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ESTERASE ACTIVITY IN RAT HEPATOCYTES

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Abstract—Hydrolysis of acetylsalicylate, benorylate, phenetsal, fluazifop butyl and paraoxon has been studied with freshly isolated rat hepatocytes maintained as a monolayer. Acetylsalicylate and paraoxon were the poorest substrates for hydrolysis whereas benorylate was hydrolysed one hundred times faster. Phenetsal and fluazifop butyl were both hydrolysed at one-tenth of the rate of benorylate. Inhibitor studies with paraoxon, BNPP and physostigmine indicated the involvement of different carboxylesterase isozymes. Studies with acetylsalicylate indicated that uptake of the substrate into the hepatocyte may influence the rate of formation of the hydrolysis product. Studies of hydrolysis in hepatocytes more closely reflect *in vivo* hepatic hydrolysis than subcellular fractions as cytosolic and microsomal esterases can act in parallel.

Liver carboxylesterases (EC 3.1.1.1) are a group of B-esterases which hydrolyse a wide range of xenobiotic ester substrates. They limit the activity of many ester drugs and reduce the toxicity of pesticides to animals and to man [1, 2]. Recently their activity has been utilized by the pharmaceutical industry for the liberation of active drugs from pro-drug esters. Carboxylesterases are present both within the endoplasmic reticulum and the cytosol. Microsomal carboxylesterases have been the most extensively characterized [3], however, the cytosolic carboxylesterases may be relatively more important for metabolism of some substrates. Differing specificities and activities suggest that different carboxylesterase isozymes may be present in microsomes and cytosol [4, 5].

Membrane bound microsomal carboxylesterase activity has been studied generally following solubilization with detergents, and the enzymes have been classified on the basis of their isoelectric point and by inhibition by organophosphate inhibitors such as paraoxon and BNPP, into four main groups [6, 7].

However, in the *in vivo* situation, substrates which enter the intact hepatocyte will first encounter cytosolic esterases and then must gain access to the endoplasmic reticulum lumen for metabolism by microsomal carboxylesterases. Studies in isolated hepatocytes in which microsomal and cytosolic carboxylesterases can act in parallel, may more closely represent the *in vivo* situation than studies in subcellular fractions. This paper reports studies of the hydrolysis of the analgesics aspirin (acetylsalicylate), benorylate (*p*-acetamidophenyl acetylsalicylate), phenetsal (*p*-acetamidophenyl salicylate) and the herbicide fluazifop butyl (butyl 2-(4-(5-trifluoromethyl-2-pyridyloxy)phenoxy)propanoate) by freshly isolated rat hepatocytes with and without esterase inhibitors. Hydrolysis of paraoxon was also followed. Paraoxon hydrolysis is mediated by a cysteine containing A-esterase [8].

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MATERIALS AND METHODS

Chemicals

Benorylate and phenetsal were gifts from Sterling Winthrop Research Europe, and fluazifop butyl and fluazifop from Central Toxicology Laboratory, I.C.I. All other chemicals were obtained from the Sigma Chemical Co.

Preparation of hepatocytes

Adult male Wistar rats (220 g) were used. Hepatocytes were isolated by a modification of the method of Berry and Friend [9]. The livers were perfused *in situ* with 0.1 M Hepes-buffered saline pH 7.5 containing EDTA (80 μM) and potassium chloride (6.7 μM) followed by the same Hepes buffer without EDTA but containing calcium chloride (10 mM) and 120 mg collagenase (Sigma) per 1000 mL buffer. Parenchymal cells were washed and separated from nonparenchymal cells, debris and nonviable cells by centrifugation from Percoll: Hanks Balanced salt solution (modified) 10× (9:1). Cells were suspended in Williams E medium containing gentamycin (50 μg/mL), insulin (0.5 μg/mL) and fetal calf serum (10%). Greater than 90% of cells obtained were viable as assessed by Trypan blue exclusion.

Hepatocytes were diluted to 7×10^5 cells/mL and 3-mL aliquots plated into plastic Petri dishes and allowed to form a monolayer in a humidified incubator at 37° at 5% CO_2 and 95% air. After 3 hr the culture medium was replaced with 3 mL Williams E with no fetal calf serum and the monolayers held overnight in the incubator. Incubations with esterase substrates were carried out the following morning. All metabolism studies were carried out in the absence of fetal calf serum as albumin may act as an esterase [10].

Hydrolysis studies

For determination of esterase activities, hepatocytes were incubated with acetylsalicylic acid (1 mM) added in 15 μL methanol, benorylate (0.3 mM) added in 15 μL acetone, phenetsal

(0.3 mM) in 15 μL acetone, paraoxon (0.17 mM) in 15 μL methanol and fluazifop butyl (0.19 mM added in 15 μL acetonitrile). Acetylsalicylate was freshly dissolved prior to assay and paraoxon was freshly diluted from stock. Hydrolysis of acetylsalicylate was monitored by measuring salicylate production. Benorylate disappearance and phenetsal, salicylate and paracetamol production were measured; the rate of hydrolysis was expressed in terms of benorylate disappearance. Hydrolysis of fluazifop butyl and paraoxon were measured by the formation of fluazifop acid and *p*-nitrophenol, respectively.

Hydrolysis of the ester to free acid was followed with time for up to 3 hr for aspirin. Hydrolysis of the other substrates which were more rapid was followed for 10 min. Two plates were used for each time point. Following incubation samples of medium (200 μL) were removed to an equal volume of perchloric acid (6%) and stored on ice until analysis by HPLC the same day. Spontaneous hydrolysis of the esters in Williams E at 37° was monitored in parallel. Viability of the cells during the study was monitored by Trypan blue exclusion and LDH leakage. Long-term maintainance of esterase activity was assessed using acetylsalicylate as substrate. On days 2 to 4 following plating, hydrolysis of acetylsalicylate (0.3 mM) was determined by sampling medium at 0, 15, 40 and 60 min. Fresh Williams E medium was applied to the cells each 24 hr.

Inhibitors

Paraoxon, BNPP and physostigmine were added to the cells at the following concentrations: paraoxon 170 μM, BNPP 460 and 46 μM and physostigmine 170 μM. The cells were preincubated for 20 min and the substrate then added. Rates of hydrolysis were measured as previously described.

Hepatocyte uptake

Uptake of acetylsalicylate into the hepatocyte was measured in the presence and absence of paraoxon. Acetylsalicylate (2.0 mM) was added to hepatocytes and at intervals following addition of substrate, 200 μL of medium was removed and added to an equal volume of 6% perchloric acid. The rest of the medium was removed and the cells were washed with 2 × 1 mL isotonic saline. Triton X-100 (1 mL 0.1%) was added, the cells stood for 2 min on ice, then ruptured cells were scraped from the plate and the membranes separated by centrifugation at 2000 *g* for 5 min. Aliquots of the Triton solution containing cell cytosol were mixed with perchloric acid and assayed to give the levels of salicylate. These were compared to the levels in the medium. All preparations were kept on ice and injected onto the HPLC as soon as possible. Protein content of the monolayer was determined by the method of Lowry *et al.* [11], on cells dissolved in 1 M sodium hydroxide.

HPLC assays

All assays were carried out on a Kontron HPLC using a Spherisorb ODS (5 μ, 15 cm) column, or Jones Apex ODS (5 μ, 15 cm) for fluazifop butyl analysis. A 100-μL sample mixed with perchloric acid was injected directly onto the column without neutralization and compared to standards prepared in a similar manner. Details of the methods for acetylsalicylate, salicylate, benorylate and phenetsal have previously been described [4, 5]. Mobile phases and detection conditions were as follows:

Acetylsalicylate hydrolysis. Mobile phase: 50:50 methanol: orthophosphoric acid (0.072%, w/v), flow rate 1 mL/min, UV detection 234 nm. Retention times acetylsalicylate 2.0 min and salicylate 2.8 min.

Benorylate and phenetsal hydrolysis. Mobile phase: continuous gradient from 20 to 60% methanol in orthophosphoric acid (0.072%, w/v), flow rate 1 mL/min, UV detection 234 nm. Retention times paracetamol 4.6 min, acetylsalicylate 9.4 min, salicylate 9.9 min, benorylate 11.5 min and phenetsal 14.4 min.

Paraoxon hydrolysis. Mobile phase: 50:50 methanol: orthophosphoric acid (0.072%, w/v), flow rate 1 mL/min, detection 330 nm (*p*-nitrophenol), 270 nm (paraoxon). Retention times *p*-nitrophenol 4.0 min and paraoxon 10.0 min.

Fluazifop butyl hydrolysis. Mobile phase: 70:30 acetonitrile: orthophosphoric acid (0.072%, w/v), flow rate 1 mL/min, 270 nm. Retention times: fluazifop 2.5 min and fluazifop butyl 7.5 min.

Table 1. Hydrolytic activity of rat hepatocytes

Substrate	Concentration (mM)	Activity*
Benorylate	0.3	340.0 ± 47.03 (3)
Fluazifop butyl	0.19	38.2 ± 5.9 (4)
Phenetsal	0.3	24.3 ± 0.95 (3)
Paraoxon	0.17	2.2 ± 0.5 (3)
Acetylsalicylate	1.0	3.1 ± 0.96 (3)

* Results are expressed as mean ± SE (N), nmol product/2.1 × 10^6 cells/min except for benorylate where the result is nmol substrate hydrolysed/2.1 × 10^6 cells/min.

RESULTS

Rat hepatocytes formed confluent monolayers on plastic under the conditions used. If higher concentrations of cells were used not all the cells attached to the plastic. Trypan blue staining with microscopic examination and measurement of LDH leakage, indicated that the cells remained viable for up to and including the 4 days studied. None of the substrates or their vehicles were toxic to the cells at the concentrations used, however paraoxon and BNPP caused the monolayer to lift after 1 hr.

Hydrolytic activity of rat hepatocytes was expressed as nmol product formed/min/2.1 × 10^6 cells except for benorylate where further hydrolysis of the intermediate product necessitated expressing activity as nmol substrate hydrolysed/min/2.1 × 10^6 cells and results are shown in Table 1. Acetylsalicylate was the poorest substrate for hepatocyte esterases, whereas hydrolysis of benorylate to phenetsal proceeded at 10–100 times the rate for other substrates, followed more slowly by further hydrolysis to paracetamol and salicylate. Hydrolysis of

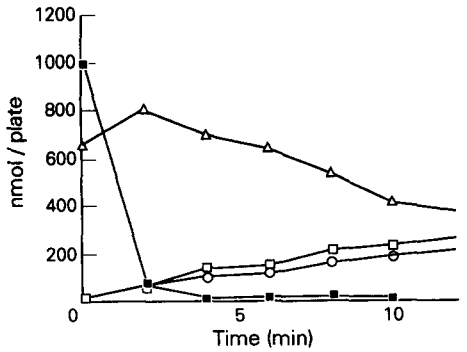

Fig. 1. Hydrolysis of benorylate (0.3 mM) by rat hepatocytes. Results are expressed as nmol formed/plate for phenetsal —△—, salicylate —○—, paracetamol —□— and for benorylate disappearance —■—.

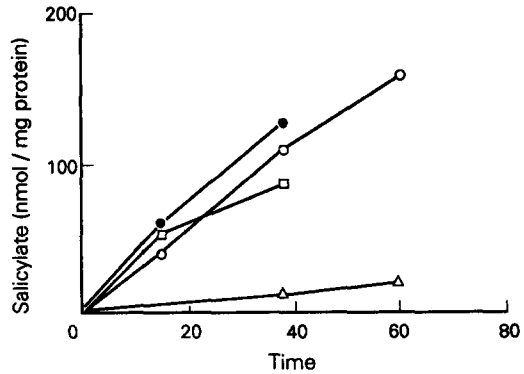

Fig. 2. Hydrolysis of acetylsalicylate (0.3 mM) to salicylate by rat hepatocytes maintained in culture for 1 —●—, 2 —○—, 3 —□— day. Spontaneous hydrolysis of acetylsalicylate in medium is also shown —△—.

benorylate is shown in Fig. 1. Hydrolysis of benorylate to phenetsal was so rapid that a high concentration of phenetsal was detected even in the zero time sample for which perchloric acid was added to the hepatocytes as soon as possible after the substrate. The initial concentration of benorylate was obtained by extrapolation to zero. Phenetsal alone was hydrolysed to paracetamol and salicylate. Fluazifop butyl was hydrolysed to fluazifop at a similar rate to phenetsal and paraoxon was hydrolysed at a similar rate to aspirin. Acetylsalicylate was spontaneously hydrolysed to salicylate in culture medium. Spontaneous hydrolysis to salicylate contributed 11% of the total hydrolysis (Fig. 2). This was considered in estimating the contribution from hepatocyte esterases. The other esters did not spontaneously hydrolyse to a significant extent during the assay.

The rate of acetylsalicylate hydrolysis did not decrease over 3 days indicating stability of the esterase enzymes in hepatocytes maintained as a monolayer on plastic (Fig. 2). Results here are expressed in terms of cellular protein; there was a good correlation between number of cells per plate and cellular protein. Further metabolism of paracetamol and salicylate was not investigated in these studies as conditions were not optimal for conjugation.

Inhibition

The effects of inhibitors on hepatocyte esterase activity are shown in Table 2 where results are expressed as a percentage of the uninhibited activity. Paraoxon strongly inhibited hydrolysis of all substrates, the least effected was phenetsal for which 17% activity remained. At the highest concentration BNPP was nearly as effective an inhibitor except with acetylsalicylate. Phenetsal and fluazifop butyl hydrolysis were slightly inhibited by physostigmine which had greater effects on acetylsalicylate and benorylate hydrolysis.

Cellular uptake of acetylsalicylate

Acetylsalicylate was used at a concentration of 2 mM rather than 1 mM to ensure detectable levels of salicylate within the cells. Previous studies had shown linear production of salicylate in the medium up to a substrate concentration of 4 mM (results not shown). The total amount of salicylate within the monolayer and in the incubation medium at different times after addition of 2 mM acetylsalicylate are shown in Table 3. When hepatocytes were incubated with acetylsalicylate the amount of salicylate increased with time within the cell and in the medium.

The volume of a rat hepatocyte is 1.15×10^{-5} μL [12] so the total volume of the monolayer of 2.1×10^{6} cells is 24.2 μL. The concentration of salicylate within the hepatocyte was apparently higher than the medium although the difference did not achieve statistical significance at any of the time points studied.

In the presence of paraoxon, which strongly inhibits acetylsalicylate hydrolysis, only a small amount of salicylate was detected in the cells and

Table 2. Effects of inhibitors on hepatocyte esterase activity

Inhibitor	Substrate			
	Acetylsalicylate	Benorylate	Phenetsal	Fluazifop butyl
Paraoxon	<1	6.7 ± 2.0	17.3 ± 1.8	<1
BNPP 0.046 mM	70 ± 4.0	19.0 ± 2.3	47.5 ± 0.3	23 ± 6.1
0.46 mM	50 ± 3.4	12.7 ± 2.6	26.3 ± 2.7	8.0 ± 1.4
Physostigmine	30 ± 6.0	30.6 ± 2.3	89.0 ± 3.6	74.5 ± 3.6

Results are expressed as percentage of control activity (100%) measured in the presence of inhibitor (mean ± SE, N = 3).

Table 3. Distribution of salicylate between medium and hepatocytes, alone and in the presence of paraoxon (0.17 mM) following addition of 2 mM acetylsalicylic acid

	Control		Paraoxon	
Time (min)	Cells (mM)	Medium (mM)	Cells (mM)	Medium (mM)
0	<0.005	<0.005	<0.005	<0.005
60	0.63 ± 0.3	0.38 ± 0.08	0.04 ± 0.03	0.05 ± 0.003
120	0.86 ± 0.3	0.73 ± 0.1	0.15 (N = 2)	0.10 ± 0.006

Salicylate concentrations mean ± SE (N = 3).
Cell concentration (mM) based on cell volume 1.15×10^{-5} μL.

this probably resulted from spontaneous hydrolysis. Production of salicylate in the medium paralleled spontaneous hydrolysis.

In one experiment acetylsalicylate levels were measured in the cells (Table 4). No unchanged acetylsalicylate was detected in the cell at any time except in the presence of paraoxon when the concentration was approximately half the medium. A high acetylsalicylate concentration was detected even in the zero time sample in which medium was removed from the cells as rapidly as possible after addition of substrate and mixing. This indicated a very rapid uptake of acetylsalicylate into the cells which in the presence of paraoxon accumulated and in the controls was metabolized by esterases.

DISCUSSION

The hydrolytic activity of freshly isolated rat hepatocytes varied considerably between substrates. Benorylate was very rapidly hydrolysed to phenetsal and then more slowly to paracetamol and salicylate. This directly paralleled the observation in man that no benorylate or phenetsal were detected in the plasma following an oral dose [13]. Phenetsal and fluazifop butyl were hydrolysed at about one-tenth of the rate of benorylate. For both of these substrates following an oral dose in man little unchanged ester is seen *in vivo* [13, 14]. Acetylsalicylate was less rapidly hydrolysed by rat hepatocyte esterases and this correlates with the *in vivo* situation in man in which acetylsalicylate is detected within the plasma for 90 min following an oral dose [15]. Similarly, paraoxon is detected in the blood following exposure and this correlates with the relatively slow hydrolysis of paraoxon by liver esterases [16]. Therefore the *in vivo* first pass liver hydrolysis of esters in man correlates well with the observations seen in isolated rat hepatocytes. *In vivo* the liver is the major site for hydrolysis although there is also a contribution from plasma cholinesterase, A-esterase and carboxylesterases to the overall fate of the ester [1].

Table 4. Distribution of acetylsalicylate (added at 2 mM) between medium and hepatocytes, alone and in the presence of paraoxon (0.17 mM)

	Control		+Paraoxon	
Time (min)	Cells (mM)	Medium (mM)	Cells (mM)	Medium (mM)
0	<0.004	2.0	0.82	1.9
80	<0.004	1.5	1.14	2.0
120	<0.004	1.45	1.51	2.0

Acetylsalicylate concentrations in single experiment.
Cell concentration (mM) based on cell volume 1.15×10^{-5} μL.

Studies with acetylsalicylate, which was hydrolysed at the slowest rate revealed no build up of substrate in the cell. Passage of acetylsalicylate and salicylate across the hepatocyte membrane may limit the observed rate of hydrolysis.

Strong inhibition of all hydrolytic activity by paraoxon indicated that the enzymes involved were carboxylesterases with serine at the active centre (EC 3.1.1.1, B-esterases). The degree of inhibition of the serine containing microsomal carboxylesterases by BNPP has been used previously as a classification by Brant *et al.* [6] who classified microsomal carboxylesterases both on the basis of BNPP inhibition and on substrates specificity [6]. Results presented here suggest that acetylsalicylate and phenetsal may be hydrolysed by the same esterase identified as acylglycerol lipase (EC 3.1.1.23) by Heymann [7]. Benorylate and fluazifop butyl are hydrolysed at a greater rate and inhibition by BNPP is greater suggesting that a different isoenzyme may be involved.

Physostigmine specifically inhibits cholinesterases and exerts a pharmacological effect as the result of inhibition of acetylcholinesterase. This occurs as the result of specific binding to the serine within the specific configuration of the cholinesterase active site. Physostigmine therefore also inhibits serine containing carboxylesterases but in a more variable manner depending on the isozyme.

Cytosolic esterases involved in xenobiotic hydrolysis have been much less extensively investigated than microsomal esterases and it has been suggested by some authors that they are merely contamination from the microsomal enzymes. However White and Hope [17] identified a specific cytosolic esterase in the guinea pig liver capable of hydrolysing acetylsalicylate with different properties from the microsomal enzyme and Mentlein *et al.* [3] has identified some cytosolic activity with differing electrophoretic properties. Our previous studies

have indicated the importance of the cytosolic component in the hydrolysis of aspirin and benorylate, particularly in human liver [4, 5]. The nature of these cytosolic enzymes involved are being investigated.

Studies in hepatocytes give information which is more readily comparable to the *in vivo* situation and particularly relevant for assessing the importance of hydrolytic enzymes in influencing the effect of xenobiotics.

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