyloside, probably because of the extra negative charge. This assumption is justified, because in isolated mitochondria there is no corresponding lag period (Vignais *et al.*, 1973). After 5min the ATP content in the presence of carboxyatractyloside fell progressively to about 80% of the initial value. With atractyloside the rate of decrease of the ATP concentration was more or less steady, but much slower. The rate of glucose synthesis remained higher with carboxyatractyloside throughout the incubation period. The differences between atractyloside and carboxyatractyloside were maintained during the 60min.

Effects of atractyloside and carboxyatractyloside on glucose synthesis from dihydroxyacetone

Dihydroxyacetone is a substrate that does not require mitochondrial ATP for conversion into glucose. One molecule of cytosolic ATP is required per molecule of dihydroxyacetone converted into glucose. This can be supplied by the formation of lactate, which on balance produces 1 molecule of ATP per molecule of lactate. If glucose synthesis were entirely dependent on cytosolic ATP production from lactate formation, then the expected ratio (lactate+pyruvate formed)/(glucose formed) would be 2. In fact the ratio rose to 10 (Table 5). This suggests that ATP was used for other energy-requiring processes such as the maintenance of concentration gradients. This view is supported by the finding that, at the higher concentrations (20μM and above) of the inhibitors, the K^+ content of the cells tended to be about 15% higher in the presence of dihydroxyacetone than in the absence of added substrate (results not shown in Table 5). Both inhibitors decreased glucose formation by about the same extent (50%), but atractyloside caused the greater increase in lactate production (Table 5). The rate of O_2 consumption was also decreased by both inhibitors and the inhibition increased slightly with increasing concentrations of these substances. Lactate formation also increased relatively little with increasing concentrations of inhibitors. Because lactate production was less dependent than glucose production on the inhibitory concentrations, the ratio (lactate+pyruvate formed)/(glucose formed) rose with increasing inhibitor concentrations.

Effects of atractyloside and carboxyatractyloside on the adenine nucleotide content of hepatocytes

There was a striking difference between the effects of atractyloside and carboxyatractyloside on the ATP and AMP content of the hepatocytes (Table 6). In the presence of dihydroxyacetone, atractyloside at 10, 20 and 40μM decreased the ATP content by about 25%, whereas carboxyatractyloside at 40μM decreased it by about 80%. The content of AMP was slightly increased by atractyloside, but almost quadrupled by 40μM-carboxyatractyloside. The effects of both substances on ADP were relatively small.

The total adenine nucleotides changed very little in the presence of dihydroxyacetone, but decreased markedly in the presence of lactate, NH_4Cl, oleate, ornithine and pyruvate, when no source of cytosolic ATP was available.

In spite of the large changes in [ATP] and [AMP], especially in the presence of carboxyatractyloside, the mass-action ratio of the adenylate kinase system ($[ATP][AMP]/[ADP]^2$) was maintained within the near-equilibrium range even in the presence of high concentrations of the inhibitors. This range depends on the concentration of Mg^{2+}, which in turn depends on the relative concentrations of ATP and AMP.

Under conditions where glucose synthesis and urea synthesis occurred at high rates, the effects of the

Table 5. *Effects of atractyloside and carboxyatractyloside on O_2 uptake and on glucose and lactate production from dihydroxyacetone*

Experimental details are as described in the text. The results are expressed as μmol/min per g wet wt. with means±S.E.M. when there are three or more observations (given in parentheses). The initial concentration of dihydroxyacetone was 10mM.

Inhibitor added	Concn. (μM)	O_2 uptake	Inhibition (%)	Glucose formed	Inhibition (%)	Lactate (plus pyruvate) formed	Lactate+pyruvate / Glucose
None	—	4.08±0.27 (5)		2.25±0.07 (7)		0.69±0.08 (6)	0.31
Atractyloside	10	1.98 (1)	52	0.91±0.09 (3)	60	1.81±0.01 (3)	1.99
	20	1.75 (1)	57	0.56±0.02 (3)	75	2.10±0.05 (3)	3.75
	40	1.64±0.15 (5)	60	0.39±0.05 (5)	83	2.61±0.16 (5)	4.7
	60	1.45±0.09 (3)	64	0.24±0.04 (3)	89	2.49±0.23 (3)	10.4
Carboxyatractyloside	10	1.92 (1)	53	1.15±0.10 (3)	49	0.85±0.04 (3)	0.74
	20	2.19 (1)	46	0.72±0.12 (3)	68	0.80±0.02 (3)	1.11
	40	1.57±0.14 (5)	62	0.29±0.04 (4)	87	1.16±0.18 (5)	4.0
	60	1.36±0.07 (3)	66	0.18±0.01 (3)	92	0.86±0.04 (3)	4.8

Table 6. *Effects of atractyloside and carboxyatractyloside on the adenine nucleotide content of hepatocytes*

Experimental details are as described in the text. Results are expressed as μmol/g, as means ±S.E.M. with the numbers of observations in parentheses. The initial concentration of dihydroxyacetone, lactate and NH_4Cl was 10mM, and that of ornithine, oleate and pyruvate 1mM.

Inhibitor	Dihydroxyacetone					Lactate, NH_4Cl, ornithine, oleate, pyruvate				
	ATP	ADP	AMP	Total adenine nucleotides	$\frac{[ATP][AMP]}{[ADP]^2}$	ATP	ADP	AMP	Total adenine nucleotides	$\frac{[ATP][AMP]}{[ADP]^2}$
None	1.95 ±0.10 (7)	0.91 ±0.10 (6)	0.38 ±0.02 (7)	3.24	0.89	2.13 ±0.07 (9)	0.61 ±0.04 (9)	0.30 ±0.05 (9)	3.04	1.72
Atractyloside										
10μM	1.47 ±0.13 (4)	0.75 ±0.16 (3)	0.42 ±0.09 (3)	2.64	1.1	1.53 ±0.13 (5)	0.92 ±0.08 (5)	0.56 ±0.11 (5)	3.01	1.01
20μM	1.42 ±0.09 (4)	0.95 ±0.21 (3)	0.43 ±0.09 (3)	2.80	0.68	1.09 ±0.16 (3)	0.69 ±0.09 (3)	0.78 ±0.09 (3)	2.56	1.79
40μM	1.49 ±0.09 (5)	1.08 ±0.07 (5)	0.50 ±0.03 (5)	3.07	0.64	0.49 ±0.09 (4)	0.57 ±0.12 (4)	0.50 ±0.04 (4)	1.56	0.75
Carboxyatractyloside										
10μM	0.86 ±0.28 (4)	0.97 ±0.20 (3)	0.85 ±0.23 (3)	2.68	0.78	0.98 ±0.18 (5)	0.70 ±0.10 (5)	0.90 ±0.03 (5)	2.58	1.80
20μM	0.58 ±0.18 (4)	0.98 ±0.23 (3)	0.98 ±0.24 (3)	2.54	0.59	0.33 ±0.04 (3)	0.62 ±0.11 (3)	1.35 ±0.11 (3)	2.30	1.16
40μM	0.27 ±0.06 (5)	0.77 ±0.05 (5)	1.51 ±0.13 (5)	2.55	0.69	0.18 ±0.05 (3)	0.44 ±0.06 (3)	1.34 ±0.16 (3)	1.96	1.25

inhibitors were somewhat similar to those seen in the presence of dihydroxyacetone, in that carboxyatractyloside caused greater changes than atractyloside.

Effects of atractyloside and carboxyatractyloside on the phosphorylation state of the adenine nucleotides in the cytosolic and mitochondrial compartments of hepatocytes

Zuurendonk & Tager (1974) have reported the effects of atractyloside on the [ATP]/[ADP] ratio in cytosol and mitochondria, using their separation technique. In our own experiments, atractyloside (and also carboxyatractyloside) decreased the [ATP]/[ADP][P_i] ratio in the cytosol and did not significantly affect the ratio in the mitochondria. The decrease in the cytosolic value was between 10- and 15-fold at 20μM inhibitor (results not shown in Tables); thus the effect observed in the intact cells reflects the change in the cytosol.

Adenine nucleotide translocator as a rate-limiting factor for ATP-requiring reactions in the cytosol

The following experiments were undertaken to test whether the adenine nucleotide translocator limits the flux to the cytosol of ATP generated in the mitochondria and therefore is a rate-limiting step for ATP-requiring reactions in the cytosol, e.g. glucose synthesis (see Akerboom *et al.*, 1977). The effects of the atractylosides were examined at different rates of O_2 uptake and of translocation. Different rates were obtained by varying the nature of the substrates. The rates of translocation were calculated on the assumption that 4mol of ATP is translocated/mol of glucose formed and 2mol of ATP/mol of urea formed. The inhibition by both atractylosides at 10μM was in the region of 50% for both O_2 uptake and translocation, irrespective of the control rate of O_2 uptake, which varied between 3 and 9.65μmol/min per g, or of translocation, which varied between 1.2 and 16.8μmol/min per g (Table 7). At the lowest rate the inhibition appears a little lower (33%), but the calculation of the rate of translocation at these low rates was not accurate enough to consider these differences significant. This rate-independence of the degree of inhibition is a remarkable phenomenon. It is unexpected because under conditions where a 'catalyst' is not used to full capacity (at saturating substrate concentrations) the inhibition should be less than when the catalyst is used fully; when under-used the reserve capacity should make good a partial inhibition. The validity of this concept was tested for cellular O_2 consumption with HCN as inhibitor. The inhibition at the given HCN concentration increased with the rate of O_2 uptake (and translocation) (Table 8).

Thus the inhibition of O_2 uptake by HCN and the inhibition of the translocator by atractyloside

Table 7. *Effects of atractyloside and carboxyatractyloside at different rates of respiration and translocation*
Experimental details are as described in the text. The rates of translocation are calculated assuming 4mol of ATP translocated/mol of glucose formed and 2mol of ATP translocated/mol of urea formed. The data are taken from either Table 1 or representative experiments and expressed as μmol/min per g wet wt. The concentration of the inhibitors was 10μM.

Condition	Inhibitor	O_2 uptake	Inhibition (%)	Translocation	Inhibition (%)
Lactate, NH_4Cl, ornithine, oleate, pyruvate	None	9.65		16.8	
	Atractyloside	5.53	43	8.5	51
	Carboxyatractyloside	5.53	43	10.2	40
Alanine, oleate	None	5.7		5.0	
	Atractyloside	3.3	42	2.8	44
	Carboxyatractyloside	3.0	47	2.2	56
No added substrate	None	3.0		1.2	
	Atractyloside	2.0	33	0.8	33
	Carboxyatractyloside	1.55	48	0.8	33

Table 8. *Effects of HCN at different rates of respiration and translocation*
Experimental details are as described in the text. The rates of translocation are calculated as described in Table 7, and the data are taken from a representative experiment and expressed as μmol/min per g wet wt.

Condition	HCN (mM)	O_2 uptake	Inhibition (%)	Translocation	Inhibition (%)
Lactate, NH_4Cl, ornithine, oleate, pyruvate	None	10.5		19.8	
	0.1	5.9	44	10.8	45
	0.2	3.8	64	5.3	73
Alanine, oleate	None	5.1		4.4	
	0.1	4.3	16	4.0	9
	0.2	4.0	22	3.2	27
No added substrate	None	2.06		—	
	0.1	1.89	8	—	

exhibit fundamentally different characteristics. The unexpected behaviour of the atractyloside inhibition was investigated by a series of further experiments in which two different inhibitors, HCN and atractyloside, were added at the same time.

In these experiments (see Table 9) the inhibitions by atractyloside were the same, irrespective of the inhibition by HCN, a further illustration of a special feature of the action of atractyloside. As a consequence, inhibitions by atractyloside and HCN are additive.

Discussion

Why does atractyloside inhibit gluconeogenesis to a greater extent than urea synthesis?

As stated above, both gluconeogenesis and urea synthesis depend on cytosolic ATP. Addition of atractyloside is not the only situation where the liver responds in this differential way. When slices of liver are incubated, urea synthesis is also much better preserved than gluconeogenesis. Rates of urea synthesis in liver slices are about half those found in hepatocytes [compare data of Krebs & Henseleit (1932) and Table 1], whereas rates of glucose synthesis are about one-tenth of those found in hepatocytes [compare data of Hastings *et al.* (1952) and Table 1]. Slicing, like atractyloside treatment, is accompanied by a loss of cytosolic ATP (Krebs *et al.*, 1974). Thus the question to be answered is why a loss of ATP affects the two processes to different degrees. In urea synthesis cytosolic ATP is needed for the argininosuccinate synthetase reaction; in gluconeogenesis it is needed for the 3-phosphoglycerate kinase reaction and for the formation of GTP from GDP, GTP being the intermediate phosphate donor for the phosphoenolpyruvate carboxykinase reaction. Loss of adenine nucleotides is likely to be paralleled by loss of other nucleotides, such as the guanine nucleotides.

These considerations lead to the conclusion that loss of ATP affects argininosuccinate synthetase less than the cytosolic kinases of gluconeogenesis. This may be due either to differences in K_m values for ATP or to loss of GDP (since the rate of the nucleoside diphosphate kinase reaction depends both on ATP and GDP).

Table 9. *Effects of atractyloside on O_2 uptake and translocation at various rates of respiration in the absence and presence of HCN*

Experimental details are as described in the text. The rates of translocation are calculated as described in Table 7. Results, which are from representative experiments, are expressed as μmol/min per g wet wt.

Condition	Inhibitor: HCN (μM)	Inhibitor: Atractyloside (μM)	O_2 uptake	Inhibition by atractyloside in the presence of HCN (%)	Inhibition by HCN in the presence of atractyloside (%)	Translocation	Inhibition by atractyloside in the presence of HCN (%)	Inhibition by HCN in the presence of atractyloside (%)
Lactate, NH_4Cl, ornithine, oleate, pyruvate	None	None	10.5			19.8		
	None	10	6.2			9.5		
	100	None	5.9			10.8		
	100	10	4.6	22	26	7.0	35	26
Alanine, oleate	None	None	5.1			4.4		
	None	10	3.4			2.9		
	100	None	4.3			4.0		
	100	10	3.2	26	6	2.2	44	24
	200	None	4.0			3.2		
	200	10	3.0	25	12	1.8	44	38

Is the adenine nucleotide translocator rate-limiting for oxidative phosphorylation?

Akerboom *et al.* (1977), experimenting with isolated hepatocytes, found that the synthesis of glucose from lactate, which depends on the availability of cytosolic ATP and therefore on translocation, is inhibited by atractyloside, and they concluded from this observation that translocation is rate-limiting *in vivo*, i.e. in the absence of atractyloside. This argument is not valid. In any sequence of reactions (where every step depends on the substrate supply by the preceding step) inhibition of any single step is liable to cause an inhibition of the overall process. Hence the parallelism between the inhibition of an intermediary step and the overall rate is not necessarily evidence of rate limitation by the individual step under normal conditions. Whether a step is rate-limiting can be settled by the assay of the catalytic capacity under near-physiological conditions and the comparison of the assayed rate with the overall rate of the reaction sequence *in vivo*. By this criterion the translocator is not rate-limiting *in vivo* under physiological conditions.

Indeed, the capacity of the translocator as measured by exchange of labelled adenine nucleotides (Klingenberg, 1976) in isolated mitochondria is higher than the maximum measured rate of oxidative phosphorylation. As the K_m value of ADP for the translocator is between 1 and 10 μM (Vignais, 1976; Klingenberg, 1976) and the cytosolic ADP content is between 300 and 600 μM (Siess & Wieland, 1976), the translocator could operate at full capacity and thus in physiological situations the capacity well exceeds the requirement.

Further, near-equilibrium exists between the first two sites of oxidative phosphorylation and the cytosolic phosphorylation state (Wilson *et al.*, 1974*a*,*b*) according to the following equation:

$$K = \frac{[NAD^+][\text{cytochrome } c^{2+}]^2[ATP]^2}{[NADH][\text{cytochrome } c^{3+}]^2[ADP]^2[P_i]^2}$$

where [ATP], [ADP] and [P_i] are cytosolic values and [NAD^+] and [NADH] are free mitochondrial values. Thus translocation, which is an obligatory intermediate step in the reaction sequence, cannot be rate-limiting.

Finally, in hepatocytes the rate of respiration changes in parallel with changes in [ATP]/[ADP][P_i] but not in parallel with changes in the [ATP]/[ADP] ratio (see Erecińska *et al.*, 1977, especially Table 2).

These considerations indicate that the translocator does not limit the rate of oxidative phosphorylation under physiological conditions. However, it is an open question why the atractylosides inhibit translocation even when the demand is low. It would be expected, by analogy with the HCN inhibition of cytochrome oxidase, that inhibitions would be

greater at the higher than at the lower initial rates. For the different pattern of inhibition by the atractylosides we can offer no satisfactory explanation.

The obligatory coupling of electron transport with the synthesis of ATP implies under most conditions (when the rate is not maximal) that the rate of supply of ADP to the electron-transport chain is the rate-limiting factor. Most of the ADP arises in the cytosol as a result of ATP-consuming processes. In the 'resting' state the rate of electron transport is no more than 30% of the maximum rate which occurs when the liver is flooded with lactate, NH_4Cl, ornithine and other cofactors promoting gluconeogenesis and urea synthesis. The ADP formed in the cytosol has to be translocated in exchange for ATP, and, because translocation is essential, one could argue that the transfer of ATP to the mitochondria, as effected by the translocator, is the rate-limiting step. One must distinguish, however, when considering rate control generally by enzymes or carriers between rate limitation by the capacity of the enzyme or carrier on the one hand, and the availability of the substrate on the other hand. Thus in the case of translocation the capacity of the carrier or the rate of supply of the substrate can limit the rate of translocation. It is in this sense that we conclude that the translocator is normally not rate-limiting, any more than, say, malate or succinate dehydrogenase control the rate of the tricarboxylic acid cycle. In any complex sequence of reactions it is the feeding into the sequence of intermediates from outside that determines the overall rate. In oxidative phosphorylation the decisive factor is the rate at which ADP (and P_i) are fed into the sequence, and this depends on the sum of the rates of ATP-consuming reactions. These ideas are of course contained in the well-established concept of the coupling between electron transport and phosphorylation.

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