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The Specificity and Metabolic Implications of the Inhibition of Pyruvate Transport in Isolated Mitochondria and Intact Tissue Preparations by α-Cyano-4-hydroxycinnamate and Related Compounds

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1. Effects of α-cyano-4-hydroxycinnamate and α-cyanocinnamate on a number of enzymes involved in pyruvate metabolism have been investigated. Little or no inhibition was observed of any enzyme at concentrations that inhibit completely mitochondrial pyruvate transport. At much higher concentrations (1 mM) some inhibition of pyruvate carboxylase was apparent. 2. α-Cyano-4-hydroxycinnamate (1–100 μM) specifically inhibited pyruvate oxidation by mitochondria isolated from rat heart, brain, kidney and from blowfly flight muscle; oxidation of other substrates in the presence or absence of ADP was not affected. Similar concentrations of the compound also inhibited the carboxylation of pyruvate by rat liver mitochondria and the activation by pyruvate of pyruvate dehydrogenase in fat-cell mitochondria. These findings imply that pyruvate dehydrogenase, pyruvate dehydrogenase kinase and pyruvate carboxylase are exposed to mitochondrial matrix concentrations of pyruvate rather than to cytoplasmic concentrations. 3. Studies with whole-cell preparations incubated *in vitro* indicate that α-cyano-4-hydroxycinnamate or α-cyanocinnamate (at concentrations below 200 μM) can be used to specifically inhibit mitochondrial pyruvate transport within cells and thus alter the metabolic emphasis of the preparation. In epididymal fat-pads, fatty acid synthesis from glucose and fructose, but not from acetate, was markedly inhibited. No changes in tissue ATP concentrations were observed. The effects on fatty acid synthesis were reversible. In kidney-cortex slices, gluconeogenesis from pyruvate and lactate but not from succinate was inhibited. In the rat heart perfused with medium containing glucose and insulin, addition of α-cyanocinnamate (200 μM) greatly increased the output and tissue concentrations of lactate plus pyruvate but decreased the lactate/pyruvate ratio. 4. The inhibition by cyanocinnamate derivatives of pyruvate transport across the cell membrane of human erythrocytes requires much higher concentrations of the derivatives than the inhibition of transport across the mitochondrial membrane. α-Cyano-4-hydroxycinnamate appears to enter erythrocytes on the cell-membrane pyruvate carrier. Entry is not observed in the presence of albumin, which may explain the small effects when these compounds are injected into whole animals.

α-Cyano-4-hydroxycinnamate and its derivatives are extremely potent inhibitors of mitochondrial pyruvate transport (Halestrap & Denton, 1974; Halestrap, 1975). α-Cyano-4-hydroxycinnamate itself almost totally inhibits mitochondrial pyruvate oxidation at concentrations of about 50 μM whereas α-cyanocinnamate and compound UK 5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate] exert almost total inhibition at 1 μM or less (Halestrap, 1975). The transport of pyruvate and lactate across the erythrocyte membrane is also inhibited by α-cyano-4-hydroxycinnamate (Halestrap & Denton, 1974). The studies reported in the present paper were carried out to examine whether the effects of α-cyano-4-hydroxycinnamate were restricted to inhibition of membrane pyruvate transport or whether other enzymes involved in pyruvate metabolism were also inhibited. The possible use of α-cyano-4-hydroxycinnamate and its derivatives in the study of pyruvate metabolism in intact tissue preparations was also investigated. We report that the effects of low concentrations of the inhibitor are almost entirely restricted to mitochondrial pyruvate transport and that the inhibition can be readily demonstrated in a number of intact tissue preparations.

Experimental

Materials

Rats. Epididymal fat-pads, hearts and livers were obtained from male albino Wistar rats (approx. 200 g) allowed free access to water and a stock laboratory diet (modified 41B; Oxoid Ltd., London S.E.1, U.K.). The animals, well matched for

age and weight in any one experiment, were killed by decapitation. Kidney cortex was obtained from similar animals starved for 24h before death.

Chemicals. These were obtained as given in Halestrap (1975) except for crystalline insulin which was a gift of Boots Pure Drug Co. Ltd., Nottingham, U.K. and was dissolved in 3mM-HCl to yield a stock solution of 10units/ml. Acetyl-CoA was prepared by the method of Simon & Shemin (1953).

Media. Fat-pads and kidney-cortex slices were incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5). Additions were made as indicated in the text and Tables.

Mitochondria. Mitochondria were prepared from rat liver (Chappell & Hansford, 1972), fat cells (Martin & Denton, 1970), blowfly flight muscle (Chappell & Hansford, 1972) and rat heart (Halestrap, 1975). Mitochondrial protein concentrations were determined by a biuret method (Gornall *et al.*, 1949).

Methods

Enzyme assays. All enzyme assays were performed at 30°C. Pyruvate carboxylase (EC 6.4.1.1) was assayed in crude homogenates of liver by incorporation of $H^{14}CO_3^-$ into acid-stable material (Wimhurst & Manchester, 1970). The assay medium contained Tris–HCl (0.1M), pH7.4, $MgCl_2$ (5mM), ATP (2mM), $KH^{14}CO_3$ (15mM; 0.25μCi/μmol), acetyl-CoA (0.25mM), acetyl phosphate (2mM), phosphotransacetylase (EC2.3.1.8) (1unit/ml), citrate synthase (EC 4.1.3.7) (1unit/ml) and pyruvate at the required concentration. Assays, carried out in a final volume of 0.5ml in sealed plastic tubes, were started by the addition of enzyme and terminated by the addition of 5M-HCl (0.1ml). Samples (0.4ml) were transferred into scintillation vials, taken to dryness at 100°C, dissolved in water (0.2ml) and counted for radioactivity in a Nuclear–Chicago Isocap scintillation counter after addition of 10ml of scintillator [toluene (600ml), methoxyethanol (400ml), naphthalene (80g) and 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6g)]. Samples were also carried through the assay procedure in the absence of pyruvate to correct for non-specific acid-stable radioactivity.

Pyruvate dehydrogenase (EC 1.2.4.1) was assayed spectrophotometrically by coupling the reaction to arylamine acetyltransferase (Coore *et al.*, 1971). The total activity of pyruvate dehydrogenase was determined after activation of the enzyme by incubation with pig heart pyruvate dehydrogenase phosphate phosphatase (Severson *et al.*, 1974). Pyruvate dehydrogenase kinase was assayed as described by Cooper *et al.* (1974) and pyruvate dehydrogenase phosphate phosphatase by the method of Severson *et al.* (1974). Citrate synthase (EC 4.1.3.7) was assayed by the method of Srere *et al.* (1963) with both oxaloacetate and acetyl-CoA at a concentration of 50μM. Malate dehydrogenase (decarboxylating) ($NADPH^+$) (EC 1.1.1.40) was assayed as described by Martin & Denton (1970) with malate at 50μM. Pyruvate kinase (EC 2.7.1.40) was assayed as described by Pogson (1968) with phosphoenolpyruvate at 50μM. Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Kornberg (1955).

Carboxylation of pyruvate by intact mitochondria. This was measured by a modification of the method of Walter & Stucki (1970). Liver mitochondria (approx. 0.5mg of protein) were incubated for 10min in 0.5ml of medium containing KCl (110mM), potassium phosphate (10mM), $KH^{14}CO_3^-$ (10mM; 0.1μCi/μmol), Tris–HCl (5mM), $MgCl_2$ (5mM) and pyruvate (5mM). The assay was started by the addition of mitochondria and terminated by the addition of 0.1ml of HCl (5M). The acidified medium was centrifuged for 1min in an Eppendorf 3200 centrifuge, the supernatant transferred to a scintillation bottle and evaporated to dryness at 100°C. After dissolution of the residue in water (0.2ml) the toluene–methoxyethanol scintillator (10ml) was added and the sample counted for radioactivity in a Nuclear–Chicago Isocap scintillation counter. Some assays were done in the absence of pyruvate to correct for pyruvate-independent carboxylation. A linear time-course for the incorporation of $H^{14}CO_3^-$ into acid-stable material was observed for at least 10min.

Measurement of the rate of fatty acid synthesis and lactate and pyruvate outputs by epididymal fat-pads. After incubation with [U-^{14}C]glucose, fat-pads were frozen and ground in liquid N_2 and about 1g of the frozen powder was dissolved in 6ml of 5M-KOH at 80°C for 10min. After addition of 6ml of ethanol, saponification was continued for 1h with occasional shaking. The saponified mixture was then cooled on ice, acidified with 9M-H_2SO_4 (4ml) and fatty acids were extracted with 3×10ml of light petroleum (b.p. 40–60°C). After evaporation of the light petroleum with an overdraught, the fatty acid residue was dissolved and transferred into scintillation bottles with a total of 15ml of the toluene–methoxyethanol scintillator before assay of ^{14}C and/or ^{3}H in a Nuclear–Chicago Isocap scintillation counter. The output of lactate and pyruvate was determined as described by Denton & Halperin (1968).

Whole tissue ATP concentrations in fat-pads. Frozen powdered fat-pads were extracted in 5% (w/v) $HClO_4$ and diethyl ether (Denton & Halperin, 1968), and ATP was assayed in the neutralized extract by the method of Lamprecht & Trautschold (1963).

Gluconeogenesis in kidney-cortex slices. This was studied by the method of Krebs *et al.* (1963). Kidney-cortex slices were cut free by the method of Deutsch (1935) and transferred to Krebs & Henseleit's (1932) bicarbonate medium at 0°C before incubation as described in the legends to the Tables. After incubation, 0.1 ml of acidified medium was assayed for glucose with glucose oxidase (Bergmeyer & Bernt, 1963) by using a Boehringer blood-glucose test kit. Control experiments indicated that α-cyano-4-hydroxycinnamate did not interfere with the assay.

Results and Discussion

Effects of α-cyano-4-hydroxycinnamate on some enzymes involved in pyruvate metabolism (Table 1)

To determine whether the inhibitory effects of α-cyano-4-hydroxycinnamate were limited to pyruvate transport, other enzymes involved in pyruvate metabolism were tested for inhibition by this compound. α-Cyano-4-hydroxycinnamate absorbs very strongly at 340nm (ε about 22litre·mol^{-1}·cm^{-1}) and makes the assay of certain enzymes rather difficult at high concentrations of the inhibitor. In these cases α-cyanocinnamate, which does not absorb at 340nm but is a powerful inhibitor of pyruvate transport (Halestrap, 1975), was also tested on the enzymes. Even at low substrate concentration (50μM) and high inhibitor concentration (2.0mM) no appreciable inhibition of citrate synthase, lactate dehydrogenase, pyruvate kinase, pyruvate dehydrogenase, pyruvate dehydrogenase kinase, pyruvate dehydrogenase phosphate phosphatase or NADP$^+$–malate dehydrogenase was observed. However, pyruvate carboxylase did show some inhibition at high concentrations of α-cyanocinnamate and α-cyano-4-hydroxycinnamate. This inhibition was investigated further by using a partially purified preparation of the rat liver enzyme which showed similar kinetic properties to those reported by other workers (Seufert *et al.*, 1971; McLure, 1969; McLure *et al.*, 1971). The K_m for pyruvate was 0.1mM and the inhibition by α-cyano-4-hydroxycinnamate competitive with respect to pyruvate (K_i 2.3mM). By comparison, the K_i value of this inhibitor for mitochondrial pyruvate transport is 6μM (Halestrap, 1975). Initially it appeared that α-cyano-4-hydroxycinnamate inhibited pyruvate dehydrogenase, but further investigation showed that this apparent inhibition was due to inhibition of arylamine acetyltransferase, the enzyme used to follow the reaction. This inhibition was competitive with respect to the substrate *p*-(*p*-aminophenylazo)-benzenesulphonate and it is possible that α-cyano-4-hydroxycinnamate may act as a substrate for the enzyme. α-Cyanocinnamate contains no aryl hydroxyl group and does not inhibit the enzyme.

Effects of α-cyano-4-hydroxycinnamate on pyruvate metabolism by isolated mitochondria

Fig. 1 shows the effect of α-cyano-4-hydroxycinnamate on the rate of pyruvate carboxylation by intact liver mitochondria incubated at 30°C in the presence of pyruvate, phosphate and HCO_3^-. Inhibition was half-maximal at approx. 6μM; no inhibition of isolated pyruvate carboxylase is observed under these conditions. Complete inhibition of carboxylation was not observed even with 1 mM inhibitor. This may be due to a slow rate of carrier-independent diffusion of pyruvate across the

Table 1. *Effects of α-cyano-4-hydroxycinnamate and α-cyanocinnamate on some enzymes of pyruvate metabolism*

Enzymes from fat cells were assayed in samples of an extract of epididymal fat-pads prepared at 0°C by homogenizing the pads in 0.1M-potassium phosphate buffer, pH7.2, containing 2mM-EDTA and centrifuging for 2min at 15000*g*. Inhibition is expressed as a percentage of the rate in the absence of added α-cyano-4-hydroxycinnamate or α-cyanocinnamate. Results are the mean of at least two determinations. Further details of assay conditions are given under 'Methods'.

Enzyme	Concentration (mM)		Inhibition (%)	Concentration (mM)		Inhibition (%)
	α-Cyano-4-hydroxycinnamate	Pyruvate		α-Cyano-cinnamate	Pyruvate	
Citrate synthase (fat-cell)	0.25	—	0	2.0	—	0
Pyruvate kinase (rabbit muscle)	0.05	—	0	2.0	—	25
Pyruvate carboxylase (fat-cell)	2.00	0.2	25	2.0	0.1	65
Lactate dehydrogenase (fat-cell)	0.05	0.05	0	2.0	0.1	0
NADP$^+$–malate dehydrogenase (fat-cell)	0.05	—	15	2.0	—	23
Pyruvate dehydrogenase (heart muscle)	2.00	0.2	0	2.0	0.1	14
Pyruvate dehydrogenase kinase (heart muscle)	0.50	—	0	—	—	—
Pyruvate dehydrogenase phosphate phosphatase (heart muscle)	0.05	—	0	—	—	—
Mitochondrial pyruvate transport	0.05	1.4	>90	0.05	2.0	>90

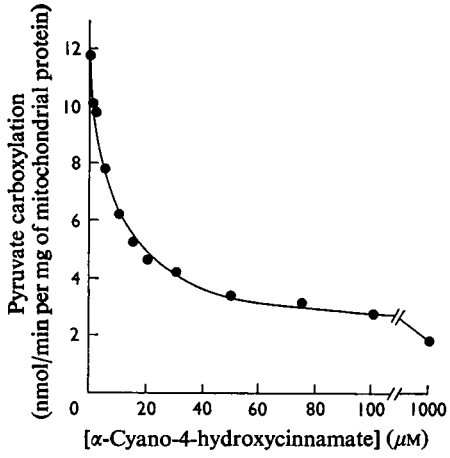

Fig. 1. *Inhibition of pyruvate carboxylation in intact liver mitochondria by α-cyano-4-hydroxycinnamate*

Pyruvate carboxylation was measured by pyruvate-dependent fixation of $H^{14}CO_3^-$ into acid-stable material as described under 'Methods'. Pyruvate was present at 5mM.

membrane at these high concentrations (2mM) of pyruvate. Such carrier-independent pyruvate transport has been detected by proton flux at high pyruvate concentrations (Halestrap, 1975). The K_i of pyruvate transport into rat liver mitochondria for α-cyano-4-hydroxycinnamate is also 6μM. This would imply that under these conditions pyruvate transport may limit the rate of pyruvate carboxylation. Further, the rate of pyruvate carboxylation at 30°C (11.8nmol/min per mg of mitochondrial protein) may represent a rate of pyruvate transport of about 20nmol/min per mg of mitochondrial protein since some pyruvate oxidation also occurs under these conditions (Walter & Stucki, 1970; Stucki *et al.*, 1972). This is in fact close to the maximum rate of pyruvate transport at 30°C of about 17 nmol/min per mg of mitochondrial protein (Halestrap, 1975).

Fig. 2(*a*) shows the effects of α-cyano-4-hydroxycinnamate on pyruvate and 2-oxoglutarate oxidation by rat heart mitochondria. Pyruvate-dependent-O_2 uptake was almost totally inhibited by 25μM-α-cyano-hydroxycinnamate whereas oxidation of 2-oxoglutarate remained unaffected. Other experiments (not shown) showed no appreciable effects of 0.5mM-α-cyano-4-hydroxycinnamate on the oxidation of succinate, glutamate, acetyl carnitine and palmitoyl carnitine. Similar specific inhibition of pyruvate oxidation could be observed with mitochondria from rat brain, adipose tissue or kidney cortex and from blowfly flight muscle (Fig. 2*b*).

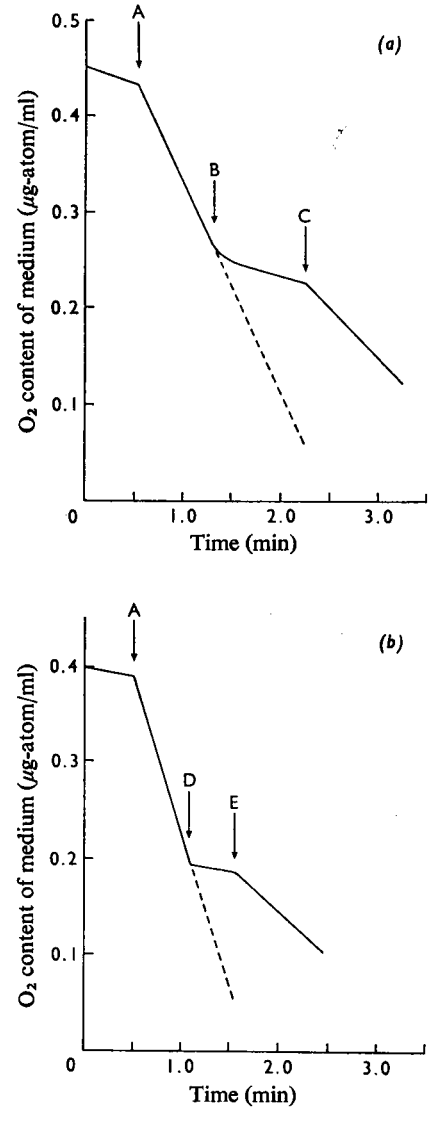

Fig. 2. *Effect of α-cyano-4-hydroxycinnamate on pyruvate oxidation by rat heart and blowfly flight-muscle mitochondria*

Mitochondria from rat heart (approx. 0.5mg of protein) (*a*) or blowfly muscle (approx. 0.15mg of protein) (*b*) were suspended in 1ml of KCl (125mM)–Tris–HCl (20mM) medium, pH7.4, at 30°C in an oxygen electrode. In (*a*) phosphate was present at 2mM, malate at 0.5mM and pyruvate at 2.5mM. In (*b*) phosphate was present at 25mM, aspartate at 5mM and pyruvate at 1mM. Other additions were made as indicated: A, ADP (2μmol); B, α-cyano-4-hydroxycinnamate (25nmol); C, 2-oxoglutarate (5μmol); D, α-cyano-4-hydroxycinnamate (100nmol); E, proline (5μmol). ----, O_2 uptake if no inhibitor was added.

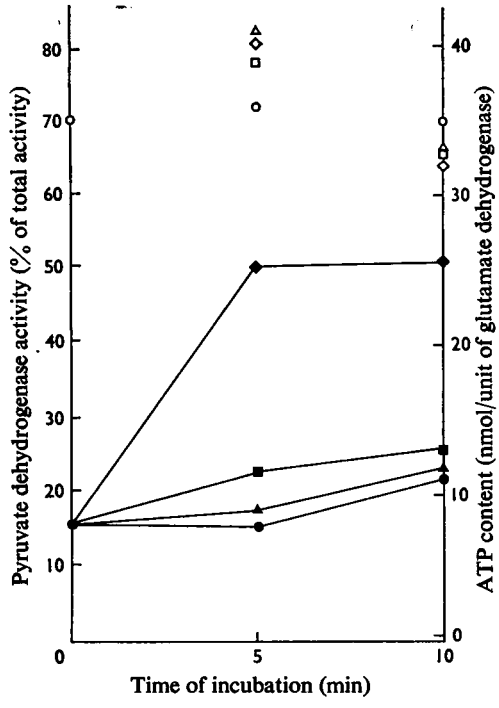

Fig. 3. *Inhibition of the pyruvate-dependent activation of pyruvate dehydrogenase in fat-cell mitochondria by α-cyano-4-hydroxycinnamate*

Mitochondria (approx. 1mg of protein/ml) were pre-incubated for 10min at 30°C in medium [KCl (125mM)–Tris–HCl (20mM)–phosphate (2mM), pH7.4] containing 2-oxoglutarate (5mM) and malate (0.5mM) to convert much of the pyruvate dehydrogenase in the mitochondria into the inactive phosphorylated form. Mitochondria were then incubated in the same medium without further additions (●, ○), or with additions of 0.1mM-α-cyano-4-hydroxycinnamate (▲, △), or 1mM-pyruvate (◆, ◇) or both α-cyano-4-hydroxycinnamate and pyruvate (■, □) for 5 or 10min. Samples were taken for the assay of ATP content (○, △, ◇, □) and the activity of pyruvate dehydrogenase (expressed as % of total pyruvate dehydrogenase activity) (●, ▲, ◆, ■), and of glutamate dehydrogenase as in Severson *et al.* (1974). Results are the mean of two observations on separate preparations of mitochondria which agreed within 10%.

Incubation of fat-cell mitochondria with pyruvate in the presence of 2-oxoglutarate and malate results in an increase in pyruvate dehydrogenase activity (Martin *et al.*, 1972) presumably because the kinase which causes inactivation of pyruvate dehydrogenase by phosphorylation is inhibited by pyruvate (Linn *et al.*, 1969; Martin *et al.*, 1972; Cooper *et al.*, 1974). This pyruvate-dependent activation of pyruvate dehydrogenase in adipose-tissue mitochondria can be prevented by the addition of 0.1mM-α-cyano-4-hydroxycinnamate (Fig. 3). There is no appreciable change in the mitochondrial content of ATP under these conditions and no effect of α-cyano-4-hydroxycinnamate has been observed on either the kinase or the phosphatase involved in pyruvate dehydrogenase regulation (Table 1).

Effects of α-cyano-4-hydroxycinnamate on pyruvate metabolism by isolated tissue preparations

Table 2 shows the effects of α-cyano-4-hydroxycinnamate on the metabolism of epididymal fat-pads under a variety of incubation conditions. The rate of fatty acid synthesis from glucose (in the presence and the absence of insulin) or from fructose is greatly inhibited by α-cyano-4-hydroxycinnamate, whereas the output of lactate and pyruvate is substantially increased in the presence of inhibitor. No changes in the concentrations of ATP in the whole tissue were observed in the presence of α-cyano-4-hydroxycinnamate. Values of all parameters measured in control experiments agree well with the data of other workers (Denton & Halperin, 1968; Coore *et al.*, 1971; Saggerson & Greenbaum, 1970; Saggerson, 1972).

The output of lactate plus pyruvate was increased in all the inhibited incubations, but the effect of α-cyano-4-hydroxycinnamate on the ratio of lactate/pyruvate released into the medium varied. In the presence of glucose and insulin, α-cyano-4-hydroxycinnamate had no effect on the lactate/pyruvate ratio. Under conditions of less-rapid fatty acid synthesis, the inhibitor caused a decrease in the lactate/pyruvate ratio. This was most noticeable in the absence of any exogenous substrate but was also apparent when tissues were incubated with glucose or fructose in the absence of insulin. It would appear that under these conditions the transfer of cytoplasmic reducing equivalents into the mitochondria is enhanced. This would suggest that the oxidation of fatty acids is not sufficient to supply all the reducing equivalents oxidized within the mitochondria when the supply of pyruvate is severely restricted.

If α-cyano-4-hydroxycinnamate only prevents mitochondrial metabolism of pyruvate, it would be expected that, in the presence of glucose to allow the generation of NADPH through the operation of the pentose phosphate pathway, the incorporation of acetate into fatty acids should not be decreased in the presence of inhibitor. The results shown in Table 3 support this prediction. The incorporation of ^{14}C from [U-^{14}C]glucose into fatty acids was substantially decreased by α-cyano-4-hydroxycinnamate, whereas [^{3}H]acetate incorporation was actually increased slightly but significantly. This result again indicates the specificity of the inhibition of α-cyano-4-hydroxycinnamate and makes it unlikely that

Table 2. *Effects of α-cyano-4-hydroxycinnamate on the metabolism of rat epididymal fat-pads*

In each experiment fat-pads were paired between control and experimental flasks (four pads/flask) containing 10ml of Krebs' bicarbonate-buffered medium (Krebs & Henseleit, 1932). After gassing with O_2+CO_2 (95:5), the pads were preincubated for 30min at 37°C, removed, blotted and transferred to fresh medium containing additions as indicated. Where present U-^{14}C-labelled substrates were at 10mM and 0.1 μCi/ml. After gassing, the pads were incubated for a further 30min and then removed, blotted, weighed and frozen and ground in liquid N_2. Incorporation of substrate into fatty acids, the outputs of pyruvate and lactate into medium and the tissue concentration of ATP were measured as described under 'Methods'. Results are given as the mean±S.E.M. for four separate experiments except where no error is given, when the result is the mean of two observations agreeing within 10%. Effect of α-cyano-4-hydroxycinnamate: $*P<0.02$; $**P<0.01$.

Additions to incubation medium	Concentration of α-cyano-4-hydroxycinnamate (mM)	Incorporation of ^{14}C into fatty acids (μg-atoms/h per g)	Lactate output (μmol/h per g)	Pyruvate output (μmol/h per g)	Lactate output / Pyruvate output	Whole tissue concentration of ATP (nmol/g)
1. [U-^{14}C]Glucose	0	4.64±5.2	0.82±0.09	0.160±0.014	5.10±0.31	103.0
	0.1	2.00±0.31**	1.79±0.09**	0.580±0.046**	3.10±0.35**	95.5
2. [U-^{14}C]Glucose+insulin (1 munit/ml)	0	32.7±1.4	1.00±0.09	0.182±0.010	5.50±0.29	140.0±3.0
	0.1	9.5±0.7**	2.99±0.23**	0.516±0.040**	5.90±0.30	146.0±6.0
3. [U-^{14}C]Fructose	0	5.20±0.66	1.02±0.07	0.133±0.021	8.37±1.42	134.0
	0.2	2.45±0.30**	2.03±0.07**	0.704±0.010**	3.14±0.62*	145.0
4. None	0	—	0.31±0.04	0.034±0.002	10.2±1.3	—
	0.1	—	0.38±0.05	0.192±0.016**	2.0±0.1**	—

the inhibition of lipogenesis is by some non-specific effect. No changes in tissue ATP concentrations were seen in the presence of inhibitor (Table 2).

The inhibition of fatty acid synthesis from glucose by α-cyano-4-hydroxycinnamate can be reversed by washing out the inhibitor (Table 4). This is consistent with the reversible nature of the inhibition of pyruvate transport by α-cyano-4-hydroxycinnamate observed in isolated liver mitochondria (Halestrap, 1975).

Gluconeogenesis from both pyruvate and lactate requires the carboxylation of pyruvate in the mitochondria, which we have shown can be inhibited by α-cyano-4-hydroxycinnamate (Fig. 1). It would be expected therefore, that this inhibitor would cause a decrease in the rate of gluconeogenesis from pyruvate and lactate but not from succinate. In Table 5 we present results of experiments using kidney-cortex slices which fulfil these expectations. Rates of gluconeogenesis in the absence of inhibitor were similar to those observed by Krebs *et al.* (1963). The low rate of gluconeogenesis seen in the absence of added substrate was also slightly inhibited by α-cyano-4-hydroxycinnamate, but since the source of the glucose under these conditions is not known little can be concluded from this result. Inhibition of gluconeogenesis from pyruvate and lactate by α-cyano-4-hydroxycinnamate has also been observed in isolated parenchymal cells from mouse liver (Crisp, 1973) whereas gluconeogenesis from glycerol and fructose in these cells was little affected. The sensitivity of gluconeogenesis from pyruvate in kidney-cortex slices to inhibition by α-cyano-4-hydroxycinnamate is shown in Fig. 4. Inhibition was half-maximal at about 25μM and reached a maximum value of about 85% inhibition. This pattern of inhibition is quite close to that observed on pyruvate carboxylation by intact liver mitochondria (Fig. 1).

The possibility existed, however, that the site of inhibition of gluconeogenesis was the entry of pyruvate and lactate into the cell since α-cyano-4-hydroxycinnamate is known to inhibit pyruvate transport into erythrocytes (Halestrap & Denton, 1974). To gain more information on the transport of both pyruvate and α-cyano-4-hydroxycinnamate across cell membranes further experiments have been performed with human erythrocytes. In the presence of a Chloride gradient created as described by Halestrap & Denton (1974) the accumulation of α-cyano-4-hydroxycinnamate was studied by making use of the high absorbancy of this compound at 340nm (Table 6). Accumulation was found to be extensive and the extent was unaffected by addition of a permeant anion, acetate (2mM), but was markedly decreased in the presence of α-cyano-cinnamate (which does not absorb at 340nm).

Table 3. *Effects of α-cyano-4-hydroxycinnamate on the incorporation of ^{14}C from [U-^{14}C]glucose and ^{3}H from [^{3}H]acetate into fatty acids in rat epididymal fat-pads*

Experimental procedure was as given in the legend to Table 2 except that the incubation medium contained both 10mM-[U-^{14}C]glucose (0.1 μCi/ml) and 10mM-sodium [^{3}H]acetate (2 μCi/ml). Results are the means ± S.E.M. for four separate experiments. Effect of α-cyano-4-hydroxycinnamate compared with control value: $^{**}P<0.001$; $^{*}P<0.01$.

	Incorporation of label into fatty acids (μg-atoms of substrate/h per g)		
Source of label	Control	α-Cyano-4-hydroxycinnamate (0.1 mM)	Rate with inhibitor (% of control value)
[U-^{14}C]Glucose	21.84 ± 5.28	8.76 ± 1.98	40.6 ± 1.8**
[^{3}H]Acetate	2.32 ± 0.36	2.76 ± 0.46	118.2 ± 2.0*

Table 4. *Reversibility of α-cyano-4-hydroxycinnamate inhibition of fatty acid synthesis in rat epididymal fat-pads*

Fat-pads from six rats were evenly distributed between four flasks (three pads/flask) containing 10ml of bicarbonate-buffered medium. After gassing with O_2+CO_2 (95:5) the pads were incubated for 30min at 37°C, blotted and transferred to fresh medium containing insulin (1munit/ml) and 10mM-glucose. In two incubations [U-^{14}C]glucose was also present at 0.1 μCi/ml. One of these incubations contained 0.1mM-α-cyano-4-hydroxycinnamate as did one of the incubations containing unlabelled glucose. After gassing and 30min further incubation (first incubation), the pads were removed and blotted. Those incubated with [U-^{14}C]glucose were frozen in liquid N_2 and weighed. The two other sets of pads were washed in bicarbonate-buffered medium, gassed and incubated in fresh medium containing 10mM-glucose for 45min at 37°C and then transferred to medium containing insulin (1munit/ml) and 10mM-glucose (0.1 μCi/ml). After gassing, incubation was continued (second incubation) for 30min and the pads were blotted, frozen and weighed. The incorporation of [U-^{14}C]glucose into fatty acids was measured as described under 'Methods'. Results are the mean of four separate experiments. Effect of α-cyano-4-hydroxycinnamate compared with control value: $^{*}P<0.001$.

	Incorporation of ^{14}C into fatty acids (μg-atoms/h per g)		
Incubation	Control	α-Cyano-4-hydroxycinnamate in first incubation (0.1 mM)	Rate with inhibitor (% of control value)
First incubation (before removal of inhibitor)	29.6 ± 1.5	11.01 ± 1.25	38.2 ± 3.8*
Second inhibition (after removal of inhibitor)	38.0 ± 1.1	33.0 ± 1.8	87.4 ± 6.0

Table 5. *Effects of α-cyano-4-hydroxycinnamate on gluconeogenesis from various substrates by rat kidney-cortex slices*

Slices of rat kidney cortex were prepared as described under 'Methods'. Each slice was divided equally between control and experimental flasks containing 4ml of bicarbonate-buffered medium, each flask having a final weight of kidney cortex of approximately 10–20mg dry wt. The flasks were gassed with O_2+CO_2 (95:5) and preincubated for 10min at 37°C before the appropriate substrate was added as a 1M solution in bicarbonate-buffered medium. Incubation was continued for 1h more, when the slices were removed, blotted, freeze-dried and weighed, and the medium was acidified with 0.4ml of HCl (4M) before assay of glucose. Results are given as the mean ± S.E.M. of three separate observations. Effect of α-cyano-4-hydroxycinnamate compared with control: $^{*}P<0.01$; $^{**}P<0.001$.

	Glucose production (μmol/h per g)		
Substrate (mM)	Control	α-Cyano-4-hydroxycinnamate (0.1 mM)	Rate with inhibitor (% of control value)
None	11.4 ± 0.7	7.56 ± 0.6	65.3 ± 2.9*
Pyruvate (10)	201.3 ± 22.1	32.4 ± 2.0	16.4 ± 1.8**
Lactate (10)	58.1 ± 12.2	13.1 ± 4.9	20.6 ± 5.8**
Succinate (10)	114.6 ± 4.4	97.1 ± 9.7	84.3 ± 5.4

This analogue like α-cyano-4-hydroxycinnamate and compound UK 5099 inhibited pyruvate entry into the erythrocyte (Table 7; Halestrap & Denton, 1974). It would therefore seem likely that α-cyano-4-hydroxycinnamate enters the erythrocyte on the pyruvate transporter. This is in contrast with the mitochondrial pyruvate carrier, which does not appear to transport α-cyano-4-hydroxycinnamate (Halestrap, 1975).

The sensitivity of erythrocyte pyruvate transport to inhibition by α-cyano-4-hydroxycinnamate was considerably less than that of the mitochondrial carrier (Table 7). The latter process is almost totally inhibited by 25 μM inhibitor whereas no significant

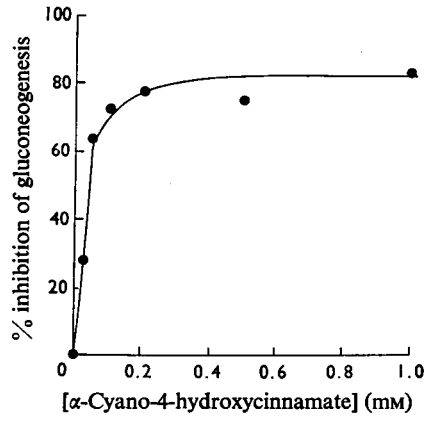

Fig. 4. *Concentration-dependence of α-cyano-4-hydroxycinnamate inhibition of gluconeogenesis from pyruvate in kidney-cortex slices*

The experimental procedure was that described in Table 5, pyruvate being present at 10mM concentration and α-cyano-4-hydroxycinnamate as indicated.

Table 6. *Uptake of α-cyano-4-hydroxycinnamate by erythrocytes*

Erythrocytes were prepared in citrate-based medium (84mM-sodium citrate–10mM-Tris adjusted to pH7.4 with 1M-NaH_2PO_4) and then incubated in the same medium containing α-cyano-4-hydroxycinnamate (0.2mM), [6,6′-^{3}H]sucrose (1mM; 1μCi/ml) as extracellular marker and other additions as indicated. After 5min (Expt. 1) or 2.5min (Expt. 2), the cells were separated by centrifugation. The uptake of α-cyano-4-hydroxycinnamate into the cells was calculated from the disappearance of α-cyano-4-hydroxycinnamate from the supernatant. This was determined from the reduction in absorption at 340nm in supernatant samples diluted tenfold compared with supernatants from cells centrifuged immediately after addition of α-cyano-4-hydroxycinnamate (0.2mM). (The percentage reduction in control values was in the range 0.25–0.30; the molar extinction of α-cyano-4-hydroxycinnamate at 340nm is 22.0litre·mol^{-1}·cm^{-1}). Extracellular and intracellular volumes were calculated as by Halestrap & Denton (1974). Results are the mean±S.E.M. of four separate observations; *P<0.001 compared with control values.

Expt.	Additions	α-Cyano-4-hydroxycinnamate accumulation as: intracellular concentration / extracellular concentration
1	None (control)	4.11±0.21
	Albumin (20mg/ml)	0.15±0.08*
2	None (control)	2.68±0.19
	Acetate (2mM)	2.41±0.10
	α-Cyanocinnamate (0.2mM)	1.36±0.10*

Table 7. *Sensitivity of erythrocyte pyruvate transport to inhibition by α-cyano-4-hydroxycinnamate and related compounds*

Human erythrocytes were prepared in citrate-based medium (84mM-sodium citrate–10mM-Tris adjusted to pH7.4 with 1M-NaH_2PO_4) and then incubated at 22°C in the same medium containing [3-^{14}C]pyruvate (1mM; 0.1μCi/ml) and [6,6′-^{3}H]sucrose (1mM; 1μCi/ml) and other additions as indicated. After 5min the cells were separated by centrifugation and the [^{14}C]pyruvate accumulated was determined. Further details were as given by Halestrap & Denton (1974). Results are the mean±S.E.M. of four separate determinations; *P<0.001 compared with control value.

Additions	Pyruvate accumulation by erythrocytes: intracellular concentration / extracellular concentration
None (control)	5.06±0.14
α-Cyano-4-hydroxycinnamate (0.5mM)	3.84±0.23*
α-Cyano-4-hydroxycinnamate (0.025mM)	4.90±0.12
α-Cyanocinnamate (0.2mM)	3.56±0.05*
Compound UK 5099 (0.1mM)	3.11±0.11*
Albumin (20mg/ml)	4.82±0.19
Albumin (20mg/ml)+α-cyano-4-hydroxycinnamate (0.5mM)	4.73±0.10

inhibition of erythrocyte pyruvate transport was detected at this concentration. Similarly, although albumin (20mg/ml) does not prevent inhibition of mitochondrial pyruvate transport by α-cyano-4-hydroxycinnamate (Halestrap, 1975) it does prevent inhibition of the erythrocyte system. Entry of inhibitor into the cell is also prevented by albumin (Table 6), which implies that albumin binds the inhibitor making it unavailable for cell-membrane transport.

We have not obtained conclusive evidence that pyruvate or lactate entry into cells other than erythrocytes is inhibited by α-cyano-4-hydroxycinnamate or its analogues. The inhibition of gluconeogenesis from pyruvate and lactate in kidney-cortex slices is demonstrable at concentrations of inhibitor that cause little or no inhibition of the entry of pyruvate or lactate into the erythrocyte (Fig. 4). The markedly increased outputs of pyruvate and lactate by epididymal fat-pads incubated with glucose or fructose when exposed to α-cyano-4-hydroxycinnamate (100μM) (Table 2) show that, if a lactate/pyruvate transport system does exist in these cells, flux through the system is not inhibited under these conditions. In hearts perfused with medium containing 1mM-glucose and 25munits of insulin/ml as described by Randle *et al.* (1970), we were also unable to show evidence for inhibition of pyruvate or

lactate transport out of the heart by α-cyanocinnamate. The presence of α-cyanocinnamate (200μM) increased the output of lactate and pyruvate respectively from 4.29±0.49 and 1.05±0.10 to 4.70±0.10 and 5.51±0.58μmol/min per g dry wt. (each result the mean±S.E.M. of four observations). Under the same conditions the internal concentrations of lactate and pyruvate respectively altered from 1.12±0.16 and 0.068±0.012mM to 0.62±0.04 and 0.27±0.04mM. Thus both in heart and adipose tissue the effects of α-cyanocinnamic acid analogues at the concentrations used in this study appear to be restricted to inhibition of pyruvate transport across the mitochondrial membrane. The sensitivity of the inhibition of gluconeogenesis from lactate and pyruvate suggests that this is also the case for kidney-cortex slices.

General conclusions

It is clear from the results reported by Halestrap (1975) that α-cyano-4-hydroxycinnamate and its analogues α-cyanocinnamate and compound UK5099 are very potent inhibitors of the transport of pyruvate into and out of mitochondria. The studies in the present paper on erythrocytes confirm that these compounds inhibit the transfer of pyruvate into erythrocytes but much higher concentrations are required. The effects of α-cyano-4-hydroxycinnamate and α-cyanocinnamate on glucose metabolism in adipose and heart tissue and gluconeogenesis in kidney-cortex slices appear to be solely confined to inhibition of the transport of pyruvate into the mitochondria. No enzymes of pyruvate metabolism showed appreciable inhibition by these compounds except pyruvate carboxylase and the inhibition of this enzyme was only apparent at concentrations of inhibitor far in excess of those required to inhibit pyruvate transport. No effects were detected on other metabolic pathways in the tissues studied: thus neither gluconeogenesis from succinate nor fatty acid synthesis from acetate was diminished.

In conclusion the attributes of potency, specificity and the ability to enter cells demonstrated by these two inhibitors indicates that they are likely to be very useful tools in the study of the role of mitochondrial pyruvate metabolism in intact cell preparations. α-Cyanocinnamate is probably the inhibitor of choice for most studies since it is more powerful than α-cyano-4-hydroxycinnamate and does not absorb at 340nm. The entry of α-cyano-4-hydroxycinnamate into erythrocytes was greatly diminished in the presence of albumin. Attempts to demonstrate inhibition of pyruvate metabolism with α-cyano-4-hydroxycinnamate *in vivo* have not been successful and it is possible that binding of this inhibitor to albumin and other plasma proteins greatly decreased its uptake into cells.

The near complete inhibition of both pyruvate oxidation and pyruvate carboxylation by α-cyano-4-hydroxycinnamate is strong evidence that the pyruvate-binding sites of both the pyruvate decarboxylase component of the pyruvate dehydrogenase complex and pyruvate carboxylase are within the inner mitochondrial membrane. Similarly the ability of α-cyano-4-hydroxycinnamate to prevent the increased conversion of the pyruvate dehydrogenase complex into the active non-phosphorylated form demonstrates that any regulation of pyruvate dehydrogenase kinase by pyruvate that may occur physiologically requires the prior transport of pyruvate across the mitochondrial membrane. Thus the possibility must be considered that the rate of carboxylation or decarboxylation of pyruvate to give oxaloacetate and acetyl-CoA respectively might be regulated in certain physiological circumstances by alterations in the activity of the mitochondrial pyruvate-transporting system. The $V_{max.}$ value of the system, particularly in liver, may not be greatly in excess of maximum rates of pyruvate metabolism in the intact tissue. Circumstances in which regulation of pyruvate transport might be important include the effects of hormones on gluconeogenesis in the liver (Adam & Haynes, 1969; Halestrap, 1975) and the effects of increased plasma and tissue concentrations of phenylpyruvate and 2-oxo-4-methyl pentanoate in phenylketonuria and maple-syrup urine disease (Halestrap *et al.*, 1974).

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