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International Journal of Pharmaceutics 108 (1994) 225–232

international journal of pharmaceutics

Drug delivery via active transport at the blood-brain barrier: II. Investigation of monocarboxylic acid transport in vitro

Ian Walker *, Dave Nicholls, William J. Irwin, Sally Freeman

Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK

(Received 17 November 1993; Modified version received 27 January 1994; Accepted 14 February 1994)

Abstract

Monocarboxylic acids such as pyruvate and L-lactate are actively transported at the blood-brain barrier (BBB). The possibility that a diester of the antiviral agent phosphonoformate (PFA) may mimic a monocarboxylic acid, and be actively transported, was investigated. A diester of PFA (sodium methyl methoxycarbonylphosphonate) was synthesised and characterised. Active monocarboxylic acid transport was studied in vitro using monolayers of porcine brain microvessel endothelial cells (BMEC). Confluent monolayers were obtained after 4–5 days in culture, and alkaline phosphatase activity and the presence of factor VIII antigen were demonstrated histochemically. The transport of [^{14}C]pyruvate was shown to be temperature- and concentration-dependent and transport constants were calculated to be $K_m = 1.50$ mM and $V_{max} = 64.5$ nmol/h per insert by non-linear regression. The anti-convulsant valproate and other monocarboxylic acids were found to inhibit the transport of [^{14}C]pyruvate, consistent with their transport by the monocarboxylic acid transporter in vivo. The diester of PFA, which was stable under the experimental conditions, did not inhibit [^{14}C]pyruvate transport, indicating that it is not a substrate for active transport at the BBB.

Key words: Active transport; Monocarboxylic acid; Blood-brain barrier; Brain delivery; Cell culture; Phosphonoformate

1. Introduction

The brain microvessel endothelium provides a barrier to the passive transport of hydrophilic drugs into the brain (for reviews, see Oldendorf, 1977; and Brightman, 1989). Phosphonoformate (Foscarnet, PFA) is a useful antiviral agent, showing activity against most retroviruses, including HIV (Oberg, 1989). However, PFA is precluded from entering the brain, presumably because it is tri-anionic at physiological pH (pK_a values 0.49, 3.41 and 7.27) (Warren and Williams, 1971). Lipophilic triesters of phosphonoformate, which were designed to improve transport properties, are unsuitable prodrugs due to their rapid and complicated hydrolysis, involving competitive P-O and P-C bond cleavage reactions (Krol et al., 1991; Mitchell et al., 1991, 1992; Krol and Thatcher, 1993). In contrast, diesters of phosphonoformate are more stable towards hydrolysis, but are considered to be too polar for passive

* Corresponding author.

SSDI 0378-5173(94)00071-C

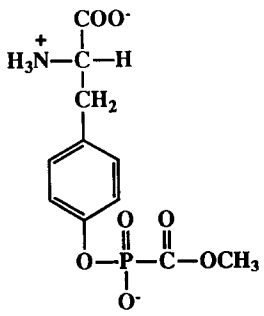
diffusion into the CNS. We have recently shown, however, that the L-tyrosine conjugate of PFA (**1**) inhibits the transport of L-[^{3}H]tyrosine indicating that (**1**) may be a substrate for active transport at the BBB (Walker et al., 1994). Here, the ability of a PFA analogue to utilise the monocarboxylic acid (MCA) transporter was investigated.

The carrier-mediated transport of short chain monocarboxylic acids (MCA) across the BBB has been characterised in vivo using the brain uptake index technique (Oldendorf, 1973). The carrier was shown to be consistent with an active transport process. Cross-inhibition was demonstrated for pyruvate with acetate, propionate, L-lactate and butyrate but not with octanoate and decanoate or di- and tricarboxylic acids. Cross-inhibition was not observed with pyruvate and L-phenylalanine, L-arginine or D-glucose indicating the carrier system to be distinct from amino acid and monosaccharide transporters. It has been shown that α-keto acids such as pyruvate show a high affinity for the MCA carrier system (Oldendorf, 1973; Conn and Steele, 1982; Steele 1986). A non-saturable component (approx. 15% for pyruvate) of MCA transport is present in vivo (Conn and Steele, 1982). The contribution of this pathway to the total transport causes problems in the estimation of kinetic parameters. As a result, large error bars are apparent in the values reported (see Table 1; Pardridge, 1983).

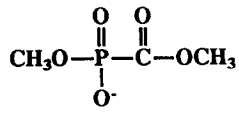
The biological importance of the MCA transport system is not certain. It may function to increase the flux of monocarboxylic acids into the brain where they may serve as metabolic substrates, or may be a bidirectional system to allow efflux of organic acids, for example L-lactate, from the brain in normal or hypoxic states (Oldendorf, 1973). The role of this carrier in the BBB transport of therapeutic agents has been investigated in vivo (Kang et al., 1990) and in vitro using bovine brain microvessel endothelial cells (BMEC) in primary culture (Terasaki et al., 1991). Valproate and salicylate were shown to inhibit the uptake of [^{3}H]acetic acid, a model substrate, into BMEC monolayers, suggesting carrier-mediated transport of these drugs at the BBB.

Table 1
Transport constants for monocarboxylic acids at the adult rat blood-brain barrier (Pardridge, 1983)

MCA	K_m (mM)	V_{max} (nmol min^{-1} g^{-1})	k_d (ml min^{-1} g^{-1})
L-Lactate	1.8 ±0.6	91 ± 35	0.019 ± 0.004
Pyruvate	0.57 ± 0.16	88 ± 26	0.034 ± 0.008
Butyrate	0.70 ± 0.20	86 ± 11	0.041 ± 0.002



(**1**)



(**2**)

In order to assess the ability of diesters of PFA to utilise the MCA carrier system, it is necessary to demonstrate its presence in porcine BMEC monolayers. The porcine BMEC were cultured on 25 mm Falcon Cyclopore inserts and the active transport of [^{14}C]pyruvate was established. Other monocarboxylic acids and the PFA diester (**2**) were then evaluated as potential inhibitors of [^{14}C]pyruvate transport.

2. Materials and methods

High-field NMR spectra, ^{1}H (300 MHz), ^{13}C (75.5 MHz) and ^{31}P (121.5 MHz), were recorded on a Bruker AC spectrometer. ^{1}H- and ^{13}C-NMR spectra were referenced to tetramethylsilane and ^{31}P-NMR spectra to 85% H_3PO_4; positive chemical shifts are downfield from the reference. ^{31}P-NMR spectra are ^{1}H decoupled unless otherwise stated. Infrared spectra were recorded on a Perkin-Elmer 1310 infrared spectrometer. Melting points were measured on an Gallenkamp electrothermal melting point apparatus and are

not corrected. Elemental analyses were recorded by Butterworth Laboratories Ltd, Teddington, Middlesex. Chemicals were obtained from Aldrich or Sigma Chemical Co. Tetrahydrofuran was dried by heating under reflux with sodium, using benzophenone as an indicator, followed by distillation.

2.1. Synthesis of the phosphonoformate diester (2)

Dimethyl methoxycarbonylphosphonate was prepared analogously to the method described by Noren et al. (1983) for the preparation of diethyl 4-methoxyphenoxycarbonylphosphonate. 86% (58 g, 0.34 mol); b.p. 70–74°C (0.5 mmHg); ^{1}H-NMR ($CDCl_3$) δ 3.91 (6H, d, $J_{PH} = 11$ Hz, $2 \times POCH_3$), 3.82 (3H, d, $J_{PH} = 1$ Hz, $COCH_3$); ^{31}P-NMR ($CDCl_3$) δ −2.75 (s); ^{31}P-NMR (^{1}H coupled, $CDCl_3$) δ −2.75 (sept q, $J_{PH} = 11$ Hz, $J_{PH} = 1$ Hz); ^{13}C-NMR ($CDCl_3$) δ 54.5 (d, $J_{PC} = 6$ Hz, $2 \times POCH_3$), 52.5 (d, $J_{PC} = 4$ Hz, $COCH_3$), C = O not observed; IR (liquid film) 1720 cm^{-1} (C = O), 1280 (P = O), 1040 (P-O).

Sodium methyl methoxycarbonylphosphonate (**2**) was prepared analogously to the method of Noren et al. (1983) described for the preparation of sodium methyl benzyloxycarbonylphosphonate. 60% (0.19 g, 1.0 mmol); m.p. 180–182°C; ^{1}H-NMR (D_2O) δ 3.58 (3H, s, $COCH_3$), 3.47 (3H, d, $J_{PH} =$ 11 Hz, $POCH_3$); ^{31}P-NMR (D_2O) δ −1.11 (s); ^{31}P-NMR (^{1}H coupled, D_2O) δ −1.11 (q, $J_{PH} =$ 11 Hz); ^{13}C-NMR (D_2O) δ 55.9 (d, $J_{PC} = 6$ Hz, $POCH_3$), 54.6 (d, $J_{PC} = 4$ Hz, $COCH_3$), C = O not observed; IR (Nujol mull) 1720 cm^{-1} (C = O), 1280 (P = O), 1040 (P-O). Found: C, 20.44; H, 3.40%; $C_3H_6O_5PNa$ requires C, 20.47; H, 3.44%.

2.2. Isolation and culture of porcine brain microvessel endothelial cells

Porcine brain microvessel endothelial cells (BMEC) were isolated and cultured as previously described (Walker et al., 1994) employing methods similar to those of Audus and Borchardt (1986). Confluent BMEC monolayers were formed after 4–5 days in culture and used in transport studies on day 7 of culture. Alkaline phosphatase activity (using Sigma kit no. 85) and the presence of factor VIII antigen (using a Dako primary antibody and Vectastain reagents) were demonstrated on 7-day-old confluent BMEC monolayers, by histochemical methods.

2.3. Transport studies

Three 7-day-old confluent BMEC monolayers in Falcon 25 mm inserts (Becton Dickinson) were removed from their six-well plate and the medium removed by aspiration. Transport medium (Hank's Balanced Salt Solution buffered to pH 7.4 with 14 mM Hepes, 1.5 ml) containing 0.2 μCi of [^{3}H]mannitol (19.1 Ci/mmol, Amersham) and 0.2 μCi of [^{14}C]pyruvate (28.9 mCi/mmol, Amersham) were added to each insert. Each insert was placed in a six-well plate containing transport medium (2.5 ml) in each well. At the end of the incubation period, the inserts were removed and the solutions from the inserts individually added to Optiphase HiSafe 3 scintillation cocktail (LKB, 10 ml). The contents of the wells were also individually added to scintillation cocktail (10 ml). The membrane was removed from the insert with a scalpel and added to scintillation cocktail (10 ml). All samples were then counted using a Packard Tri-Carb 1600TR liquid scintillation counter and the transport of [^{3}H]mannitol and [^{14}C]pyruvate determined.

2.4. Temperature dependence

The transport of 0.2 μCi of [^{3}H]mannitol (19.1 Ci/mmol, Amersham) and 0.2 μCi of [^{14}C]pyruvate (28.9 mCi/mmol, Amersham) through the BMEC monolayers was monitored at 37 and 4°C. After 30 min, the inserts were moved to other wells containing fresh transport buffer. This was repeated at 30 min intervals up to a total incubation period of 120 min. The transport medium from the wells in the six-well plates were individually counted and the cumulative transport with time was calculated.

2.5. Concentration dependence

The transport of 0.2 μCi of [^{3}H]mannitol (19.1 Ci/mmol) and 0.2 μCi of [^{14}C]pyruvate (28.9

mCi/mmol) was monitored at 37°C for 60 min, in the presence of a range of pyruvate concentrations (0.025–2 mM) in the apical chamber. Kinetic parameters were calculated using Enzpack 3 (Biosoft Ltd), Fig P (Biosoft Ltd) and NONREG (Irwin, 1990).

2.6. Inhibition profile

The transport of 0.2 μCi of [^{3}H]mannitol (19.1 Ci/mmol) and 0.2 μCi of [^{14}C]pyruvate (28.9 mCi/mmol) was monitored at 37°C for 60 min, in the presence of a range of potential inhibitors of pyruvate transport (10 mM) in the apical chamber. Any inhibition observed was expressed as a percentage of the transport measured in the absence of potential inhibitor (control).

2.7. Correction of transport data

All [^{14}C]pyruvate transport data were corrected for paracellular leakage, using a method analogous to that of Audus and Borchardt (1986). In all experiments, the transport of [^{3}H]mannitol was measured simultaneously with that of [^{14}C]pyruvate to provide an estimate of paracellular diffusion (PD). The flux of [^{3}H]mannitol was multiplied by a factor (K), to account for the relative diffusion rates of mannitol and pyruvate. This was subtracted from the flux of [^{14}C]pyruvate, using the following equations:

corrected % transport of [^{14}C]pyruvate

= observed % transport of [^{14}C]pyruvate

$-(\mathrm{PD} \times K)$ (1)

or

corrected transport of [^{14}C]pyruvate (cpm)

= observed transport of [^{14}C]pyruvate (cpm)

$-$(total [^{14}C]pyruvate (cpm)

$\times \mathrm{PD} \times 0.01 \times K$) (2)

where PD denotes the estimate of paracellular diffusion (simultaneous [^{3}H]mannitol % transport).

The value of K was determined experimentally from the ratio of the relative diffusion rates of [^{14}C]pyruvate (34.44%/h) and [^{3}H]mannitol (24.65%/h) across a cyclopore membrane (Falcon 25 mm insert) and was found to be 1.40. A theoretical method of calculating K uses the ratio of the square roots of the molecular weights ($K = \sqrt{MW}_{\mathrm{man}} / \sqrt{MW}_{\mathrm{pyr}}$) (Audus and Borchardt, 1986). This method gives a value of K for [^{14}C]pyruvate/[^{3}H]mannitol of 1.44, similar to that derived experimentally.

2.8. Identification of [^{14}C]pyruvate post-transport using radio-thin layer chromatography

After a 180 min transport experiment using 1.0 μCi of [^{14}C]pyruvate, a sample of medium from the basolateral chamber was applied in spots across the width of a Merck aluminium-backed cellulose TLC plate (3.5 cm wide and 4.5 cm long). The plate was eluted with 2-propanol/5% formic acid (13:7). The solvent was allowed to run 3 cm up the plate before it was removed from the solvent tank and dried. The plate was then cut into ten 3 mm horizontal strips from the baseline to the solvent front. Each strip was individually added to Optiphase HiSafe 3 scintillation cocktail (10 ml) and counted using a Packard Tri-Carb 1600TR liquid scintillation counter. The distance from the baseline was then plotted against dpm. This was repeated using [^{14}C]pyruvate, not subjected to transport, for use as a reference.

2.9. Identification of sodium methyl methoxycarbonylphosphonate (2) post-transport

A transport study was performed using 10 mM of (**2**) in the apical transport medium. After 180 min at 37°C, a sample of the basolateral medium (1 ml) was removed and its ^{31}P-NMR spectrum recorded, using a D_2O inner lock. This was compared with a spectrum of (**2**) dissolved in transport medium.

3. Results and discussion

The diester of PFA, sodium methyl methoxycarbonylphosphonate (**2**), was prepared using

methods similar to those described by Noren et al. (1983). Dimethyl methoxycarbonylphosphonate was synthesised by an Arbuzov reaction between trimethyl phosphite and methyl chloroformate. The triester of PFA was then selectively demethylated using sodium iodide to give (2). All ^{1}H-, ^{13}C- and ^{31}P-NMR and IR spectra were consistent with dimethyl methoxycarbonylphosphonate and (2). Compound (2) was further characterised by elemental analysis.

Seeding of 50 000 cells/cm^2 resulted in confluent monolayers of spindle-shaped cells after 4–5 days in culture. The monolayers provided the greatest barrier to paracellular diffusion on day 7 of culture as as indicated by [^{3}H]mannitol transport, therefore transport studies were performed on day 7 (Walker et al., 1994). The stability of [^{14}C]pyruvate to the transport conditions was investigated by radio-TLC, which showed a high degree of coincidence between the histograms for the sample taken from the basolateral chamber, after a 3 h transport experiment at 37°C, and a [^{14}C]pyruvate reference (Fig. 1). This confirmed that the [^{14}C]label detected in the basolateral chamber was [^{14}C]pyruvate. As controls, acetate and L-lactate were run on the same TLC system and found to have R_f values of 0.18 and 0.32, respectively. As little activity is apparent at these values, metabolism of pyruvate to acetate or lactate was not observed.

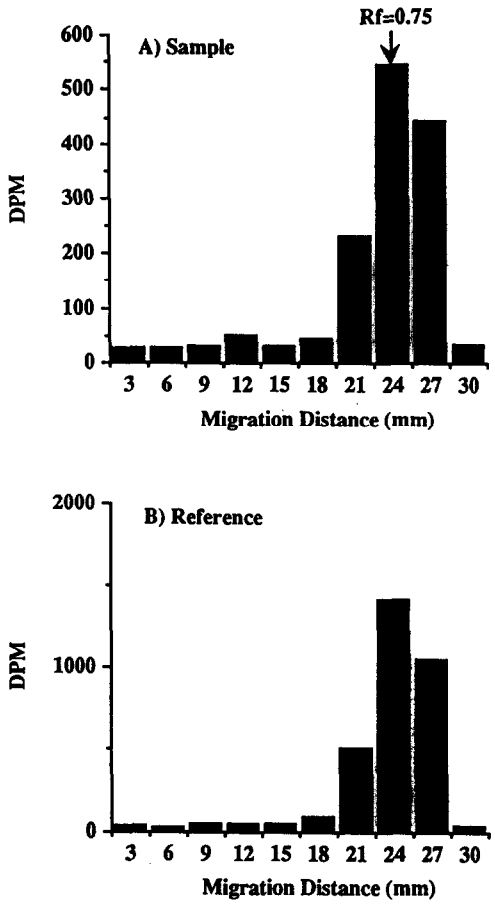


Fig. 1. Identification of [^{14}C]pyruvate post-transport using radio-TLC.

Transport of pyruvate across the BMEC monolayers may entail mediation by an active carrier system, passive transcellular and paracellular diffusion. As shown in Eq. 3, the monocarboxylic acid flux (J), from a substrate solution of concentration [S], is thus dependent upon the Michaelis-Menten parameters (K_m and V_{max}) for the active component, and the transcellular (k_d) and paracellular (k_p) diffusion rates.

$$J = \frac{V_{max} \cdot [S]}{K_m + [S]} + k_d[S] + k_p[S] \qquad (3)$$

The correction with mannitol effectively eliminates the paracellular component of transport due to a leaky monolayer (Walker et al., 1994). In order to assess the importance of the other two pathways, and to identify any saturable component of [^{14}C]pyruvate transport, the effect of increasing unlabelled pyruvate concentration upon [^{14}C]pyruvate transport was studied. Fig. 2 shows a plot of the corrected data of radiolabelled and total pyruvate flux, and demonstrates a non-linear dependence upon donor concentration with a maximum plateau value being approached. Non-linear regression of the transport data to Eq. 3 ($k_p = 0$) confirms that transcellular diffusion is inconsequential ($k_d \approx 0$). Thus, pyruvate transport across the BMEC monolayer is saturable; this is characteristic of Michaelis-Menten kinetics (Eq. 3; $k_p = 0$, $k_d = 0$) and indicates the availability of a finite number of transporters at the cell surfaces.

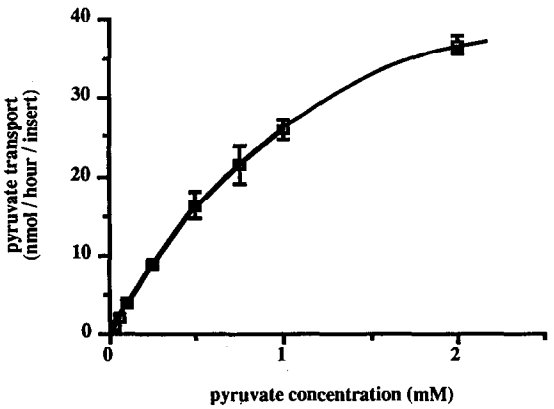


Fig. 2. Concentration dependence of pyruvate transport.

Using a variety of linear transformations (Eadie-Hofstee, Lineweaver-Burk, Hanes-Woolf), the transport constants in Table 2 were calculated. Kinetic constants were also calculated from the direct linear method (Enzpack 3, Biosoft) and from non-linear regression (Fig P, Biosoft and NONREG, Irwin, 1990) to Eq. 3. The values obtained by these methods are also recorded in Table 2 and (with the exception of the inferior Lineweaver-Burk transformation) show close agreement. The K_m values are comparable with that for [^{14}C]pyruvate transport calculated for the rat BBB in vivo of 0.57 ± 0.16 mM (Table 1; Pardridge, 1983). Also for comparison, Terasaki et al. (1991) calculated the K_m of acetic acid to be 3.41 ± 1.87 mM in primary cultured bovine BMEC.

A comparison of the transport of [^{14}C]pyruvate was made at 4 and 37°C (Fig. 3). It can be seen that, after correcting for the paracellular component, the transport of [^{14}C]pyruvate is decreased at 4°C which is a feature expected of transport utilising an active carrier system.

To provide a profile for the specificity of the pyruvate transporter, the effect of various monocarboxylic acids upon the transport of [^{14}C]pyruvate was investigated. A range of competitors at concentrations of 10 mM, which far exceeded that of pyruvate (4.6×10^{-3} mM), was studied and the inhibition profile is shown in Fig. 4.

Stereospecificity of the transporter can be seen as L-lactate inhibits transport ($39.64 \pm 8.46\%$) while D-lactate does not ($2.89 \pm 13.30\%$). This is in agreement with results in vivo (Oldendorf, 1973) and in vitro using bovine BMEC (Terasaki et al., 1991) where the natural enantiomer has a greater affinity for the carrier system. The anti-epileptic drug valproate [$(Pr)_2CHCOO^-$] inhibited [^{14}C]pyruvate transport ($69.80 \pm 4.92\%$) to a similar extent to that of unlabelled pyruvate ($65.24 \pm 4.21\%$), an observation consistent with valproate being transported by the monocarboxylic acid system. Terasaki et al. (1991) demonstrated the inhibition of [^{3}H]acetic acid uptake by valproate to be twice that seen with pyruvate. It has previously been demonstrated that the active transport of valproate in vivo occurs at a higher rate in the brain-to-blood than blood-to-brain direction (Cornford et al., 1985). It has been reported that aromatic α-keto acids such as phenylpyruvate are not substrates for the carrier system and do not inhibit the transport of other α-keto acids (Steele, 1986), however, here

Table 2
Michaelis-Menten parameters for the transport of [^{14}C]pyruvate across BMEC monolayers (standard deviations in parentheses)

Method	K_m (mM)	V_{max} (nmol/h per insert)	r
Eadie-Hofstee	1.39	61.1	0.982
Lineweaver-Burk	0.91	42.0	0.999
Hanes-Woolf	1.48	63.8	0.996
Direct method	1.41	62.6	–
Non-linear regression	1.50 (0.13)	64.5 (3.25)	–

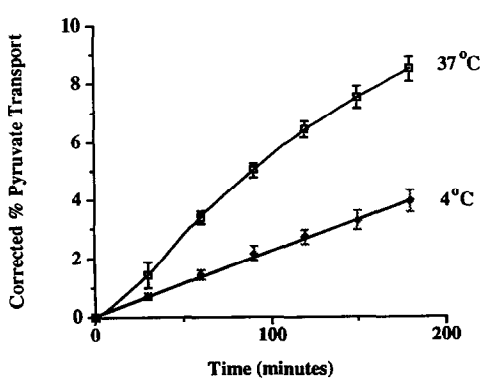


Fig. 3. Temperature dependence of pyruvate transport.

phenylpyruvate was shown to inhibit pyruvate transport (72.65 ± 4.92%). Other work in vivo (Kang et al., 1990) and in vitro (Terasaki et al., 1991) has shown inhibition of monocarboxylic acid transport by aromatic acids such as benzoic and salicylic acid. L-Phenylalanine also did not show any inhibition of pyruvate transport (3.27 ± 5.13%) consistent with previous reports that the monocarboxylic acid system is distinct from the L-amino acid carrier (Oldendorf, 1973; Terasaki, 1991). The effect of temperature, concentration and the inhibition profile of pyruvate transport across porcine BMEC monolayers provide evidence for a short-chain monocarboxylic acid carrier similar to those previously described in vivo and in vitro, and attention is now turned to the transport of PFA and the PFA diester (**2**).

The substition of a phosphonate group for a carboxylate is a common isosteric replacement in medicinal chemistry. Indeed PFA, as a pyrophosphate analogue, indicates the interchangable nature of carboxylates and phosphonates (Oberg, 1989). Diesters of PFA may be considered structural analogues of α-keto acids, such as pyruvate, and therefore, possibly, show affinity for the MCA transport system. Two types of PFA diester may be envisaged; the sodium alkyl methoxycarbonylphosphonates, of which (**2**) is an example and the sodium dialkyl carboxyphosphonates. This latter type, with a free carboxylate group, are unstable due to spontaneous decarboxylation (Krol et al., 1991; Mitchell et al., 1991, 1992; Krol and Thatcher, 1993). In contrast, the stability of the diester of PFA (**2**) in the cell culture model was confirmed post-transport. The identification of (**2**) in the basolateral transport medium was demonstrated by ^{31}P-NMR spectroscopy which gave a single peak at δ −2.60 ppm. The chemical shift was confirmed with an authentic sample in transport medium. However, PFA and the diester (**2**), showed no inhibition of pyruvate transport (−5.70 ± 4.57 and −6.55 ± 11.81%, respectively) and are therefore not substrates for the MCA carrying system.

Acknowledgements

We thank the MRC AIDS Directed Programme for a studentship (I.W.), a travel award (D.N.) and project grants, and the Lister Institute for a fellowship (S.F.). We would also like to thank Professors R.T. Borchardt and K.L. Audus for allowing D.N. to study cell culture methods at the University of Kansas.

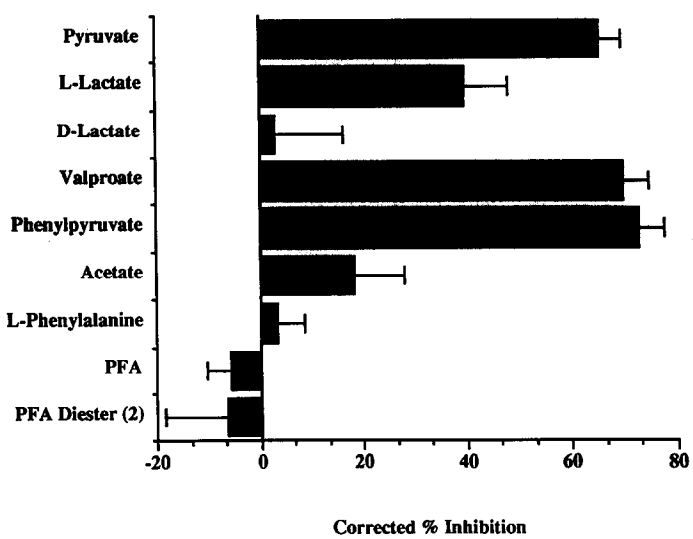


Fig. 4. Inhibition of [^{14}C]pyruvate transport with 10 mM of other acids.

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