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Specific Inhibition of Pyruvate Transport in Rat Liver Mitochondria and Human Erythrocytes by α-Cyano-4-hydroxycinnamate

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α-Cyano-4-hydroxycinnamate greatly inhibits the transport of pyruvate but not that of acetate or butyrate in liver mitochondria and erythrocytes. In the latter, lactate uptake is also inhibited. It is concluded that a specific carrier is involved in membrane transport of pyruvate and that the plasma-membrane carrier may also be involved in lactate transport.

Previous studies have been unable to establish with any certainty whether or not there is a specific carrier system involved in the transport of pyruvate across either the plasma membrane of cells or the inner membrane of mitochondria (Watts & Randle, 1967; Watts, 1969; Papa *et al.*, 1971; Zahlten *et al.*, 1972). The principal reason for this uncertainty has been the absence of a specific inhibitor of pyruvate transport (Klingenberg, 1970*a*,*b*). In this communication, we report studies which demonstrate the specific inhibition of pyruvate transport across both the plasma membrane of erythrocytes and also the inner membrane of rat liver mitochondria by α-cyano-4-hydroxycinnamate. This is taken to imply that there is a specific carrier involved in pyruvate transport across both membranes. Evidence is presented that lactate may also be transported across the plasma membrane of erythrocytes by the same carrier.

The inhibitory properties of α-cyano-4-hydroxycinnamate became apparent during a survey of analogues of the enol form of pyruvate aimed at identifying specific inhibitors of enzymes involved in pyruvate metabolism. The possibility that this compound inhibited the mitochondrial transport of pyruvate was suspected when it was observed that rates of pyruvate oxidation by mitochondria from blowfly flight muscle and rat heart, pyruvate-dependent CO_2 fixation in liver mitochondria and pyruvate inhibition of pyruvate dehydrogenase kinase in fat-cell mitochondria were all greatly diminished on addition of low concentrations of α-cyano-4-hydroxycinnamate (less than 200μM) to the mitochondrial incubation media. However, at concentrations of up to 2mM, the compound had little or no inhibitory effects on the appropriate enzymes prepared from extracts of mitochondria (A. P. Halestrap & R. M. Denton, unpublished work).

Methods

Rat liver mitochondria prepared by the method of Chappell & Hansford (1972) were incubated in medium (125mM-KCl–20mM-Tris–HCl, pH7.4) with additions as indicated in the text and the Tables. Mitochondria were separated from incubation medium by centrifugation at 15000*g* for 1min in an Eppendorf 3200 centrifuge. The pellet was immediately suspended in 200μl of water and $HClO_4$ was added to 2% (w/v) final concentration; $HClO_4$ was also added to the supernatant to give the same final concentration. These acid extracts of pellet and supernatant were assayed for radioactivity and pyruvate after centrifugation at 15000*g* for 30s.

Human erythrocytes were prepared by centrifuging fresh heparinized blood for 10min at 3000*g* and were washed with 3×3vol. of citrate-based medium (84mM-sodium citrate–10mM-Tris adjusted to pH7.4 with 1M-NaH_2PO_4). The cells were finally suspended in 2vol. of the citrate-based medium and used at this concentration in all experiments. Cells were separated from medium by centrifugation at 15000*g* for 30s. The pellet of erythrocytes was immediately suspended in 0.5ml of water, $HClO_4$ was added to 2% (w/v) final concentration and the extract was vigorously mixed by vortex mixer; $HClO_4$ was also added to the supernatant to 2% (w/v) and radioactivity, pyruvate or lactate was assayed in the acid extracts after centrifugation. All experiments (involving mitochondria and erythrocytes) were carried out at room temperature (20–22°C).

Pyruvate and lactate were assayed enzymically by the methods of Hohorst (1963) and Bücher *et al.* (1963) respectively. Simultaneous assay of ^{14}C and ^{3}H was performed in methoxyethanol–toluene (2:3, v/v) containing 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6g/l) and naphthalene (80g/l) with a Nuclear–Chicago Isocap scintillation counter, care being taken that corrections for quenching, calculated by external standardization, were similar for all samples.

α-Cyano-4-hydroxycinnamate was obtained from Ralph N. Emanuel, Wembley, Middx. HA0 1PY, U.K., and was added as a 100mM solution in ethanol; ethanol alone was added to controls. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks, U.K.; [U-^{14}C]pyruvate

was dissolved in water on arrival and samples containing 2.5 μCi were freeze-dried in separate tubes, and stored at −15°C until use.

Results

α-Cyano-4-hydroxycinnamate (0.5 mM) greatly inhibited the accumulation of pyruvate by mitochondria incubated in the presence of succinate and rotenone, without having any appreciable effect on the accumulation of acetate, lactate or butyrate (Table 1). All four monocarboxylate anions were accumulated to much the same extent under the conditions used.

Radioactive and enzymic assays of pyruvate gave very similar results, indicating that as expected little pyruvate is metabolized in mitochondria incubated with rotenone and in the absence of phosphate. The presence of succinate with rotenone allowed marked accumulation of pyruvate; if the succinate was omitted and antimycin (5 μg/ml) was added, pyruvate uptake was decreased to about 20% of the uptake in the presence of succinate. However, α-cyano-4-hydroxycinnamate (0.5 mM) still inhibited uptake of pyruvate significantly ($P<0.02$) under these conditions although errors were necessarily larger.

Further confirmation of the inhibition of mitochondrial transport of pyruvate was obtained in experiments designed to study the effect of α-cyano-4-hydroxycinnamate on the efflux of pyruvate from pre-loaded mitochondria. Mitochondria were preincubated for 5 min with rotenone (5 μg/ml), succinate (2.5 mM), ^3H_2O and pyruvate (1.4 mM) and then centrifuged in the presence or absence of α-cyano-4-hydroxycinnamate (0.5 mM). The mitochondrial pellets were resuspended in medium containing rotenone with or without added α-cyano-4-hydroxycinnamate (0.5 mM). After 2 min incubation at room temperature the pyruvate content of the mitochondria was determined after centrifugation as described for Expt. 1 of Table 1. In one experiment in which the pyruvate retained in the mitochondria was calculated from enzymic assays, the presence of α-cyano-4-hydroxycinnamate increased the pyruvate retained from 0.95 to 2.35 nmol/μl of intramitochondrial space (values are the mean of two separate observations). In another experiment using [U-^{14}C]pyruvate and α-cyano-4-hydroxycinnamate (0.2 mM), the equivalent values (expressed as the mean ± S.E.M. for four observations) were 1.17 ± 0.13 and 1.91 ± 0.07 nmol/μl of intramitochondrial space respectively. Even when 50 μM-α-cyano-4-hydroxycinnamate was used it was possible to demonstrate an inhibition of the efflux of pyruvate; the pyruvate retained increased from 1.05 to 1.70 nmol/μl of intramitochondrial volume (means of two observations).

Previous studies have shown that human erythrocytes readily accumulate pyruvate and other monocarboxylate anions when incubated in a citrate-based medium containing no chloride (Watts, 1969). Under these conditions α-cyano-4-hydroxycinnamate (0.5 mM) markedly diminished pyruvate uptake (calculated on the basis of either enzymic or radioactive

Table 1. *Effect of α-cyano-4-hydroxycinnamate on the transport of pyruvate, lactate, acetate and butyrate into rat liver mitochondria*

Mitochondria (8 mg of protein) were preincubated for 5 min in medium (1 ml) containing succinate (2.5 mM), rotenone (5 μg/ml) and where appropriate: ^3H_2O (2 μCi/ml) as total water space marker; [6,6′(n)-^{3}H]sucrose (2 μCi/ml) or [U-^{14}C]sucrose (0.2 μCi/ml) as extramitochondrial space marker; and α-cyano-4-hydroxycinnamate (0.5 mM). Substrate (1.4 mM, and, where added as ^{14}C-labelled substrate, 0.2 μCi/ml) was then added and after 2 min further incubation the mitochondria were separated from the medium by centrifugation. Samples of the acid extracts of pellet and supernatant were analysed for ^{3}H, ^{14}C and pyruvate (see under 'Methods'). Intramitochondrial volume was calculated from observations on mitochondria incubated with both [^{14}C]sucrose and ^3H_2O. Mitochondrial substrate content was calculated by correcting the pellet substrate content for substrate in the extramitochondrial space determined from the distribution of added markers: [U-^{14}C]sucrose and ^3H_2O (Expt. 1a); ^3H_2O (Expt. 1b); [6,6′(n)-^{3}H]sucrose (Expt. 2). Intramitochondrial water volumes (μl/mg of protein) were 0.93 ± 0.06 (Expt. 1, eight observations) and 0.65 ± 0.04 (Expt. 2, four observations). In Expt. 1(b) and Expt. 2 it is assumed that all ^{14}C assayed was present as the relevant substrate. All results are given as means ± S.E.M. for four observations.

Expt. no.	Substrate	Substrate content of mitochondria incubated (nmol/μl of intramitochondrial space)	
		Without α-cyano-4-hydroxycinnamate	With α-cyano-4-hydroxycinnamate
1(a)	Pyruvate	2.43 ± 0.24	0.90 ± 0.16*
(b)	[U-^{14}C]Pyruvate	2.44 ± 0.27	0.77 ± 0.32*
2	[U-^{14}C]Pyruvate	5.23 ± 0.23	2.27 ± 0.12*
	[U-^{14}C]Lactate	6.50 ± 0.14	6.75 ± 0.48
	[U-^{14}C]Acetate	6.50 ± 0.32	6.62 ± 0.27
	[U-^{14}C]Butyrate	7.18 ± 0.46	6.38 ± 0.54

* $P<0.01$ versus control incubated in the absence of α-cyano-4-hydroxycinnamate.

assay of pyruvate) while having no significant effect on the uptake of acetate or butyrate (Table 2). Lactate was taken up to a lesser extent than pyruvate, acetate or butyrate; however, this uptake was also sensitive to inhibition by α-cyano-4-hydroxycinnamate, although perhaps to a smaller degree than that of pyruvate.

Discussion

The specific inhibition of pyruvate transport by α-cyano-4-hydroxycinnamate implies that a specific carrier is involved in the transport of pyruvate across plasma membranes and also across the inner membrane of mitochondria. The presence of such a carrier explains the rapid transfer of pyruvate across plasma and mitochondrial membranes at physiological pH and pyruvate concentrations. The carrier in plasma membranes may also be involved in the transport of lactate. The structure of the inhibitor might suggest that it is the enol form of pyruvate that binds to the carrier.

α-Cyano-4-hydroxycinnamate appears to be a rather potent inhibitor of pyruvate transport. Preliminary studies (not shown) have indicated that accumulation of pyruvate by mitochondria is complete in less than 30s at 20°C, and yet inhibition of accumulation was clearly demonstrable in mitochondria incubated for 2min and then centrifuged for a further 1min at this temperature (Table 1). Moreover, some of the pyruvate measured may have been bound to sites on the outside of the mitochondria (Zahlten *et al.*, 1972). It should be stressed that in all the experiments in this study the concentration of inhibitor was considerably less than that of pyruvate and effects were demonstrable on mitochondria at concentrations of inhibitor only 4% of those of pyruvate. The potency of the inhibitor should allow the use of 'inhibitor stop' techniques (Pfaff & Klingenberg, 1968; Quagliariello *et al.*, 1969; Robinson & Williams, 1970) to study in detail the kinetics, specificity, temperature-dependence and mechanism of membrane pyruvate transport.

α-Cyano-4-hydroxycinnamate appears to be a rather specific inhibitor. It does not appreciably inhibit a number of enzymes involved in pyruvate metabolism such as pyruvate dehydrogenase, pyruvate carboxylase, pyruvate kinase and lactate dehydrogenase at concentrations of pyruvate and α-cyano-4-hydroxycinnamate used in this study. Moreover, the compound at concentrations of up to 2mM has little or no effect on the oxidation of succinate, glutamate and malate or oxoglutarate and malate by coupled liver mitochondria incubated in the presence

Table 2. *Effect of α-cyano-4-hydroxycinnamate on the transport of pyruvate, lactate, acetate and butyrate into human erythrocytes*

Erythrocytes were incubated for 5min in medium (total vol. 1.0ml) containing ^3H_2O (2mCi/ml) and [U-^{14}C]sorbitol (0.2mg/ml and 0.2μCi/ml) (Expt. 1) or [^{3}H]inulin (0.05mg/ml and 2.0μCi/ml) (Expt. 2); where added, α-cyano-4-hydroxycinnamate was present at 0.5mM. Substrate (approx. 1.5mM and, in Expt. 2, 0.2μCi/ml) was added and after a further 2min of incubation the erythrocytes were separated by centrifugation. The acidified supernatant and pellet extracts were analysed for ^{3}H, ^{14}C and pyruvate or lactate (see under 'Methods'). Intracellular substrate concentrations were determined from the substrate content of the pellet corrected for extracellular contamination calculated from the total pellet water content and the distribution of the extracellular marker [U-^{14}C]sorbitol (Expt. 1) or [^{3}H]inulin (Expt. 2). In both experiments the pellet volume was 0.23ml, of which 0.05ml was extracellular and 0.14ml was intracellular. In Expt. 2 it is assumed that all ^{14}C assayed was present in the relevant substrate. Results are given as means ± S.E.M. for four observations (Expt. 1) or as means of duplicate determinations (Expt. 2).

Expt. no.	Substrate	α-Cyano-4-hydroxycinnamate	Extracellular substrate concentration (mM)	Intracellular substrate concentration (mM)	Concentration ratio $\frac{\text{intracellular}}{\text{extracellular}}$
1	Pyruvate	−	0.61 ± 0.03	3.66 ± 0.04	6.01 ± 0.27
	Pyruvate	+	1.07 ± 0.05*	1.49 ± 0.13*	1.39 ± 0.09*
	Lactate	−	2.52 ± 0.05	2.96 ± 0.05	1.18 ± 0.05
	Lactate	+	2.82 ± 0.04*	1.80 ± 0.06*	0.64 ± 0.02*
2	[U-^{14}C]Pyruvate	−	0.78	4.07	5.2
	[U-^{14}C]Pyruvate	+	1.32	1.68	1.3
	[U-^{14}C]Lactate	−	1.64	2.67	1.6
	[U-^{14}C]Lactate	+	1.91	0.86	0.5
	[U-^{14}C]Acetate	−	0.72	4.75	6.6
	[U-^{14}C]Acetate	+	0.71	4.55	6.4
	[1-^{14}C]Butyrate	−	0.63	3.78	6.0
	[1-^{14}C]Butyrate	+	0.63	3.92	6.2

* $P < 0.01$ versus control incubated in the absence of α-cyano-4-hydroxycinnamate.

of ADP (A. P. Halestrap & R. M. Denton, unpublished work). The dual attributes of specificity and potency would suggest that α-cyano-4-hydroxycinnamate may be used for specifically inhibiting the mitochondrial transport of pyruvate in intact tissue preparations.

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Bücher, Th., Czok, R., Lamprecht, W. & Latzko, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 253–259, Verlag Chemie, Berlin

Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components—Preparation and Fractionation* (Birnie, G. D., ed.), 2nd edn., pp. 77–91, Butterworths, London

Hohorst, H.-J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 266–270, Verlag Chemie, Berlin

Klingenberg, M. (1970*a*) *FEBS Lett.* **6**, 145–154

Klingenberg, M. (1970*b*) *Essays Biochem.* **6**, 119–159

Papa, S., Francavilla, A., Paradies, G. & Meduri, B. (1971) *FEBS Lett.* **12**, 285–288

Pfaff, E. & Klingenberg, M. (1968) *Eur. J. Biochem.* **6**, 66–79

Quagliariello, E., Palmieri, F., Prezioso, G. & Klingenberg, M. (1969) *FEBS Lett.* **4**, 251–254

Robinson, B. H. & Williams, G. R. (1970) *Biochim. Biophys. Acta* **216**, 63–70

Watts, D. J. (1969) Ph.D. Thesis, University of Bristol

Watts, D. J. & Randle, P. J. (1967) *Biochem. J.* **104**, 51P

Zahlten, R. N., Hochberg, A. A., Stratman, F. W. & Lardy, H. A. (1972) *FEBS Lett.* **21**, 11–13