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Identification of the protein responsible for pyruvate transport into rat liver and heart mitochondria by specific labelling with [^{3}H]*N*-phenylmaleimide

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1. *N*-Phenylmaleimide irreversibly inhibits pyruvate transport into rat heart and liver mitochondria to a much greater extent than does *N*-ethylmaleimide, iodoacetate or bromopyruvate. α-Cyanocinnamate protects the pyruvate transporter from attack by this thiol-blocking reagent. 2. In both heart and liver mitochondria α-cyanocinnamate diminishes labelling by [^{3}H]*N*-phenylmaleimide of a membrane protein of subunit mol.wt. 15000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 3. Exposure of mitochondria to unlabelled *N*-phenylmaleimide in the presence of α-cyanocinnamate, followed by removal of α-cyanocinnamate and exposure to [^{3}H]*N*-phenylmaleimide, produced specific labelling of the same protein. 4. Both labelling and kinetic experiments with inhibitors gave values for the approximate amount of carrier present in liver and heart mitochondria of 100 and 450 pmol/mg of mitochondrial protein respectively. 5. The turnover numbers for net pyruvate transport and pyruvate exchange at 0°C were 6 and 200 min^{-1} respectively.

Pyruvate enters mitochondria by a transport mechanism that is specifically inhibited by α-cyanocinnamate derivatives (Halestrap & Denton, 1974; Halestrap, 1975, 1976, 1978*a*; Halestrap *et al.*, 1980). It has been reported that pyruvate transport may also be inhibited by thiol-blocking reagents such as iodoacetate and *N*-ethylmaleimide (Papa & Paradies, 1974; Halestrap, 1976; Paradies & Papa, 1978), and Halestrap (1976, 1978*a*) and Halestrap *et al.* (1980) have proposed that α-cyanocinnamate also inhibits by binding reversibly to an intramitochondrial thiol group.

Identification of several mitochondrial carriers has been achieved by specific labelling techniques (Klingenberg, 1976*a*; Klingenberg *et al.*, 1978; Aquila *et al.*, 1978; Heaton *et al.*, 1978; Wohlrab, 1979; Hofmann & Kadenbach, 1979). In the case of the adenine nucleotide translocase the protection that carboxyatractylate affords the carrier from attack by thiol-blocking reagents and 8-azido-ATP can be used to identify the carrier protein (Klingenberg, 1976*b*; Heaton *et al.*, 1978; Wohlrab, 1979). A similar technique has been used to identify the mitochondrial phosphate transporter by using organomercurials to protect essential thiol groups (Coty & Pedersen, 1975; Hadvary & Kadenbach, 1976).

Abbreviation used: SDS, sodium dodecyl sulphate.

In the present paper we investigate the use of α-cyanocinnamate to protect the pyruvate transporter from inactivation by thiol-blocking reagents. In addition, we demonstrate that the reversible protection afforded by α-cyanocinnamate allows identification of the carrier after labelling of heart and liver mitochondrial proteins with [^{3}H]*N*-phenylmaleimide. Separation of the labelled mitochondrial proteins by SDS/polyacrylamide-gel electrophoresis showed the protein to have a molecular weight of about 15000 and to be present at about 100 and 450 pmol/mg of protein in liver and heart mitochondria respectively. These values agree closely with those derived kinetically by titration of pyruvate transport activity with the inhibitor α-cyano-β-(1-phenylindol-3-yl)acrylate.

Experimental

Chemicals

Unless stated below, all chemicals, biochemicals and radiochemicals were obtained from the sources given previously (Halestrap 1975, 1978*a*). *N*-Phenylmaleimide was synthesized from aniline and maleic anhydride by the method of Cava *et al.* (1961). The ^{3}H-labelled compound was prepared similarly but on a micro scale from [*ring*-G-^{3}H]aniline (50 μCi/μmol) obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

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Mitochondria

Rat liver mitochondria were prepared as described by Chappell & Hansford (1972), and rat heart mitochondria by using a Polytron PT 10 tissue homogenizer as described by Halestrap (1975). The isolation medium contained 250mM-sucrose, 10mM-Tris/HCl buffer and 2mM-EGTA at pH7.4. The mitochondria always demonstrated a control ratio of greater than 3 with succinate as substrate. Mitochondrial protein was measured by a modified biuret method (Gornall *et al.*, 1949).

Measurement of mitochondrial parameters

Mitochondrial volumes and the accumulation of pyruvate and acetate by mitochondria were measured as described previously (Halestrap & Denton, 1974; Halestrap & McGivan, 1979). Rat liver mitochondria (approx. 4mg of mitochondrial protein) were suspended in 1ml of medium containing 250mM-sucrose, 20mM-4-morpholinepropanesulphonic acid, 1mM-KCl, 3mM-ascorbate, 50μM-*NNN'N'*-tetramethylphenylenediamine, 0.5mM-EGTA, 1μM-valinomycin, 1μM-rotenone and either [6,6-^{3}H]sucrose (0.15μCi/ml) or [U-^{14}C]sucrose (0.1μCi/ml), depending on the isotopic form of the compound being taken up. The medium was at pH7.0 and maintained at 6°C. The mitochondria were preincubated for 3min before the addition of either [2-^{14}C]pyruvate (0.15mM, 0.1μCi/ml), [^{3}H]acetate (0.15mM, 0.5μCi/ml) or ^3H_2O (1μCi/ml). The mitochondria were separated from the medium after the required incubation period by rapid centrifugation at 9000*g* for 1min in an Eppendorf 3200 centrifuge. The pellet and supernatant were separated and acidified with $HClO_4$ (final concn. 2%, w/v), and the mitochondrial contents of pyruvate, acetate or water were determined by assay of ^{3}H and ^{14}C as described previously (Halestrap & Denton, 1974). Oxidation of pyruvate by rat heart mitochondria was studied polarographically as described by Halestrap (1975).

Treatment of mitochondrial proteins for electrophoresis

After incubation of mitochondria with *N*-phenylmaleimide under the required conditions (see the legends to Figures and Tables), mitochondria were disrupted by freezing and thawing twice in 100mM-sodium phosphate/2mM-EDTA buffer, pH7.2. Separation of a crude inner-membrane fraction was achieved by centrifugation for 50000*g*-min. The protein concentration of this fraction was measured on a parallel membrane preparation from the same labelled mitochondria by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The pellet, which contained all the mitochondrial succinate dehydrogenase activity but only 50% of the initial protein, was dissolved in sample buffer (20mM-sodium phosphate, 40mg of SDS/ml, 700mM-2-mercaptoethanol, 0.2mg of Bromophenol Blue/ml and 200mg of sucrose/ml, pH7.0), and proteins were separated by discontinuous SDS/polyacrylamide-gel electrophoresis by using the method of Laemmli (1970). Slab gels (14cm × 14cm) of 1cm track width and 12.5% (w/v) polyacrylamide were used, and each track was loaded to an identical protein concentration. After electrophoresis at 35mA/slab for 3–4h, when the dye front reached 1cm from the bottom of the gel, the gel was fixed for 30min in 50% (v/v) methanol containing 1M-trichloroacetic acid before staining of the proteins with Coomassie Brilliant Blue. After being destained, gels were prepared for fluorography by soaking in scintillator [20% (w/v) 2,5-diphenyloxazole in dimethyl sulphoxide] for 4h as described by Bonner & Laskey (1974) before being dried down and exposed to pre-activated film (Kodak X-Omat) at −70°C (Laskey & Mills, 1975). Purified proteins were used for molecular-weight determinations, and for assessment of the ^{3}H radioactivity (d.p.m.) represented by bands in the fluorograph [^{3}H]*N*-phenylmaleimide-labelled lysozyme of known radioactivity was included. This was prepared by incubating lysozyme (5mg/ml) with [^{3}H]*N*-phenylamaleimide (1mM, 50μCi/μmol) in 20mM-Tris/HCl buffer, pH7.8, for 20min at room temperature. The incubation medium was then diluted 5-fold with water, and the protein was precipitated with trichloroacetic acid (10%, w/v). The precipitate was washed several times with trichloroacetic acid (10%, w/v) before being dissolved in sample buffer at about 1mg/ml. The amount of ^{3}H incorporated into the protein was determined by dialysing portions of the standards against fresh sample buffer and assaying the dialysis residue for ^{3}H by liquid-scintilation counting. Fluorographs were scanned at 625nm with a Gilford linear densitometer connected to a Hewlett Packard 9845S computer, which allowed highly flexible data manipulation, display and storage. Peak areas were calculated by this means and could be corrected to d.p.m. by comparison with the peak area of the ^{3}H-labelled lysozyme standard.

Results

Inhibition of pyruvate transport by thiol-blocking reagents

The data in Table 1 investigate the effects of various thiol-blocking reagents on mitochondrial pyruvate transport in order to identify a suitable labelling reagent. As reported by others (Papa & Paradies, 1974; Halestrap, 1976), various thiol-blocking reagents inhibit pyruvate uptake into the mitochondria, but it can be seen that many of them also inhibit the accumulation of acetate. Since this

Table 1. *Effects of thiol-blocking reagents on mitochondrial pyruvate and acetate uptake*
Rat liver mitochondria (8mg of protein/ml) were incubated in medium (250mM-sucrose/10mM-Tris/HCl buffer/2mM-EGTA/1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, pH 7.8) at 25°C for 10min with additions as indicated. After being washed in isolation medium at 0°C, the mitochondria (4mg of protein/ml) were resuspended, and the pyruvate and acetate uptakes after 1min were measured by the 'centrifuge-stop' technique as described in the Experimental section, except that the uptake medium contained 125mM-choline chloride in the place of 250mM-sucrose. Each value is the mean ± S.E.M. for four determinations, with significance compared with control incubations of: *$P<0.001$; †$P<0.01$; ‡$P<0.02$.

Additions	Mitochondrial uptake of carboxylate (nmol/mg of protein)		Pyruvate uptake / Acetate uptake
	Pyruvate	Acetate	
None	0.80 ± 0.04	1.19 ± 0.02	0.68 ± 0.03
Iodoacetate (4mM)	0.45 ± 0.02*	0.73 ± 0.01*	0.62 ± 0.03
N-Ethylmaleimide (2mM)	0.47 ± 0.02*	0.84 ± 0.03*	0.56 ± 0.02‡
Bromopyruvate (2mM)	0.46 ± 0.01*	1.06 ± 0.02†	0.43 ± 0.01*
N-Phenylmaleimide (1mM)	0.28 ± 0.02*	1.45 ± 0.02*	0.19 ± 0.01*

Table 2. *Protection of pyruvate transport activity from N-phenylmaleimide by α-cyanocinnamate*
Rat liver mitochondria (approx. 8mg of protein/ml) were incubated in medium (250mM-sucrose/10mM-Tris/HCl buffer/2mM-EGTA/1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, pH 7.8) at 25°C for 10min. After the mitochondria had been washed twice in isolation medium supplemented with bovine serum albumin (10mg/ml), pyruvate and acetate uptakes and mitochondrial volumes were measured at 6°C as described in the Experimental section. The rate of pyruvate uptake was corrected for the increased pH gradient in the presence of *N*-phenylmaleimide, as explained in the text. The values are the means ± S.E.M. for three separate experiments on different samples of mitochondria, with statistical significance of differences from the controls calculated by Student's *t* test: *$P<0.01$; †$P<0.05$.

Additions to incubation mixture	Rate of pyruvate uptake (nmol/min per mg of protein)	Mitochondrial volume (μl/mg of protein)	Acetate uptake (nmol/mg of protein)	ΔpH	Corrected rate (nmol/min per mg of protein)	Inhibition (%)
Control	1.16 ± 0.13	0.76 ± 0.12	1.09 ± 0.13	0.99 ± 0.02	1.16 ± 0.13	—
Control + α-cyanocinnamate (0.1mM) in uptake medum	<0.02	—	—	—	—	>98
α-Cyanocinnamate (0.1mM)	0.66 ± 11†	0.72 ± 0.11	1.04 ± 0.12	0.99 ± 0.02	0.66 ± 0.11†	43
N-Phenylmaleimide (1.0mM)	0.45 ± 0.07*	0.86 ± 0.08	2.42 ± 0.50	1.26 ± 0.09†	0.25 ± 0.05*	78
α-Cyanocinnamate (0.1mM) + *N*-phenylmaleimide (1.0mM)	1.12 ± 0.12	0.98 ± 0.07	2.33 ± 0.43	1.20 ± 0.08	0.71 ± 0.05†	39

anion enters the mitochondria as the free acid, independently of the pyruvate transporter (Halestrap, 1975), this suggests that the mitochondrial pH gradient is decreased by thiol-blocking reagents such as *N*-ethylmaleimide, iodoacetate and bromopyruvate. This conclusion was confirmed with [^{14}C]5,5-dimethyloxazolidine-2,4-dione, another molecule whose distribution across membranes depends on the pH gradient. Thus inhibition of pyruvate transport may not be by a direct effect on the carrier itself, but rather a consequence of a decreased pH gradient, towards which the carrier is very sensitive (Halestrap, 1978*a*). A similar conclusion was reached by Pande & Parvin (1978). *N*-Phenylmaleimide (1mM), however, caused an increase in acetate accumulation by mitochondria, while severely inhibiting pyruvate accumulation. The increase in acetate accumulation was partly the result of an increased mitochondrial volume (Table 2), which is a well-documented effect of some thiol-blocking reagents (Brierley *et al.*, 1973). The opposite effects of *N*-phenylmaleimide on acetate and pyruvate accumulation strongly suggest that *N*-phenylmaleimide is an inhibitor of pyruvate transport, albeit a non-specific one. The reason it differs from *N*-ethylmaleimide in this respect may well reflect the greater hydrophobicity of the phenyl derivative, since the efficacy of α-cyanocinnamate derivatives as inhibitors of pyruvate transport is critically dependent on the hydrophobic moiety of

the molecule (Halestrap, 1975, 1976). Higher concentrations of *N*-phenylmaleimide than 1 mM appeared to cause mitochondrial damage sufficient to prevent accurate determination of the rate of pyruvate transport.

Protection of mitochondria by α-cyanocinnamate

The data of Table 2 investigate whether α-cyanocinnamate can protect the pyruvate carrier from inhibition by *N*-phenylmaleimide. Mitochondria were incubated in sucrose medium in the presence or in the absence of α-cyanocinnamate (0.1 mM) and/or *N*-phenylmaleimide (1 mM). Uncoupler was present to enhance the interaction of α-cyanocinnamate with the mitochondria (Halestrap, 1975, 1978*b*). After incubation mitochondria were washed twice to remove inhibitors, and the rate of pyruvate transport was measured. Measurements were also made of mitochondrial volume and acetate accumulation in order to calculate the change in pH gradient caused by *N*-phenylmaleimide. Perturbation of ΔpH is capable of influencing the rate of pyruvate transport, and rates were corrected for this by using the data of Halestrap (1978*a*). The correction used assumed that the rate of pyruvate transport was inversely proportional to the intramitochondrial [H+] at constant extramitochondrial pH. Thus:

$$\text{Corrected rate} = \text{Observed rate} \times \frac{[H^+]_{in}\ \text{inhibited}}{[H^+]_{in}\ \text{control}}$$

The corrected data show clearly that for α-cyanocinnamate (0.1 mM) inhibition falls from nearly 100% to about 40% on washing the mitochondria, as was expected from the reversible nature of the inhibition (Halestrap, 1975; Halestrap & Denton, 1975). This was true even when *N*-phenylmaleimide (1 mM) was present in addition to α-cyanocinnamate, although when *N*-phenylmaleimide alone was used inhibition remained at nearly 80%. Thus α-cyanocinnamate was protecting the carrier from inhibition by *N*-phenylmaleimide.

Labelling of mitochondrial proteins with [^{3}H]N-phenylmaleimide

By using conditions identical with those above, the effect of α-cyanocinnamate on the incorporation of [^{3}H]*N*-phenylmaleimide into mitochondrial membrane proteins was studied. Photographs and scans of fluorographs obtained from SDS/polyacrylamide-gel electrophoresis of proteins from liver and heart mitochondria treated in this way are shown in Figs. 1–3. Visual inspection of fluorographs and protein stains of such gels (Fig. 1) shows extensive labelling of at least 50% of the stained proteins, and the intensity of some of the labelled proteins strongly suggets multi-site labelling. Careful computer analysis (see the Experimental section) of the scans of fluorographs exposed for various times showed that α-cyanocinnamate induced no changes in labelling of any of the major protein bands. In contrast, carboxyatractylate induced a major decrease in the peak height of a protein of mol.wt. 28000 (Fig. 1), which confirms reports of others (Klingenberg, 1976*b*; Heaton *et al.*, 1978; Wohlrab, 1979) that labelling of the ATP/ADP carrier by thiol-blocking reagents is diminished by carboxyatractylate. Longer exposure of fluorographs revealed a minor peak of radioactivity, running at the same position as ^{3}H-labelled lysozyme, whose labelling was decreased by α-cyanocinnamate in both heart and liver mitochondria (Figs. 2*a* and 2*b*). However, in liver mitochondria in particular this peak appeared as a shoulder of a larger peak (Fig. 2*a*) and proved to be impossible to resolve completely.

Double-labelling experiments

Greater resolution of the protein whose labelling was protected by α-cyanocinnamate was achieved by a double treatment with *N*-phenylmaleimide. The mitochondria were first incubated with unlabelled *N*-phenylmaleimide in the presence or in the absence of α-cyanocinnamate in order to block most of the reactive thiol groups. After removal of α-cyanocinnamate the mitochondria were exposed to [^{3}H]*N*-phenylmaleimide to label any thiol groups that were previously protected or unmodified for other reasons. Scans of fluorographs from the SDS/polyacrylamide-gel electrophoresis resulting from these experiments are shown in Figs. 3(*a*) and 3(*b*). In both heart and liver mitochondria the labelling of proteins is much diminished in comparison with the single-label experiments (Fig. 1), and again the presence of α-cyanocinnamate in the first incubation affects the subsequent labelling of a protein of mol.wt. 15000. In this case, however, the resolution is much clearer, and as predicted the first incubations, which were in the presence of α-cyanocinnamate, gave greater labelling of this protein with [^{3}H]*N*-phenylmaleimide in the second incubation. In heart mitochondria, in two out of five experiments, a second protein, of mol.wt. 12000, also showed slightly enhanced labelling as a result of prior protection by α-cyanocinnamate. The absence of such a peak with liver mitochondria suggests that the pyruvate transporter has a subunit molecular weight of 15000 and that the 12000-mol.wt. component may be a proteolytic breakdown product of the transporter or an additional protein that also has a thiol group protected by α-cyanocinnamate. Inclusion of 0.1 mM-phenylmethanesulphonyl fluoride, an inhibitor of some, but not all, proteinases (Sekar & Hageman, 1979), in the preparation of

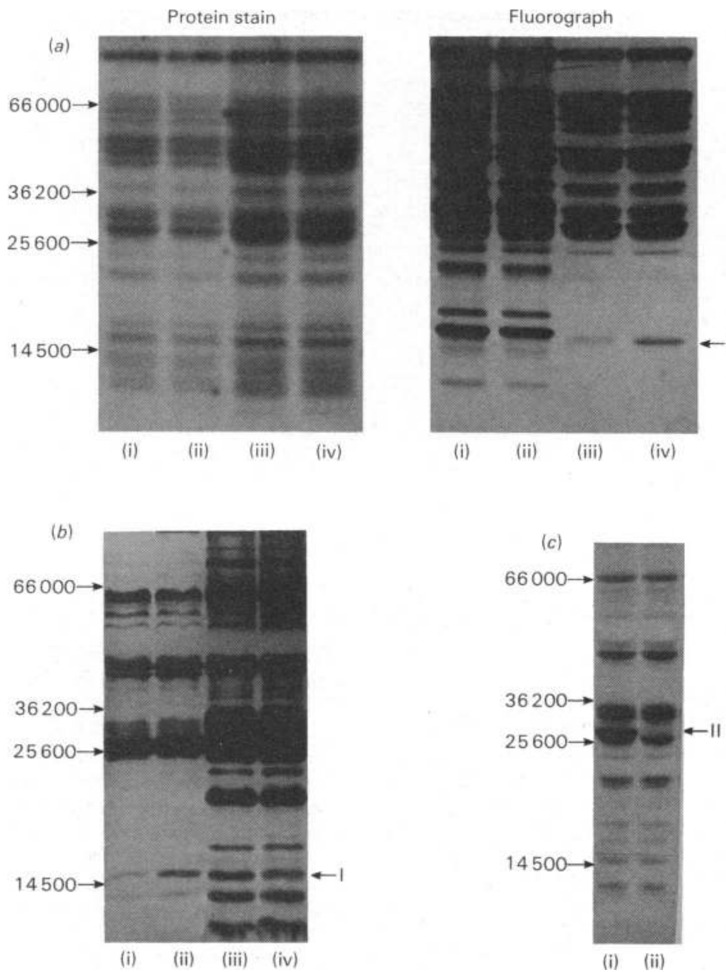

Fig. 1. *Fluorographs of liver and heart mitochondrial membrane proteins labelled with [^{3}H]N-phenylmaleimide and subjected to SDS/polyacrylamide-gel electrophoresis*

Mitochondria from liver (*a*) and heart (*b* and *c*) were labelled with [^{3}H]*N*-phenylmaleimide by using the single- or double-incubation procedures (see the text and legends to Figs. 2 and 3). Mitochondrial membranes were prepared and subjected to SDS/polyacrylamide-gel electrophoresis and fluorography as described in the Experimental section. The fluorographs were obtained after 5-week (*a* and *b*) or 1-week (*c*) exposures. In (*a*) the fluorograph is shown alongside the Coomassie Blue-stained scintillant-impregnated gel. Tracks represent the following treatments. Fluorograph (*a*): (i) single incubation control; (ii) single incubation with α-cyanocinnamate (0.1 mM); (iii) double incubation control; (iv) double incubation with α-cyanocinnamate in the first incubation. Fluorograph (*b*): (i) double incubation control; (ii) double incubation with α-cyanocinnamate in the first incubation; (iii) single incubation control; (iv) single incubation with α-cyanocinnamate. Fluorograph (*c*): (i) single incubation control; (ii) single incubation with carboxyatractylate. The positions of the following molecular-weight markers are also indicated: bovine serum albumin (mol.wt. 66000); lactate dehydrogenase (mol.wt. 36200); chymotrypsinogen (mol.wt. 25600); lysozyme (mol.wt. 14500). The positions of the proteins whose labelling is changed by α-cyanocinnamate and carboxyatractylate are indicated by arrows I and II respectively.

mitochondrial membranes was without effect on the presence or absence of this band.

By using ^{3}H-labelled lysozyme of known specific radioactivity the peak area of the ^{3}H-labelled pyruvate carrier could be converted into mol of bound [^{3}H]*N*-phenylmaleimide. In practice the presence of other labelled proteins of similar molecular weight made direct calculation impossible, but the decrease or increase in peak size caused by α-cyanocinnamate protection could be used. From the kinetic data of Table 2 the degree of protection by α-cyanocinnamate and the degree of inhibition by

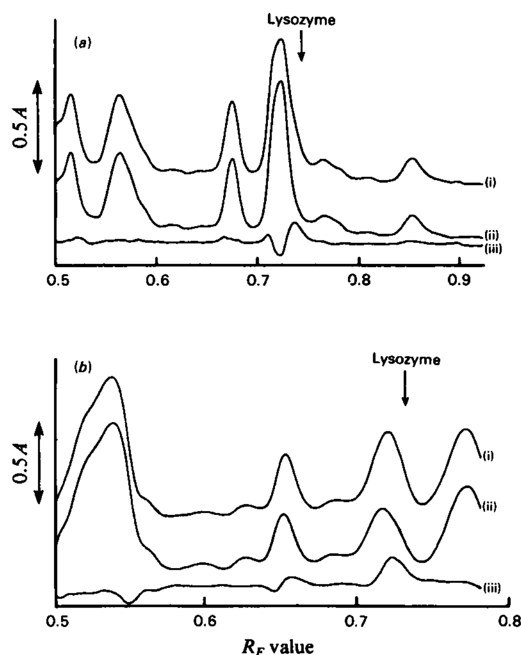

Fig. 2. *Densitometric scans of fluorographs of mitochondrial proteins labelled with [^{3}H]N-phenylmaleimide by using the single-incubation procedure*

Fluorographs of liver (*a*) and heart (*b*) mitochondrial single-labelling experiments were prepared and scanned as described in the Experimental section. Mitochondria were incubated with [^{3}H]*N*-phenylmaleimide (1 mM, 50 μCi/μmol) in the absence (i) or in the presence (ii) of α-cyanocinnamate (0.1 mM). Scan (iii) represents the difference [(i) − (ii)] between the two scans. Both fluorographs were exposed for 5 weeks. Only the lower portion of the scan is shown because of the over-exposure of the film registering the higher-molecular-weight labelled proteins (see Fig. 1). The R_F value represents the migration of the radioactive bands relative to the dye front (Bromophenol Blue). The migration of lysozyme (mol.wt. 14500) is indicated as a reference point.

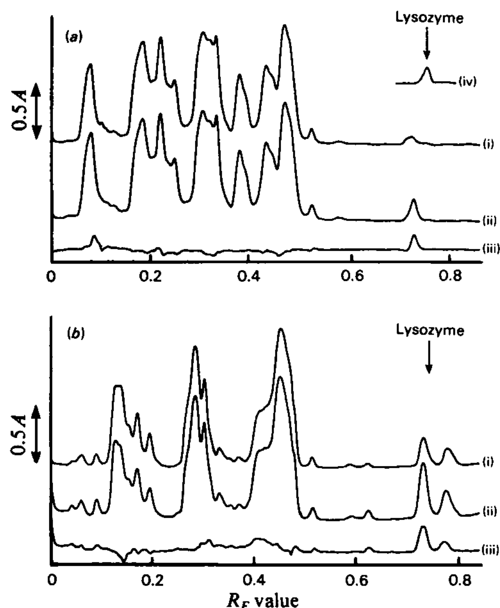

Fig. 3. *Densitometric scans of fluorographs of mitochondrial proteins labelled with [^{3}H]N-phenylmaleimide by using the double-incubation procedure.*

Densitometric scans of fluorographs from liver (*a*) and heart (*b*) mitochondrial proteins labelled by using a double-incubation procedure were prepared as described in the Experimental section. Mitochondria were incubated with *N*-phenylmaleimide (1 mM) in the absence (i) or in the presence (ii) of α-cyanocinnamate (0.1 mM). After being washed, the mitochondria were re-incubated with [^{3}H]*N*-phenylmaleimide (1 mM, 50 μCi/μmol) in the absence of α-cyanocinnamate. Scan (iii) represents the difference between the two scans [(ii) − (i)]. R_F values were calculated as explained in the legend to Fig. 2. The migration of lysozyme (mol.wt. 14500) is indicated as a reference point in (*b*), and in (*a*) the scan of [^{3}H]*N*-phenylmaleimide-labelled lysozome representing 1200 d.p.m. is shown (iv).

N-phenylmaleimide can be calculated and converted into the predicted number of carrier molecules labelled by *N*-phenylmaleimide in each incubation (Table 3). From these values, the decrease in radioactivity (d.p.m.) produced by α-cyanocinnamate and the amount of protein run on the gel, values for the pmol of pyruvate carrier/mg of mitochondrial protein can be calculated. The values assume that each molecule of α-cyanocinnamate protects one site on the pyruvate carrier from inhibition by one molecule of *N*-phenylmaleimide and that the protein content of the crude membrane fraction is 50% of the total mitochondrial protein (the mean value of several measurements). Results of a typical double-label experiment are shown in Table 4. It can be seen that these results suggest the presence of about 100 pmol of carrier protein (or subunit)/mg of protein in liver mitochondria and about 5 times as much in heart mitochondria. Mean values (±S.E.M.) for three such experiments on liver and heart mitochondria gave values of 106 ± 9 and 441 ± 75 pmol/mg of mitochondrial protein respectively.

Measurement of the kinetics of pyruvate transport into liver and heart mitochondria by the inhibitor stop method at pH 7.2 as described previously (Halestrap, 1975) gave $V_{max.}$ values (in nmol of pyruvate transported/mg of protein at 6°C, S.E. for at least six substrate concentrations, fitted by

Table 3. *Predicted extent of labelling of the pyruvate transporter by N-phenylmaleimide in double-incubation experiments*
The percentage of sites labelled was calculated from the inhibition caused by *N*-phenylmaleimide determined in Table 2, assuming that 100% labelling gave 100% inhibition. For the labelling during the second incubation period, it was assumed that *N*-phenylmaleimide labelled 78% of the sites available to it after the first incubation and washes, and that any α-cyanocinnamate remaining bound to the transporter after this treatment was not displaced by further addition of *N*-phenylmaleimide.

	Percentage of the pyruvate carrier labelled by *N*-phenylmaleimide		
	Control (I)	0.1 mM-α-Cyanocinnamate in first incubation (II)	Difference between I and II caused by α-cyanocinnamate
First incubation	78	0	78
Second incubation	17	45	28

Table 4. *Estimation of the amount of pyruvate transporter present in liver and heart mitochondria*
The data presented were calculated from the double-incubation fluorographs of liver and heart mitochondrial proteins shown in Fig. 1. The ^{3}H radioactivity (d.p.m.) in the 15000-mol.wt. peak on the fluorographs was determined by using a ^{3}H-labelled lysozyme standard of known specific radioactivity as described in the Experimental section. From knowledge of the amount of protein on the gel this was corrected to pmol of [^{3}H]*N*-phenylmaleimide bound/mg of mitochondrial protein. The data of Table 3 were then used to calculate the total amount of transport protein from the difference in labelling caused by α-cyanocinnamate.

Source of mitochondria	α-Cyanocinnamate in first incubation (mM)	[^{3}H]*N*-Phenylmaleimide in 15000-mol.wt. protein (pmol/mg of mitochondrial protein)	Difference in labelling due to α-cyanocinnamate (pmol/mg of mitochondrial protein)	Calculated amount of pyruvate transporter (pmol/mg of mitochondrial protein)
Liver	0	22.5	—	—
	0.1	52.7	30.2	108
Heart	0	168.0	—	—
	0.1	334.0	166.0	591

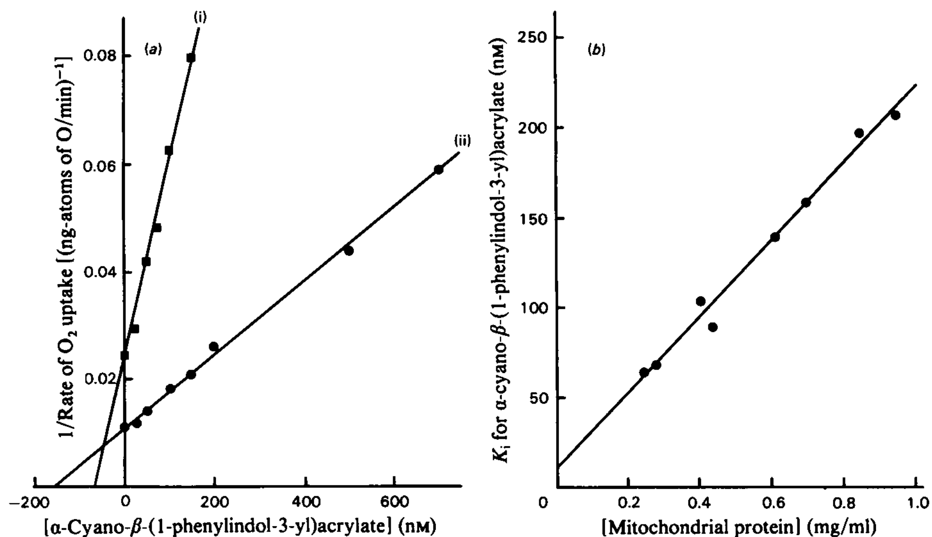

Fig. 4. *Inhibition of pyruvate oxidation in heart mitochondria by α-cyano-β-(1-phenylindol-3-yl)acrylate*
O_2 uptake was assayed by using an oxygen electrode in KCl medium (125mM-KCl/20mM-Tris/2mM-potassium phosphate/25μM-malate, pH7.4) at 30°C as described in the Experimental section. Mitochondrial protein concentrations were varied over the range 0.2–1 mg/ml. Pyruvate (1 mM) and ADP (2 mM) were added, and a steady rate of O_2 consumption was obtained. At this point inhibitor was added and the new steady rate measured. In (*a*) Dixon plots at two mitochondrial concentrations are presented: (i) 0.28 mg of protein/ml; (ii) 0.70 mg of protein/ml. In (*b*) values for the K_i calculated as described by Halestrap (1978*b*) are plotted at various protein concentrations (data are from several experiments on different mitochondrial preparations). Linear regression by least squares gave values (±S.E.) for the intercept of 8.9 ± 6.6 and the slope of 213 ± 11, with a correlation coefficient of 0.992.

least-squares regression to the Michaelis–Menten equation) of 0.79 ± 0.05 and 5.40 ± 0.46 respectively. These values differ by a similar order to the number of carrier molecules predicted by the labelling experiments. The K_m values for pyruvate were the same for both mitochondria, being 0.29 ± 0.04 and 0.25 ± 0.05 mM for liver and heart mitochondria respectively, similar to values obtained for liver mitochondria at pH 7.4 reported previously (Halestrap, 1975, 1978*a*).

A further assessment of the number of carrier molecules in heart mitochondria was made by titrating pyruvate-dependent O_2 uptake in the presence of ADP with α-cyano-*β*-(1-phenylindol-3-yl)acrylate at different protein concentrations. A Dixon plot (1/rate of O_2 uptake against inhibitor concentration) yielded a straight line (Fig. 4*a*), indicating that the pyruvate transporter was rate-limiting for respiration (see Halestrap, 1978*b*; Halestrap *et al.*, 1980). Furthermore, the calculated K_i values were linearly related to the protein concentration (Fig. 4*b*), suggesting very tight binding of the inhibitor, as predicted previously (Halestrap, 1975). The slope of this graph, 213 ± 11, gives the number of pmol of inhibitor/mg of mitochondrial protein required to give 50% inhibition. If almost all the inhibitor is bound under these conditions, it would be predicted that there are 426 pmol of carrier/mg of protein, in good agreement with the labelling experiments. The predicted dissocation constant of the inhibitor–protein complex (the extrapolated K_i value at zero protein concentration) was 8.9 ± 6.6 nM.

Discussion

The protection that α-cyanocinnamate affords the pyruvate transporter from inhibition by *N*-phenylmaleimide has allowed identification of the protein responsible for pyruvate transport. By using [^{3}H]*N*-phenylmaleimide, it was shown that in liver and heart mitochondria α-cyanocinnamate specifically protected a protein of mol.wt. 15000 from attack by this reagent. α-Cyanocinnamate also protected this protein from attack by ^{3}H- and ^{14}C-labelled iodoacetate, but the extent of labelling was substantially less than that seen with [^{3}H]*N*-phenylmaleimide (results not shown). This is consistent with the relative effects of these two reagents on the rate of pyruvate transport (Table 1).

The proposed molecular weight for the pyruvate carrier is considerably smaller than those of other carrier proteins characterized for mitochondria. By using similar labelling techniques the phosphate carrier has been reported to have a subunit molecular weight of about 32000 (Wohlrab, 1979; Hofmann & Kadenbach, 1979), the ATP/ADP carrier about 30000 (see Klingenberg, 1976*a*,*b*; Klingenberg *et al.*, 1978) and the anion channel of brown-adipose-tissue mitochondria about 32000 (Heaton *et al.*, 1978). It is quite possible that the functional form of the pyruvate carrier is composed of several subunits, which may or may not be identical, and therefore higher-molecular-weight components cannot be ruled out until purification and reconstitution of the carrier are attempted.

The amount of the carrier protein present in the membrane is similar to that for the components of the respiratory chain (Munn, 1974) and a little greater than the calculated amount of the phosphate carrier (30–60 pmol/mg of protein in rat liver mitochondria; Coty & Pedersen, 1975; Hadvary & Kadenbach, 1976; Hofmann & Kadenbach, 1979). The adenine nucleotide transporter is present in considerably larger quantities, as is the anion channel of brown adipose tissue (Riccio *et al.*, 1975; Klingenberg, 1976*a*; Boxer *et al.*, 1977; Heaton *et al.*, 1978). If it is assumed that 100 pmol of pyruvate carrier is present per mg of liver mitochondrial protein, a turnover number of the molecule can be calculated from the $V_{max.}$ of transport (Halestrap, 1975, 1978*a*). At 0°C exchange of pyruvate for pyruvate has a $V_{max.}$ of about 20 nmol/min per mg of protein, giving a turnover number of 200 min^{-1}, and for net transport at pH 7.4 the value is only about 6 min^{-1}. Even at 37°C the value for net transport rises only to about 500 min^{-1} (Halestrap, 1975, 1978*b*). These values are very much less than the value obtained for phosphate transport at 0°C of 3500 min^{-1} (Coty & Pedersen, 1975), but are nearer the value for adenine nucleotide transport at 18°C of 500 min^{-1} (Klingenberg, 1976*a*). The relatively small turnover number of the pyruvate transporter is consistent with the suggestion that pyruvate transport may limit mitochondrial pyruvate metabolism under some conditions and so be an important site of metabolic control (see Halestrap *et al.*, 1980).

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