PRELIMINARY NOTES

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Effect of uncouplers on the transport of anions in rat-liver mitochondria

The inner mitochondrial membrane appears to contain specific translocators mediating the transport of anionic substrates[1,2] In rat-liver mitochondria, exchange–diffusion reactions involving α-oxoglutarate and dicarboxylate[3,4], tricarboxylate and malate (ref. 4 and A. J. MEYER, unpublished observations), and P_i and dicarboxylate[5] have been directly demonstrated The stoicheiometry of these reactions is one to one[3–5]. Evidence has also been obtained[5] that the translocation of phosphate, but not that of malate (contrast ref. 6), can be directly coupled to an OH^- counterflux. The specificity of these reactions, and their sensitivity to inhibitors, indicate that they are mediated by separate translocators[5,7]

Uncouplers inhibit the accumulation of anionic substrates by mitochondria[8–10], the inhibition being competitive with respect to the anions[11]. VAN DAM AND SLATER[12] proposed that uncoupler anions compete with substrate anions for entry into the mitochondria, the uncoupler anion being transported *via* a non-specific anion translocator (see, however, refs. 11, 13). In view of the fact that the translocators are

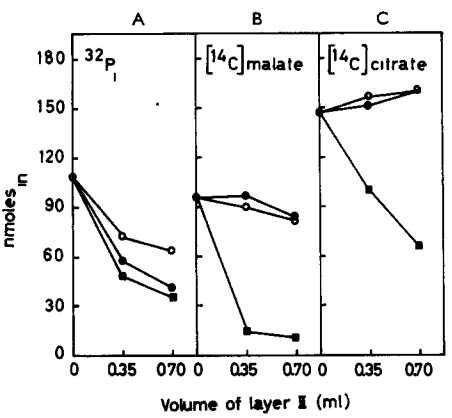

Fig 1. Effect of dicoumarol on the efflux of anionic substrates from rat-liver mitochondria Mitochondria (Expt. A, 9 2 mg protein, Expts. B and C, 7 mg protein) were preincubated in 1 ml of 150 mM sucrose, 20 mM Tris–Cl (pH 7 4), 0 5 mM EDTA, 1 mM $MgCl_2$, 1 4 μg rotenone, 0 34 μg antimycin and 10 μg oligomycin After 1 min, 1 mM $^{32}P_i$ ($9 \cdot 10^5$ counts/min; Expt A), 1 mM [^{14}C]malate ($5 \cdot 10^5$ counts/min; Expt B) or 1 mM [^{14}C]citrate ($1 \cdot 10^6$ counts/min; Expt C) were added The mitochondria in 0 35 ml of the preincubation mixture were centrifuged[14] through a washing layer containing the same components as the preincubation mixture (except labelled substrate), through a second incubation layer, and finally into $HClO_4$ Layer II contained the same components as the preincubation mixture (except labelled substrate), with no additions (○—○), 40 μM dicoumarol (●—●); 5 mM unlabelled P_i (Expt A, ■—■) or 5 mM unlabelled malate (Expts B and C, ■–·–■) The time in Layer II was about 15 sec when the volume was 0 35 ml The amount of labelled substrate remaining in the matrix space was calculated from the radioactivity in the $HClO_4$ extract The values refer to the original amount of mitochondrial protein

specific, that only phosphate exchanges with hydroxyl, and that different translocators can be linked through the anions shared, the problem arises of the specificity of the action of uncouplers on the different transport systems An insight into this has been obtained by examining the effect of uncouplers on the efflux of various anions involved in the exchanges, both in the absence and in the presence of the counter anions

Mitochondria were preloaded with a labelled substrate anion in the presence of oligomycin, antimycin, and rotenone, and then centrifuged[14] through a washing layer, through a second layer containing the substances whose effect on the efflux of the anion was to be studied, and finally into $HClO_4$. The amount of anion remaining in the matrix space of the mitochondria was determined. In Fig. 1, the efflux of $^{32}P_i$, [^{14}C]malate and [^{14}C]citrate is shown. In the controls (no additions to the second incubation layer), a substantial efflux of P_i occurred, but in contrast, no significant efflux of malate or citrate was observed. The $^{32}P_i$ efflux was increased by unlabelled P_i Unlabelled malate promoted a rapid and extensive efflux of both [^{14}C]malate and [^{14}C]citrate. Dicoumarol significantly increased the efflux of $^{32}P_i$, but had practically no effect on the matrix level of either [^{14}C]malate or [^{14}C]citrate

The data in Table I show that dicoumarol or 2,4-dinitrophenol promoted the efflux of P_i, but not that of citrate or α-oxoglutarate (*cf.* Fig. 1). Malate promoted

TABLE I

EFFECT OF UNCOUPLERS AND INHIBITORS ON THE EFFLUX OF ANIONIC SUBSTRATES FROM RAT-LIVER MITOCHONDRIA

Experimental procedure as in Fig 1 Expt 1 7 mg mitochondrial protein, preincubation with 1 mM $^{32}P_i$ ($1 \cdot 10^6$ counts/min) or 1 mM [^{14}C]citrate ($7 \cdot 10^5$ counts/min) Expt 2. 6 8 mg mitochondrial protein; preincubation with 1 mM $^{32}P_i$ or 1 mM α-oxoglutarate Expt 3. 8 4 mg mitochondrial protein, preincubation with 1 mM $^{32}P_i$. Expt 4: 11 2 mg mitochondrial protein; preincubation with 1 mM $^{32}P_i$; 300 μM mersalyl added 30 sec after addition of $^{32}P_i$ Centrifugation filtration began 3 min after addition of labelled substrate or mersalyl. Layer II contained where indicated 40 μM dicoumarol, 100 μM 2,4-dinitrophenol, 5 mM malate, and 5 mM butyl malonate The time in Layer II was about 15 sec α-Oxoglutarate was estimated enzymically

Expt No	*Additions*	*Amount (nmoles) in matrix of*		
		$^{32}P_i$	[^{14}C]*Citrate*	*α-Oxoglutarate*
1	None	47	124	
	Dicoumarol	39	127	
	Dinitrophenol	40	130	
	Malate	32	63	
2	None	66		35
	Dicoumarol	54		33
	Malate	44		2
3	None	66		
	Dinitrophenol	50		
	Butyl malonate	80		
	Dinitrophenol + butyl malonate	50		
4	None	77		
	Dinitrophenol	63		
	Mersalyl	95		
	Dinitrophenol + mersalyl	106		

the efflux of P_i, citrate and α-oxoglutarate. Butyl malonate and mersalyl inhibited the efflux of P_i that occurred in the absence of an added anion. Mersalyl, but not butyl malonate, prevented the 2,4-dinitrophenol-induced efflux of P_i, indicating that it is mediated only by the phosphate–hydroxyl translocator[7]

TABLE II

EFFECT OF DICOUMAROL ON v_{max} OF MALATE EFFLUX FROM RAT-LIVER MITOCHONDRIA AND THE K_m FOR THE COUNTER ANION

Using the experimental procedure described in Fig 1, mitochondria (8 0 mg in Expts 1 and 3, 7 0 mg in Expts 2 and 4) preloaded with [^{14}C]malate were centrifuged through the second incubation layer containing 0–1 mM counter anion with or without 100 μM dicoumarol The counter anion-induced efflux of [^{14}C]malate, *i e* the difference in the amount of [^{14}C]malate remaining in the matrix after passage of the mitochondria through Layer II in the absence and presence of counter anion, was determined These values were used to construct Lineweaver–Burk plots, from which the values for K_m and v_{max} were obtained

Expt No	*Counter anion*	v_{max} (*nmoles/15 sec*)		K_m (μ*M*)	
		Control	*+ Dicoumarol*	*Control*	*+ Dicoumarol*
1	P_i	45	48	131	400
2	Malate	67	67	23	40
3	Citrate	57	51	102	217
4	α-Oxoglutarate	51	40	56	84

The effect of uncouplers on the counter anion-driven efflux of [^{14}C]malate was examined kinetically (Table II). Dicoumarol inhibited the exchange–diffusion reactions involving malate, the inhibition being purely competitive in the case of the malate–malate and malate–phosphate antiports, and partly competitive in other cases. A similar inhibition of the malate–phosphate and malate–α-oxoglutarate exchanges was obtained with 2,4-dinitrophenol However, it had no effect on the malate–citrate or malate–malate exchanges. Possibly, the malate–phosphate, malate–α-oxoglutarate and malate–tricarboxylate translocators must all be inhibited in order to inhibit the malate–malate exchange; this appears to be the case with dicoumarol, but not with 2,4-dinitrophenol. VAN DAM AND KRAAYENHOF[15] have shown that 2,4-dinitrophenol inhibits the accumulation of citrate by freshly prepared mitochondria. However, the uptake of citrate under their conditions involves not only an exchange of citrate with intramitochondrial citrate and malate, but also a recycling of the endogenous malate and phosphate *via* the malate–phosphate translocator. Furthermore, the stimulation by 2,4-dinitrophenol of the efflux of P_i will contribute to the inhibition of [^{14}C]citrate uptake.

Our results suggest that the mechanism proposed by KRAAYENHOF AND VAN DAM[11] to explain the inhibition by uncouplers of the uptake of anions can be applicable in the case of the phosphate–hydroxyl antiporter. In the other cases studied here, the inhibition must be due at least in part, to an interaction of the uncoupler with the translocator that does not appear to involve transport of the uncoupler anion (see also ref. 6) In other experiments (to be reported elsewhere) we have found that the dinitrophenol-induced efflux of $^{32}P_i$ can be accompanied by accumulation of [^{14}C]dinitrophenol by the mitochondria, both processes being mersalyl-sensitive. Further investigation is required to elucidate the exact mechanism of this coupling

between phosphate efflux and uncoupler accumulation. The mechanisms proposed by MITCHELL[6] and by VAN DAM[11,12,15] could both, in principle, accommodate our findings.

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