Role of hepatic carbonic anhydrase in *de novo* lipogenesis

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The role of carbonic anhydrase in *de novo* lipid synthesis was examined by measuring [1-^{14}C]acetate incorporation into total lipids, fatty acids and non-saponifiable lipids in freshly isolated rat hepatocytes. Two carbonic anhydrase inhibitors, trifluoromethylsulphonamide (TFMS) and ethoxozolamide (ETZ) decreased incorporation of ^{14}C into total lipids. Both fatty acid and non-saponifiable lipid components of the total lipid were inhibited to approximately the same extent by 100 μM TFMS ($29\pm0.3\%$ and $35\pm0.3\%$ of control respectively in replicate studies). However, neither drug significantly affected ATP concentrations or the transport activity of Na^+/K^+-ATPase, two measures of cell viability. To establish the site of this inhibition, water-soluble ^{14}C-labelled metabolites from perchloric acid extracts of the radiolabelled cells were separated by ion-exchange chromatography. TFMS inhibited ^{14}C incorporation into citrate, malate, α-oxoglutarate and fumarate, but had no effect on incorporation of ^{14}C into acetoacetate. Since ATP citrate-lyase, the cytosolic enzyme that catalyses the conversion of citrate into acetyl-CoA, catalyses an early rate-limiting step in fatty acid synthesis, levels of cytosolic citrate may be rate controlling for *de novo* fatty acid and sterol synthesis. Indeed citrate concentrations were significantly reduced to $37\pm6\%$ of control in hepatocytes incubated with 100 μM TFMS for 30 min. TFMS also inhibited the incorporation of ^{14}C from [1-^{14}C]pyruvate into malate, citrate and glutamate, but not into lactate. This supports the hypothesis that TFMS inhibits pyruvate carboxylation, i.e. since all of the ^{14}C from [1-^{14}C]pyruvate converted into citric acid cycle intermediates must come via pyruvate carboxylase (i.e. rather than pyruvate dehydrogenase). Our findings indicate a role for carbonic anhydrase in hepatic *de novo* lipogenesis at the level of pyruvate carboxylation.

INTRODUCTION

Hepatocytes contain a relatively high specific activity of carbonic anhydrase due mainly to the presence of a mitochondrial matrix isoenzyme (CA V, [1–8]) and two cytosolic forms (CA II and CA III, [3, 9–11]). Metabolic roles for the hepatic isoenzymes have been investigated, particularly with regard to their possible involvement in pathways that utilize bicarbonate. Carbonic anhydrase catalyses the following readily reversible reaction:

$$H_2O + CO_2 \leftrightarrow HCO_3^- + H^+$$

The interconversion of CO_2 and HCO_3^- is important because CO bond angles change considerably when CO_2 is converted into HCO_3^- (for review see ref. [12]). Therefore, in metabolic reactions or transport processes that require bicarbonate, CO_2 is a poor substitute and vice versa [13]. Unlike many enzymically catalysed reactions, this reaction proceeds to a significant extent in the absence of enzyme. Drugs that completely inhibit carbonic anhydrase activity therefore only partially inhibit the rate of the reaction. As a result carbonic anhydrase inhibitors only partially inhibit reactions or transport processes that consume CO_2, HCO_3^- or H^+.

Sulphonamide carbonic anhydrase inhibitors (SCAIs) have been reported to inhibit *de novo* fatty acid synthesis in hepatocytes [14] and human adipose tissue [15]. In 1984, Herbert and Coulson [14] hypothesized that this inhibition might represent an interaction between acetyl-CoA carboxylase, a bicarbonate-requiring enzyme, and the cytosolic carbonic anhydrases. This idea stemmed from the inhibition *in vitro* of acetyl-CoA carboxylase by SCAIs reported earlier by Cao and Rous [16] and interactions that were being reported between carbonic anhydrase V and two other carboxylases. For instance, SCAIs had been observed to inhibit urea synthesis [2–4,17–19] and gluconeogenesis from pyruvate, but not glutamine [3,4,12,19–21]. There was good evidence that the effects of SCAIs on urea synthesis and gluconeogenesis were at the level of carbonic anhydrase V, which in turn was thought to provide additional bicarbonate for carbamoylphosphate synthetase and pyruvate carboxylase, enzymes involved in these respective processes [2–4,12,17–21].

In this report, we have examined the mechanism underlying the inhibition of fatty acid synthesis by SCAIs in freshly isolated rat hepatocytes. In agreement with Herbert and Coulson [14], who were studying reptiles, we report that SCAIs inhibit *de novo* lipid synthesis in rat hepatocytes. However, they also inhibited *de novo* synthesis of non-saponifiable lipids, which does not require acetyl-CoA carboxylase, but does share other earlier metabolic steps with *de novo* fatty acid synthesis. To determine whether or not an earlier step in the pathway might be blocked by SCAIs, the metabolic fate of [1-^{14}C]acetate and [1-^{14}C]pyruvate were examined in isolated hepatocytes.

EXPERIMENTAL

Materials

The radiolabelled compounds were from New England Nuclear. Bacto-Gelatin (gelatin) was from Difco Laboratories (Detroit, MI, U.S.A.), insulin from Eli Lilly (Indianapolis, IN, U.S.A.) and collagenase D (lot no. DHA-142) from Boehringer-Mannheim (Indianapolis, IN, U.S.A.).

Abbreviations used: TFMS, trifluoromethylsulphonamide; ETZ, ethoxozolamide; SCAI, sulphonamide carbonic anhydrase inhibitor.

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Hepatocyte isolation

Hepatocytes were prepared from Sprague–Dawley rats as previously described [22] using Vanderbilt perfusion apparatus with minor modifications. The rat liver was perfused *in situ* at 37 °C with perfusion buffer (120 mM NaCl, 4.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, 5 mM sodium pyruvate and 5 mM sodium glutamate, pH adjusted to 7.4 by constant gassing with humidified 95% O_2/5% CO_2). Once the perfusion was established, collagenase D was added to the perfusate which continued to recirculate (30 mg/150 ml of recirculating perfusate) for approx 20 min. At this time the partially digested liver was removed, gently minced in perfusion buffer containing 1.5% gelatin and shaken for 10 min at 37 °C with constant gassing (95% O_2/5% CO_2). The resulting suspension was filtered through a 250 μm nylon mesh. Hepatocytes were collected by centrifugation at 50 *g* for 2.5 min in a digital Jouan CR422 centrifuge with a swinging-bucket rotor. The cell pellet was then washed three times with gentle resuspension in physiological buffer (120 mM NaCl, 4.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, 5 mM sodium pyruvate, 5 mM sodium glutamate, 1.5% gelatin and 1.5 mM $CaCl_2$, pH adjusted to 7.4 by constant gassing with humidified 95% O_2/5% CO_2).

Radioisotope labelling

Cell suspensions (8–12% cytocrit) were incubated (5 ml aliquots in 25 ml flasks or 25 ml aliquots in 125 ml flasks) at 37 °C under a constantly replaced atmosphere of humidified 95% O_2/5% CO_2. After a 10 min incubation with TFMS, ETZ or drug vehicle (DMSO, vehicle control), [1-^{14}C]acetate or [1-^{14}C]pyruvate was added at the indicated concentrations. Insulin (100 nM) was also added at this point to stimulate lipogenesis. After a 10–60 min labelling period replicate 1 ml aliquots of the cells were centrifuged through 10 ml of ice-cold 10% sucrose. The centrifugation speed was at 800 *g* and the IEC centrifuge was fitted with a manual foot brake to allow stopping of the centrifuge in about 10 s. The tubes were returned to the ice bath, and the sucrose supernatant was removed with a Pasteur pipette attached to a vacuum. Lipids or water-soluble weakly acidic metabolic intermediates were extracted from the cell pellets and analysed as described below.

Extraction and analysis of total lipid, fatty acids and non-saponifiable lipids

Lipids were extracted by the method of Kates [23]. Briefly, water (0.8 ml) was added to each ice-cold cell pellet (approx. 0.2 ml) followed by 3.8 ml of chloroform/methanol (1:2, v/v). The pellets were vortexed and shaken for several hours. After centrifugation, the supernatant was removed and the pellet was re-extracted with chloroform/methanol/0.2 M HCl (1:2:0.8, v/v). The supernatants were pooled, combined with 2.5 ml of chloroform and 2.5 ml of water and centrifuged to form two phases. The aqueous layer was discarded. The organic phase was extracted with another 5 ml of water which was also discarded after centrifugation. An aliquot of this organic phase was measured by liquid-scintillation counting to determine the incorporation of ^{14}C into total lipid.

To measure the incorporation of ^{14}C into fatty acids and non-saponifiable lipids a fraction of the organic phase was evaporated to approx. 0.1 ml under vacuum in a Jouan RC 10.10 centrifuge. To saponify the evaporated samples, 2.5 ml of ethanol and 0.1 ml of 33% KOH was added. The capped samples were heated overnight at 75 °C. After the tubes had cooled to room temperature, the contents were combined with 1 drop of phenolphthalein indicator solution, 2.5 ml of water and 5 ml of ether. After vortexing and centrifugation, the organic phase was removed and the aqueous phase was re-extracted with ether. To determine the incorporation of ^{14}C into non-saponifiable lipids, radioactivity content of these ether extracts was quantified by liquid-scintillation counting. The aqueous phase was acidified with 0.3 ml of 6 M HCl, and fatty acids were extracted with ether as above. To determine the incorporation of ^{14}C into fatty acids, radioactivity in these ether extracts was also quantified.

Determination of [ATP] and [citrate]

Cell suspensions were incubated for 45 min with 100 nM insulin and TFMS or drug vehicle (DMSO). Aliquots of the cells (1 ml) were centrifuged through 10 ml of ice-cold 10% sucrose. The pellets were resuspended in ice-cold 2% perchloric acid using a sonicator. The perchloric acid (2%) extracts were then neutralized with a solution containing 3 M KOH, 0.5 M Mops buffer and 0.1 M EDTA. After centrifugation to remove the potassium perchlorate, dilutions of the supernatants were analysed for ATP using a commercially available bioluminescence method (Boehringer-Mannheim). The light signal was measured in an EG&G Berthold AutoLumat LB953 luminometer. Citrate was determined spectrophotometrically using the method described by Williamson and Corkey [24], which couples citrate lyase and malate dehydrogenase.

Na^+/K^+-ATPase-mediated transport

Ouabain-sensitive $^{86}Rb^+$ uptake was measured as an index of Na^+/K^+-ATPase-mediated transport activity as described by Lynch et al. [22,25]. Briefly, cell suspensions (5 ml per 25 ml flask) were incubated for 30 min in the presence or absence of 2 mM ouabain and/or SCAIs at 37 °C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2. Uptake measurements were initiated by adding ^{86}RbCl (final concentration 1.08×10^6–1.15×10^6 d.p.m./ml cell suspension). Uptake was stopped after 5 min by centrifuging cells through 10 ml of 10% sucrose/4.5 mM KCl at speeds of up to 800 *g* for 30 s. Centrifugation was rapidly terminated with a manual hand brake. Radioactivity in the cell pellets was measured by Čerenkov counting. Ouabain-sensitive $^{86}Rb^+$ uptake was taken as the difference between the uptake measured in the absence and presence of ouabain.

Extraction and analysis of water-soluble weakly acidic metabolic intermediates

For analysing weakly acidic metabolic intermediates, ice-cold hepatocyte pellets (approx. 0.2 ml and radiolabelled with [1-^{14}C]acetate or [1-^{14}C]pyruvate as described above) were resuspended in 1 ml of 2% perchloric acid using a sonicator. Perchloric acid (2%) extracts of the radiolabelled cell pellets were neutralized with a solution containing 3 M KOH, 0.5 M Mops and 0.1 M EDTA. After centrifugation to remove the potassium perchlorate, 1 ml of the supernatant was loaded on a water-equilibrated Dowex AG 1-X8 column (1 cm × 19 cm) with a flow rate of 1.75 ml/min [26–28]. The column eluate was collected in 3.5 ml fractions. The mobile phase consisted of a linear gradient formed between 125 ml of water and 125 ml of 0.05 M HCl. After fraction 60 the concentration of HCl was increased to 0.1 M. The column was run with this second linear gradient until the fractions of interest had been eluted or until fraction no. 120. Radioactivity in each fraction was determined by liquid-scintillation counting.

To identify the peaks, the elution pattern was compared with that of standard compounds run in parallel. Most of the standards were radiolabelled and quantified by liquid-scintillation counting. The only exception was acetoacetate which was determined using a commercially available kit (Miles Laboratories). The column was regenerated with 25 ml of 0.1 M HCl followed by water until the pH of the eluate was 4.0.

RESULTS

De novo total lipid synthesis in freshly isolated rat hepatocytes was measured as the incorporation of [1-^{14}C]acetate into total lipids (Figure 1). Previous studies on the effect of SCAIs on fatty acid synthesis have employed the heterocyclic SCAI, acetazolamide [3,14–16]. We used TFMS, a member of a new class of

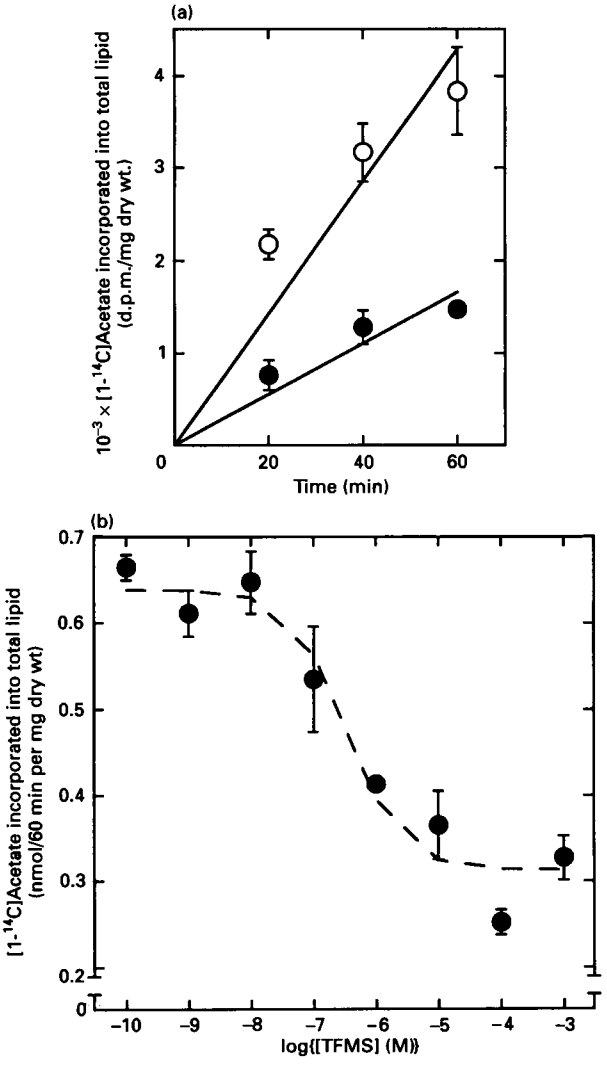

Figure 1 Effects of TFMS on *de novo* lipogenesis from [1–^{14}C]acetate

(**a**) Hepatocytes were incubated for 10 min in the presence of drug vehicle (○) (1 μl/ml DMSO) or TFMS (●) (100 μM). At time zero 1 mM [1-^{14}C]acetate (1.5×10^{12} d.p.m./mol) and 100 nM insulin were added. Triplicate 1 ml aliquots of cell suspension were taken for total lipid determination at the indicated times. (**b**) Hepatocyte suspensions were incubated with various concentrations of TFMS for 10 min and then 1 mM [1-^{14}C]acetate and 100 nM insulin were added. The cell suspensions were in triplicate for total lipid determination after 60 min as described in the Experimental section. The results are means ± S.E.M. from triplicate determinations from a single experiment that is representative of three such experiments.

Table 1 Effects of TFMS and ETZ on the incorporation of [1-^{14}C]acetate into total lipids, fatty acids and non-saponifiable lipids

Hepatocytes were incubated for 10 min in the presence of 1 μl/ml DMSO (vehicle control), 100 μM TFMS or 100 μM ETZ before the addition of 1 mM [1-^{14}C]acetate and 100 nM insulin. After 60 min quadruplicate samples were taken for determination of total lipids, fatty acids and non-saponifiable lipids as described in the Experimental section. The results shown are means ± S.E.M. from a single experiment that is representative of three such studies. Absolute values in nmol of ^{14}C incorporated/60 min per mg dry wt. averaged from three experiments are for total lipids 1.1 ± 0.2, for fatty acids 0.34 ± 0.08 and for non-saponifiable lipids 0.56 ± 0.18. *Significantly different from control as determined by Student's *t* test ($P < 0.05$).

	Incorporation of [1-^{14}C]acetate (%)		
Lipid fraction	Vehicle control	TFMS	ETZ
Total lipids	100.0 ± 11.1	39.6 ± 2.6*	31.1 ± 5.2*
Fatty acids	100.0 ± 13.0	27.6 ± 2.2*	24.4 ± 0.1*
Non-saponifiable lipids	100.0 ± 13.2	46.6 ± 7.4*	27.0 ± 3.2*

Table 2 Effect of TFMS and ETZ on Na^+/K^+-ATPase-mediated $^{86}Rb^+$ uptake and [ATP]

Cell suspensions were incubated with SCAIs or drug solvent (DMSO) for 30 min (ouabain-sensitive $^{86}Rb^+$ uptake) or 45 min ([ATP]). The results are means ± S.E.M. from triplicate determinations and are representative of two such experiments. The results were not significantly different from control as determined by Student's *t* test ($P > 0.05$).

Condition	Ouabain-sensitive $^{86}Rb^+$ uptake (nmol of $^{86}Rb^+$/5 min per mg dry wt.)	[ATP] (nmol/mg dry wt.)
Control (5 μl/ml DMSO)	10.7 ± 0.2	3.2 ± 0.4
TFMS (1 mM)	10.3 ± 0.4	3.6 ± 0.2
ETZ (1 mM)	11.2 ± 0.5	3.9 ± 0.3

very potent SCAIs [29]. It differs from classic sulphonamides, such as ETZ, in that it contains an aliphatic rather than a heterocyclic residue on the sulphonamide. As a result of their structural differences, it is unlikely that both heterocyclic and aliphatic sulphonamides would have the same non-specific effects. Incubation of the cells with 100 μM TFMS resulted in a 60–70% decrease in *de novo* lipid synthesis (Figure 1 and Table 1). The magnitude of the inhibition did not change significantly between the 20 and 60 min time point (Figure 1a). The effect of TFMS was concentration-dependent, with an IC_{50} of $3.3(\pm 1.9) \times 10^{-7}$ M (Figure 1b). The effects of ETZ on *de novo* total lipid synthesis were also examined (Table 1). Maximal concentrations of either TFMS or ETZ inhibited total lipid synthesis to approximately the same extent in replicate experiments (TFMS 61 ± 2.6% and ETZ 69 ± 5.2%, $n = 8$). TFMS and ETZ inhibited the *de novo* synthesis of both the fatty acid and non-saponifiable lipid components (Table 1). We became concerned at this point that the SCAIs might be having a cytotoxic effect on the hepatocytes. A decrease in ATP levels could explain the effects on lipid synthesis since both the synthesis of fatty acids and non-saponifiable lipids are ATP requiring. However, subsequent experiments showed that the inhibition of lipogenesis was not accompanied by decreases in the utilization of ATP by Na^+/K^+-ATPase or by a decrease in hepatocyte ATP concentrations (Table 2), arguing against this possibility.

To investigate the common metabolic steps that precede the conversion of acetate (in the mitochondria) into cytosolic acetyl-CoA (the common precursor for sterologenesis and *de novo* fatty acid synthesis), we separated the weakly acidic metabolic pre-

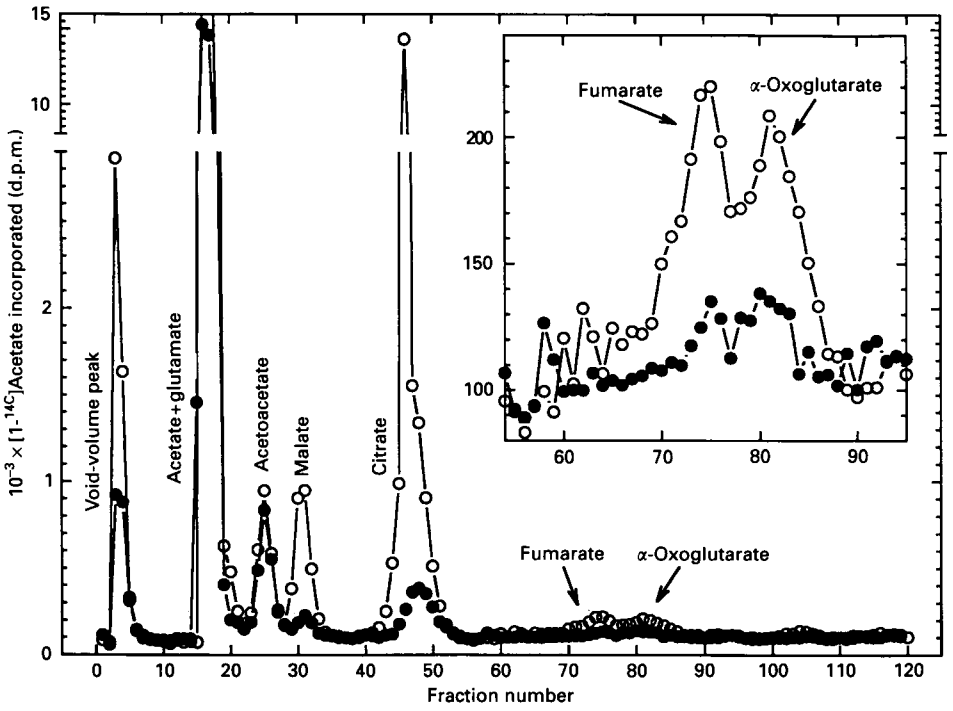

Figure 2 Effect of TFMS on the incorporation of [1-^{14}C]acetate into acidic metabolic intermediates

Hepatocytes were incubated for 10 min in the presence of drug vehicle (○) (1 μl/ml DMSO) or TFMS (●) (100 μM). At time zero [1-^{14}C]acetate was added (1.7 × 10^6 d.p.m./ml of cell suspension) to a final concentration of 1 mM. Aliquots of cell suspension (1 ml) were taken after 40 min and centrifuged through ice-cold 10% sucrose. Neutralized perchloric acid extracts of the cells were loaded on a quaternary-ammonium-ion-exchange column, and weakly acidic compounds were eluted as described in the Experimental section. The elution profiles of the compounds were compared with the elution of [1-^{14}C]- or [^{3}H]-standards as indicated (not shown). The inset shows the end of the elution profile at a different scale. The results are from a single experiment that is representative of three such studies.

cursors by ion-exchange chromatography. The radioactive peaks were identified on the basis of parallel runs with standards (Figures 2 and 3). The earliest peak from these columns represents the void volume and contains a mixture of the water-soluble neutral compounds and weak bases that do not stick to the column (e.g. glucose, urea, citrulline, alanine, glutamine, etc.). Figure 2 shows the column profiles from experiments with [1-^{14}C]acetate as the starting material. As can be seen, TFMS had no effect on the incorporation of radiolabel into acetoacetate, but was associated with a greater than 90% reduction in the radiolabelling of malate, citrate, fumarate and α-oxoglutarate. Any effect on glutamate labelling was masked by the fact that acetate and glutamate were eluted very close to each other on the columns. The effects on citrate are relevant here because cytosolic citrate is required to produce cytosolic acetyl-CoA, the common precursor for *de novo* synthesis of both fatty acids and sterols. Since changes in radiolabelling may only reflect trace changes in these compounds, we examined cellular citrate concentrations. In 30 min, citrate concentrations were reduced from 9.48 ± 0.52 to 3.51 ± 0.24 nmol/mg dry wt. To examine the possibility that pyruvate carboxylase inhibition caused these effects, we used [1-^{14}C]pyruvate (Figure 3). The carbon on the 1-position of [1-^{14}C]pyruvate is released as CO_2 during the pyruvate dehydrogenase-catalysed conversion of pyruvate into citrate. In contrast, the carbon is retained when pyruvate is converted into oxaloacetate via pyruvate carboxylase. Any [^{14}C]citrate that is formed from [1-^{14}C]pyruvate therefore comes via pyruvate carboxylase. In these experiments no significant effect was seen on the incorporation of ^{14}C from [1-^{14}C]pyruvate into lactate; however, radioisotope incorporation into glutamate, malate and citrate were reduced 65–75% in different experiments (Figure 3).

DISCUSSION

The purpose of this paper was to learn whether or not carbonic anhydrase activity is an important factor in *de novo* lipid synthesis. The results showed that inhibition of carbonic anhydrase inhibits lipid synthesis (Figure 1 and Table 1) apparently because carbonic anhydrase activity is required for optimal activity of pyruvate carboxylase rather than because it may be necessary for acetyl-CoA carboxylase activity. We considered the possibility that these effects might have reflected a cytotoxic or non-specific action of SCAIs. Until recently only heterocyclic SCAIs were available, so it was difficult to discount the possibility that effects observed with these drugs were not due to non-specific interactions of the heterocyclic residue. This is an important consideration as Beynen and Geelen [30] have reported that a number of cytotoxic cyclic compounds can inhibit lipogenesis. To address this problem, we have used TFMS, a member of a new class of aliphatic carbonic anhydrase inhibitors [29], in addition to the heterocyclic inhibitor, ETZ. Both compounds inhibited *de novo* lipogenesis (Table 1), but neither lowered ATP concentrations or the transport activity of the ATP-requiring enzyme, Na^+/K^+-ATPase (Table 2). Thus it seems unlikely that their effects on *de novo* lipogenesis represented cytotoxicity. In fact, the observation that two structurally different SCAIs have the same effect supports the conclusion that their actions are secondary to carbonic anhydrase inhibition.

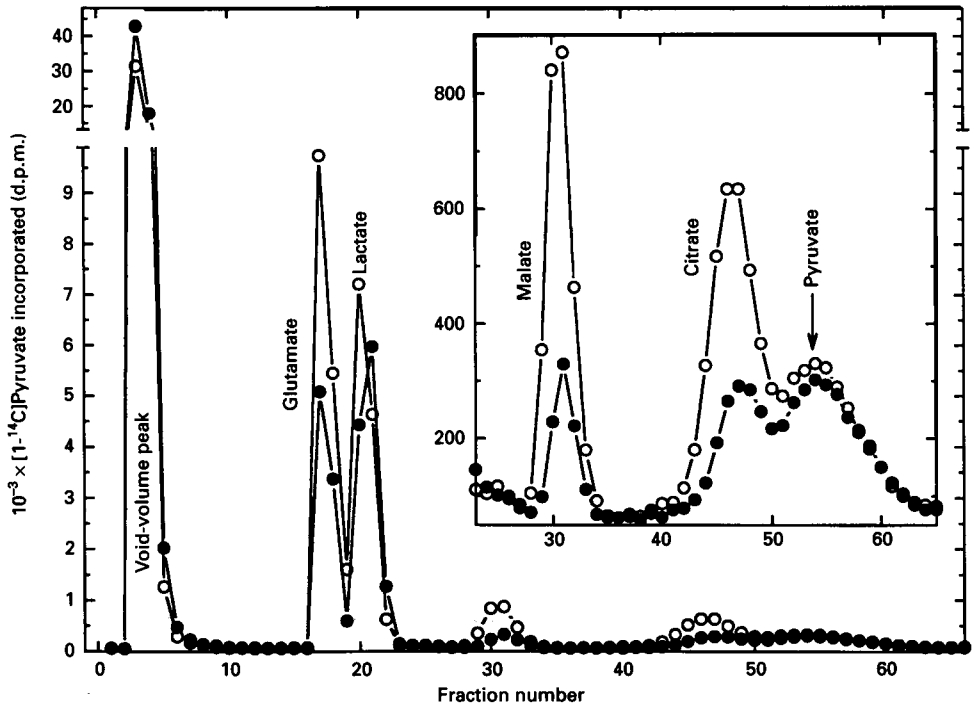

Figure 3 Effect of TFMS on the incorporation of [1-^{14}C]pyruvate into acidic metabolic intermediates

Hepatocytes were incubated for 10 min in the presence of drug vehicle (○) (1 μl/ml DMSO) or TFMS (●) (100 μM). At time zero [1-^{14}C]pyruvate was added (1.3×10^6 d.p.m./ml of cell suspension containing 5 mM unlabelled pyruvate). Aliquots of cell suspension (1 ml) were taken after 10 min and centrifuged through ice-cold 10% sucrose. Neutralized perchloric acid extracts of the cells were loaded on a quaternary-ammonium-ion-exchange column, and weakly acidic compounds were eluted. The inset shows the end of the elution profile at a different scale. The results are from a single experiment that is representative of three such studies.

TFMS and ETZ inhibited the *de novo* synthesis of both fatty acids and non-saponifiable lipids by approx. 60–70% (Table 1). It is noteworthy that the magnitude of this inhibition is at least as great as other drugs being examined as potential hypolipidaemic agents. For example, maximal concentrations of the new hypolipidaemic agent, lifibrol, inhibit *de novo* synthesis of fatty acids and sterol lipids in rat hepatocytes between 40 and 60% [31]. Further studies will be required to determine whether or not the effect of SCAIs on hepatic lipid synthesis may also lead to reductions in plasma triacylglycerols and cholesterol.

Cao and Rous [16] first observed that heterocyclic SCAIs could inhibit *de novo* fatty acid synthesis *in vitro*, but interpreted this as a direct effect on acetyl-CoA carboxylase. Later, Herbert and Coulson [14,20] performed studies *in vivo* on reptiles with heterocyclic SCAIs . They also observed inhibition of *de novo* fatty acid synthesis by SCAIs. By this time, however, several groups had reported an interaction between the mitochondrial carbonic anhydrase (isoenzyme V) and both pyruvate carboxylase and carbamoyl phosphate synthetase [2–4,12,17–21]. Herbert and Coulson [14,20] reasoned that the inhibition of *de novo* fatty acid synthesis might represent an analogous situation involving the cytosolic carbonic anhydrases and acetyl-CoA carboxylase. However, we report that SCAIs also inhibit *de novo* synthesis of non-saponifiable lipids (Table 1) which could not involve an interaction between cytosolic carbonic anhydrase isoenzymes and acetyl-CoA carboxylase, as acetyl-CoA carboxylase is not involved in the synthesis of these lipids.

In the presence of TFMS both the incorporation of [1-^{14}C]acetate into citrate and other citric acid cycle intermediates and the amount of citrate in hepatocytes were decreased by 60% or more. TFMS had no effect on the radiolabelling of acetoacetate (Figure 2). As most citrate in the liver is cytosolic and the common precursor for *de novo* fatty acid synthesis and sterologenesis, the effect of SCAIs on citrate probably explains their effects on *de novo* lipid synthesis. Table 2 indicates that the decrease in citric acid cycle intermediates is not of sufficient magnitude to impact on [ATP] or the activities of ATP-utilizing enzymes, such as Na^+/K^+-ATPase. Thus, although there may be sufficient ATP for ATP citrate-lyase after SCAI treatment (Table 2), there is insufficient citrate. The decrease in citrate is of sufficient magnitude to explain the inhibition of *de novo* lipogenesis at this step. If the effects of these drugs were on acetyl-CoA carboxylase, an increase or no change in citrate would be expected. Although inhibition of acetyl-CoA carboxylase by SCAIs may occur *in vitro*, in seems unlikely that this is the mechanism that is important *in vivo*. Interestingly, humans given SCAIs experience hypocitraturia [32,33]; however, the mechanism underlying this effect is not known.

We used [1-^{14}C]pyruvate as the radiolabel in short-term experiments to examine the role of pyruvate carboxylase in these effects. Conversion of [1-^{14}C]pyruvate into [1-^{14}C]lactate was not affected by TFMS. A significant reduction in radiolabel incorporation into citrate, malate and glutamate was observed. These findings suggest that the effects on citrate and malate arise from an inhibition of pyruvate carboxylation. SCAIs do not inhibit purified pyruvate carboxylase *in vitro*, rather their effects on this enzyme step in cells appears to arise from an inhibition of carbonic anhydrase. Thus the previously reported interaction of carbonic anhydrase V and pyruvate carboxylase [3,4,12,19–21] is not only important for gluconeogenesis but, as shown here, for *de novo* lipogenesis as well. Although it is clear that carbonic anhydrase V participates in pyruvate carboxylation, the role of

the extramitochondrial carbonic anhydrases should not be discounted. Exogenous [1-^{14}C]bicarbonate is rapidly incorporated into tricarboxylic-acid-cycle intermediates in hepatocytes. This indicates that mitochondrial CO_2 production is not the sole source of bicarbonate for pyruvate carboxylation. The transcellular movement of bicarbonate and its conversion into CO_2 for transmembrane movement into the mitochondrial matrix undoubtedly involves extramitochondrial carbonic anhydrases. This idea is supported by recent experiments that show SCAIs can inhibit pyruvate carboxylation in rat astrocytes that contain carbonic anhydrase II but lack the immunoreactive isoenzyme (V) (C. J. Lynch, A. Waheed, S. Hazen, W. Sly and K. Lanoue, unpublished work). It is likely that the presence of carbonic anhydrase V in liver is related to the comparatively high levels of carbon fixation occurring there. Thus both extramitochondrial and mitochondrial carbonic anhydrases may contribute to the effects of SCAIs on pyruvate carboxylation.

Pyruvate carboxylase is inhibited in situations where hepatic lipid synthesis is favoured. Because of this and the fact that in adipocytes most of the flux through pyruvate carboxylase ends up in malate with very little citrate formed, most texts discount the role of pyruvate carboxylase in *de novo* lipogenesis (e.g. [34]). However, ^{13}C-NMR studies show that even in the fed state (i.e. where lipogenesis is favoured) the ratio of flux through hepatic pyruvate carboxylase and pyruvate dehydrogenase is still about 1.2:1, i.e. favouring pyruvate carboxylase [35]. Our studies indicate that radiolabel incorporated into citrate is significant. It is important to remember that the carbons that are removed from the citric acid cycle via citrate for lipid synthesis are only partially returned and that demands for citric acid cycle intermediates by other metabolic processes (e.g. gluconeogenesis, ureagenesis, amino acid synthesis) may occur simultaneously, even when lipogenesis is favoured. That is where the anaplerotic activity of pyruvate carboxylase, and the mitochondrial carbonic anhydrase which assists it, becomes important. If during lipogenesis, the ratio of flux through hepatic pyruvate carboxylase and pyruvate dehydrogenase fell below 1:1 (i.e. favouring pyruvate dehydrogenase as is often depicted), we would predict that the levels of citric acid cycle intermediates would soon decline to levels sufficient for ATP production, but insufficient to maintain maximal rates of lipid synthesis, as indicated by our data.

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