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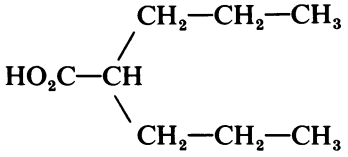
Sodium valproate inhibits the movement of secretory vesicles in rat hepatocytes

Maria E. BELLRINGER, Khalid RAHMAN and Roger COLEMAN*
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

Sodium valproate (VPA), a simple 8-carbon branched chain fatty acid, is an effective anti-epileptic drug with an occasional serious side effect of liver damage, including the accumulation of triacylglycerols within hepatocytes, and reductions in serum protein concentrations. By investigating the effects of VPA, using biliary fistula rats and isolated perfused rat livers, we have shown that secretion of triacylglycerols and rat serum albumin at the sinusoidal pole of hepatocytes, and of phospholipids, lysosomal contents, and IgA at their biliary pole, are all reduced, to somewhat different extents, by acute VPA administration. In addition, the vesicular transcytosis of exogenous protein (i.e. bovine serum albumin) from the perfusion fluid into bile is also decreased by VPA administration. To determine whether the phenomena were specific to VPA, a control series of experiments was also performed using octanoate (a straight-chain analogue of VPA). With the biliary fistula rats, octanoate did not show inhibition of secretion as compared with the saline controls; with the isolated perfused livers, however, octanoate did show such an inhibition. These phenomena suggest that VPA inhibition of secretion may be a factor in its hepatotoxicity, as the effects are apparent in both the whole animal and the isolated perfused liver, whereas octanoate is not hepatotoxic in the whole animal. Since when octanoate is administered to the isolated liver it causes an inhibition in secretion similar to that caused by VPA, it may be that the large dose of this compound reaching the liver affects a key step in liver metabolism or vesicle transport under these circumstances. Since octanoate does not normally reach the liver in such amounts, as it will normally be metabolized by other tissues, it is not hepatotoxic in the whole animal as is VPA.

INTRODUCTION

Valproate (VPA) or valproic acid, a simple eight-carbon branched chain fatty acid, is used as an effective anti-epileptic drug against most forms of epilepsy (Chapman *et al.*, 1982).



In a few cases, however, VPA has caused severe liver failure which is often fatal in children. This toxicity, although not clearly understood, appears most prevalent in those people concurrently taking other drugs (Powell-Jackson *et al.*, 1984), suffering from some types of congenital defect (Brown *et al.*, 1985), and/or those exceeding the maximum recommended daily dosage of 30 mg/kg body weight (Powell-Jackson *et al.*, 1984).

There appear to be two types of VPA toxicity; rapid onset, which is rare and can occur within the first 6 months after starting VPA therapy (this leads to death even if VPA treatment is stopped), and slow onset, in which the toxic effects are reversed if VPA therapy is stopped (Isom, 1984). The relatively frequent occurrence of minor reactions which are reversible on stopping or reducing the dose of VPA, together with the occasional severe cases, suggest the hepatotoxicity is a direct reaction of the drug itself or an immediate metabolite (Turnbull, 1983). The structural hepatotoxic effects of VPA are characterized by hepatic steatosis (Lewis *et al.*, 1982; Kesterson *et al.*, 1984), inflammation, focal necrosis (Suchy *et al.*, 1979; Gram & Bentsen, 1985), altered mitochondria (Mathis *et al.*, 1979; Hayasaka *et al.*, 1986), peroxisome proliferation, dilated rough endoplasmic reticulum, and fine granular substances within bile canaliculi (Partin *et al.*, 1983). The non-structural hepatotoxic effects of VPA are characterized by a decrease in serum albumin (Sherlock, 1983), low plasma fibrinogen concentrations (Dale *et al.*, 1978; Nutt *et al.*, 1978), asymptomatic elevations of serum aspartate aminotransferase, glutamic oxaloacetic transaminase and ammonium ions (Powell-Jackson *et al.*, 1984; Turnbull, 1983).

One of the more important structural observations noticed during VPA administration is the accumulation of lipid-filled vesicles within the hepatocytes (Lewis *et al.*, 1982; Sherlock, 1983). This steatosis is also common in other hepatic fat accumulation diseases, such as acute fatty liver of pregnancy (Bernuau *et al.*, 1983), Reye's syndrome, tetracycline toxicity, Jamaican vomiting disease and congenital defects of urea cycle enzymes (Sherlock, 1983). A fatty liver is also present in most cases of severe alcohol intake (Ryle *et al.*, 1985; Tsukamoto *et al.*, 1985; Schaffner & Thaler, 1986). It may be, therefore, that there is a common underlying factor in this group of diseases.

Abbreviation used: VPA, sodium valproate.

* To whom correspondence and reprint requests should be addressed.

Accumulation of lipid implies not only a possible overproduction but also an inability to secrete at a rate to match the production. Thus some form of inhibition of secretion may be involved in the aetiology of these conditions. The reduced concentration of albumin and fibrinogen in serum (indicated above) may also be explained in these terms since these proteins are synthesized within the hepatocyte and transported to the sinusoidal face; their reduced serum concentration could result from an interference with the transport of secretory vesicles.

In a recent study by Jezequel *et al.* (1984) it has been shown that VPA causes a dose-dependent decrease in phospholipid and cholesterol output into bile. The movement of these lipids towards the biliary pole is probably in the form of vesicles (Barnwell *et al.*, 1984; Lowe *et al.*, 1984) and thus the observation of Jezequel *et al.* (1984) may be an indication of an effect of VPA in reducing vesicle transport.

The present study was conducted to extend these initial investigations and observations to include other components secreted by the liver and hence determine whether the hepatotoxicity involves an inhibition of several secretory pathways. Furthermore, experiments were performed with octanoate (a straight chain analogue of VPA) to ascertain whether the observed effects could be a factor in VPA hepatotoxicity, or whether they were due to a general nonspecific inhibition of the secretory pathways.

MATERIALS AND METHODS

Materials

Antisera to rat IgA, rat serum albumin, rat fibrinogen and bovine serum albumin were purchased from Nordic Immunological Laboratories, Maidenhead, Berks., U.K. Sagatal was obtained from May and Baker, Dagenham, Essex, U.K. Cannulation tubing PP10 was manufactured by Portex, Hythe, Kent, U.K., and heparin was made by Weddel Pharmaceuticals, London EC1, U.K. VPA (sodium valproate) was a kind gift from Labaz Ltd., Wythenshaw, Manchester, U.K. All other fine chemicals were obtained from Sigma.

Bile fistula rats

Male Wistar rats, weighing 250–300 g, were used throughout. These had been maintained on a standard laboratory diet and under a constant light/dark cycle. Whilst under pentobarbitone (Sagatal) anaesthesia the rats were dosed intraperitoneally with VPA or octanoate [both at 500 mg/kg body weight; this amount was chosen as being a high dose similar to that used by other workers (Jezequel *et al.*, 1984)] dissolved in 0.9% sterile saline. Controls received an equal volume of saline only. Bile duct cannulations were performed 45 min after the VPA administration, using 500 mm of PP10 tubing. Animals were kept anaesthetized throughout the experiment. Bile was collected in preweighed tubes on ice for a total of 135 min (final collection was 3 h after VPA dose) in 15 min samples. A sample of blood was obtained from either a tail vein or carotid artery before administration of VPA and at the end of the 3 h. After completion of the experiment the animals were killed. The volume of bile was determined gravimetrically, a density of 1 g/ml being assumed. All bile and serum samples were stored at −20 °C until required for analysis. Aspartate aminotransferase activity was measured in the serum samples to test for liver damage. In no case was the leakage of this enzyme increased by VPA treatment.

Isolated perfused rat livers

The bile ducts of anaesthetized rats were cannulated as before and the livers were then isolated *in situ* and perfused with a recirculating medium according to Rahman & Coleman (1986). VPA or octanoate (500 mg/kg body weight) were administered dissolved in 0.9% sterile saline into the circulating perfusion fluid as soon as the liver was isolated and perfused. Controls received an equal volume of saline only. Bile was collected in 15 min samples throughout the perfusion for 90 min.

The health of the livers was monitored by analysing the extent of leakage into the perfusate of the cytosolic hepatocyte enzyme aspartate aminotransferase. In no case was the leakage of this enzyme increased by any VPA treatment.

Specific determinations

Acid phosphatase (EC 3.1.3.2) was assayed using the method employed by Hubscher & West (1965).

Aspartate aminotransferase (EC 2.6.1.1) was assayed using kits supplied by the Boehringer Corp., based on the method of Bergmeyer *et al.* (1978).

Rat serum albumin, rat fibrinogen, rat secretory IgA and bovine serum albumin in bile or perfusion fluid were determined by quantitative radial immunodiffusion by the method of Mancini *et al.* (1965) with specific antisera. Authentic rat serum albumin, rat fibrinogen and bovine serum albumin were used for standardization; secretory IgA was expressed in arbitrary units relating to the diameter of the precipitation zone.

Phospholipid present in bile was determined by the method of Bartlett (1959) after lipid extraction by the method of Bligh & Dyer (1959).

Bile salt concentrations were determined with hydroxysteroid dehydrogenase (EC 1.1.1.50) as described by Coleman *et al.* (1979).

Triacylglycerols were measured using the Peridochrom test kit from Boehringer Corp.; the assay was based on the method of Wahlefeld (1974).

RESULTS

Studies using the bile fistula rat

Bile collections were started 45 min after intraperitoneal VPA administration and the first observation was that VPA immediately increased bile flow, whereas octanoate showed no significant difference from control values (i.e. treated with saline only). The VPA-induced choleresis was prolonged throughout the time-span of the experiment (Fig. 1).

Acid phosphatase is a lysosomal marker enzyme. The activity in bile was shown to be increased by octanoate for the first 120 min, whereas VPA decreased the activity during the first 75–90 min, after which the levels returned to control values (Fig. 2*a*). A test was devised to show that the decrease in acid phosphatase activity was real rather than an inhibition of the enzyme, e.g. by a VPA metabolite. The activities of bile from a control rat and a VPA-treated rat (with decreased acid phosphatase activity) were measured. The two bile samples then were mixed together and the assay performed on the mixture;

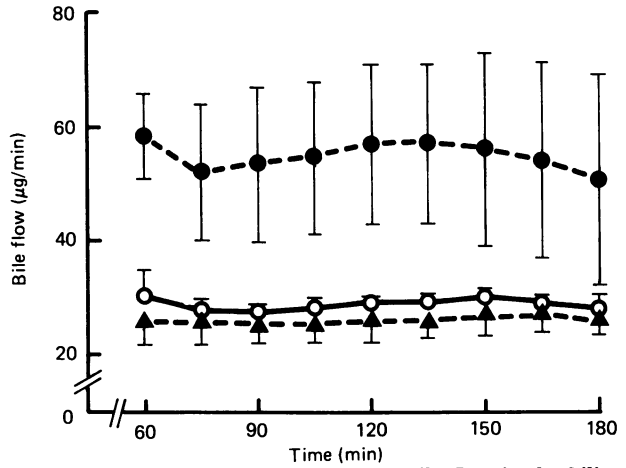


Fig. 1. Effect of VPA and octanoate on bile flow in the biliary fistula rat

For details of animal preparation and assay procedures see the Materials and methods section. VPA or octanoate was injected intraperitoneally 45 min before the collection of bile (at $t = 0$). The values on the graphs are plotted to represent the end of each collection period, and are the means for four experiments (saline control), five experiments (VPA) or six experiments (octanoate), ±S.E.M. Symbols: ○, control rats; ●, rats treated with 500 mg of VPA/kg body weight; ▲, rats treated with 500 mg of octanoate/kg body weight.

the result proved to be the sum of the individual bile activities.

VPA caused a significant decrease in the output of IgA (Fig. 2*b*), rat serum albumin (Fig. 2*c*) and phospholipid (Fig. 2*d*) into the bile, whereas octanoate showed no significant differences from the saline controls.

Total bile salt output into bile was not significantly affected by either VPA or octanoate. This shows that the increased bile flow in VPA treatment was not due to increased bile salt output, but was due to bile-salt-independent bile flow (results not shown).

The relatively large S.E.M. values in these results are probably due to animal variation due to differences in overall metabolic rates. The next stage in experimentation, therefore, was to use the isolated perfused liver in which the metabolism of the whole animal is no longer taken into account, and thus the observation of phenomena then concentrates upon liver-based parameters.

Studies using the isolated perfused liver

VPA or its corresponding control, octanoate, (both at 500 mg/kg) were dissolved in 1 ml of 0.9% sterile saline (controls received saline only), and added to the perfusion fluid as soon as the liver was isolated and being perfused.

Biochemical parameters determined in the bile. VPA initially caused bile flow to double, as with the whole

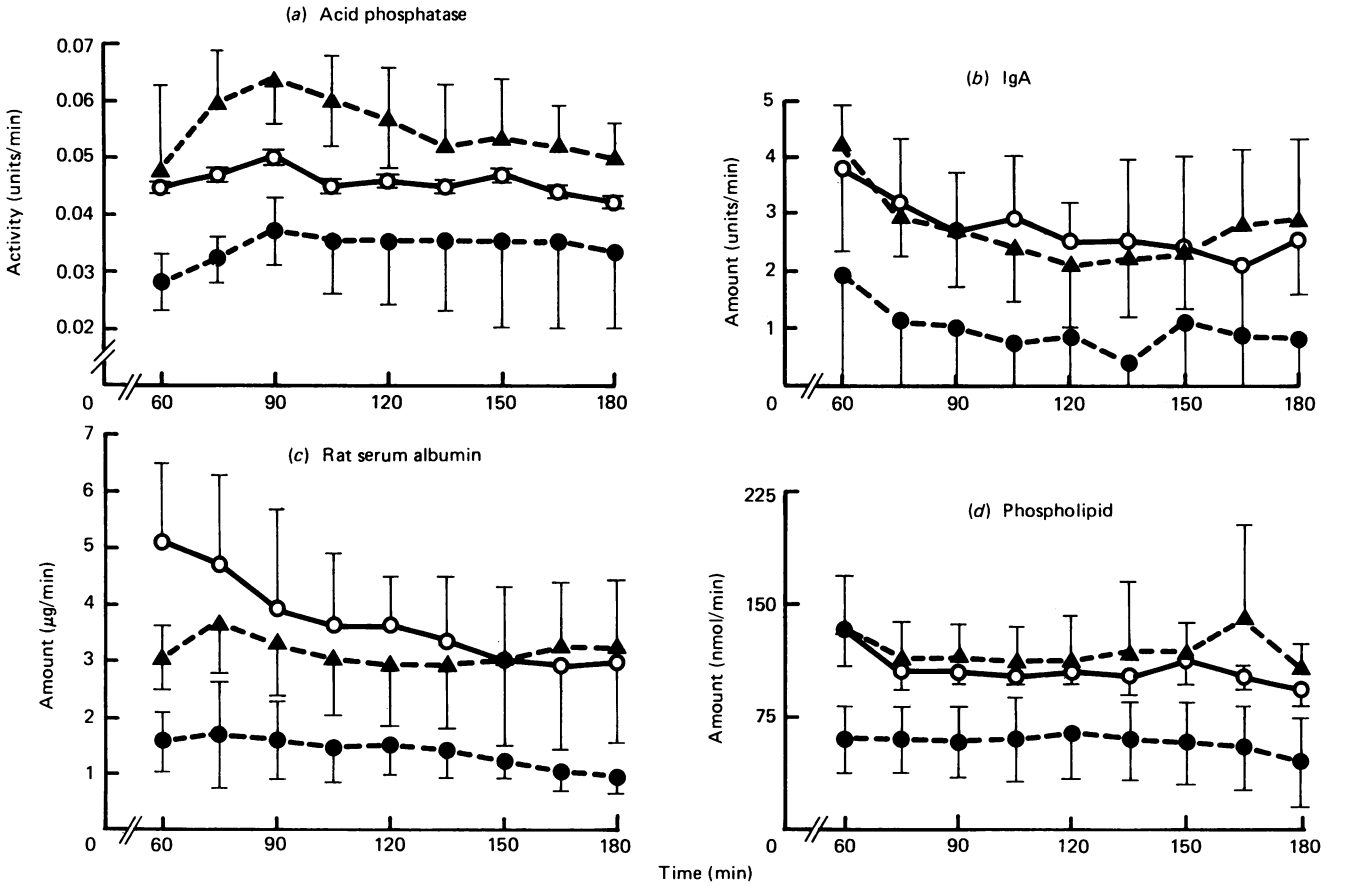


Fig. 2. Effect of VPA and octanoate on the output of acid phosphatase (*a*), IgA (*b*), rat serum albumin (*c*) and phospholipid (*d*) in the biliary fistula rat

For details see the Materials and methods section and the legend to Fig. 1. Acid phosphatase output (*a*) was measured as the activity of the enzyme in units/min (1 unit = 1 μmol of substrate hydrolysed/h at 37 °C). Rat serum albumin output (*c*) was calculated using purified rat serum albumin as a standard. IgA output (*b*) is expressed in arbitrary units.

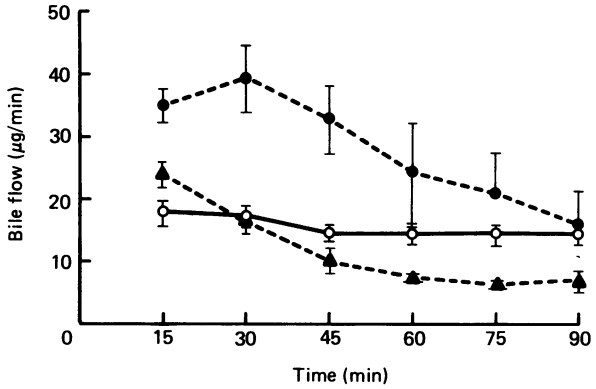


Fig. 3. Effect of VPA and octanoate on bile flow in the isolated perfused rat liver

For details of liver perfusion and assay procedures see the Materials and methods section. VPA or octanoate was administered at $t = 0$ into the recirculating perfusion fluid. The values on the graphs are plotted to represent the end of each collection period, and are the means for three experiments (control), or for four experiments (VPA and octanoate) ±S.E.M. Symbols: ○, controls; ●, VPA (500 mg/kg body weight); ▲, octanoate (500 mg/kg body weight).

animal experiments, but this flow started to return to normal amounts towards the end of the 90 min period. Octanoate caused only a very slight increase in bile flow which rapidly became a cholestasis (Fig. 3).

Both the VPA- and the octanoate-treated livers showed a significant decrease in acid phosphatase secretion rate compared with controls after about 30 min (Fig. 4*a*). This decrease was prolonged throughout the 90 min and was slightly more prominent with octanoate treatment.

Several proteins, e.g. rat serum albumin and bovine serum albumin, can enter bile from the perfusion fluid via two methods: (i) by transcytosis across the cell, and (ii) paracellularly across the tight junctions (Lowe *et al.*, 1985). As soon as the liver is isolated and perfused the supply of rat serum albumin from the blood is thus removed; hence its output into bile decreased as the supply diminished. This can be seen in the control rats. VPA treatment caused a slight reduction in this output into bile but the difference was not statistically significant. There was no difference in rate of output with octanoate treatment (Fig. 4*b*).

VPA appeared to prevent the movement of bovine serum albumin from the perfusion fluid into bile for a period of 75 min, whereas the decrease caused by octanoate was less drastic (Fig. 4*c*). To ensure that the VPA-induced decrease was real rather than an inhibition of the antibody–antigen reaction (by VPA or one of its metabolites), a standard bovine serum albumin solution was mixed with an equal volume of a bile sample showing an apparent lack of bovine serum albumin. This mixture gave precipitation rings the same size as the standard, whereas there was no precipitation with the bile sample alone. Hence, the lack of bovine serum albumin in the bile is due to a true absence rather than an interference in the assay of the proteins ($n = 2$).

Bile salt output into bile from both VPA- and octanoate-treated livers was not significantly different from control values; it was low in all cases and decreased

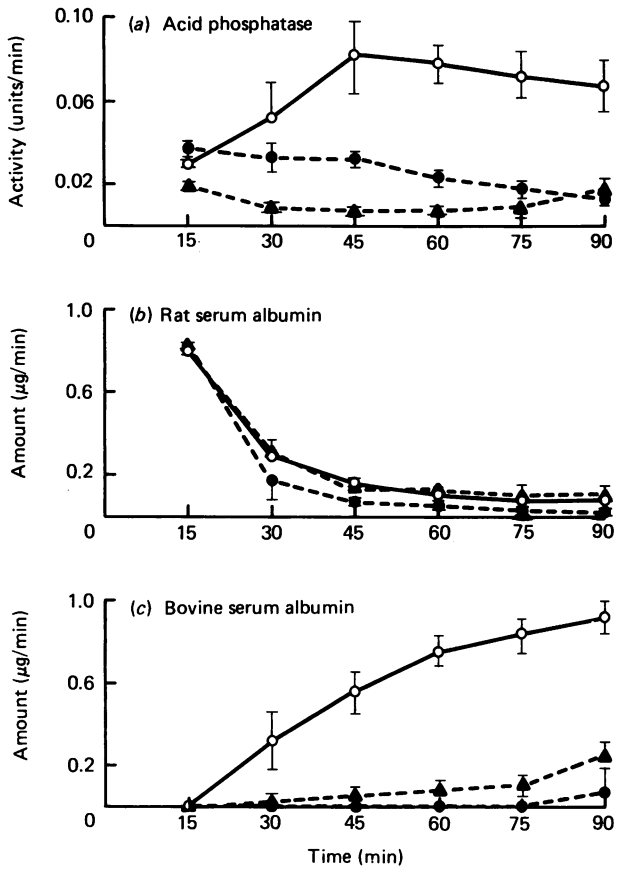


Fig. 4. Effect of VPA and octanoate on the output of acid phosphatase (*a*), rat serum albumin (*b*) and bovine serum albumin (*c*) in the isolated perfused liver

For details see the Materials and methods section and the legend to Fig. 3. Acid phosphatase and rat serum albumin outputs are calculated as described in the legend to Fig. 2. Bovine serum albumin output (*c*) was calculated using purified bovine serum albumin used as a standard.

with time due to its absence from the perfusion fluid supplying the liver (results not shown).

Biochemical parameters determined in the perfusion fluid. Rat serum albumin and triacylglycerols are synthesized within the hepatocyte and transported via the sinusoidal face into the blood stream; hence, perfusion fluid was assayed for these parameters. Both VPA and octanoate significantly decreased rat serum albumin output into perfusion fluid by approx. 30% (Fig. 5*a*). Octanoate almost completely inhibited triacylglycerol output and VPA decreased it by about 40% (Fig. 5*b*).

DISCUSSION

Effect on bile flow

Watkins & Klaassen (1982) have shown that the choleretic effect of VPA was due to the osmotic activity of VPA conjugates in the bile, mainly glucuronides of VPA metabolites. In the bile fistula rat (see Fig. 1) the administered VPA circulates in the whole animal, with the possibility of it being taken up (and metabolized) by

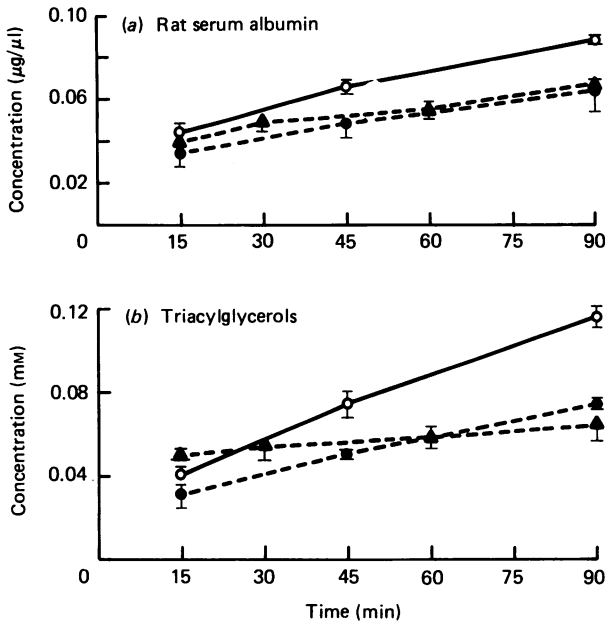


Fig. 5. Effect of VPA and octanoate on the output of rat serum albumin (*a*) and triacylglycerols (*b*) into perfusion fluid

For details see the Materials and methods section and the legend to Fig. 3. Rat serum albumin and triacylglycerol output are given as concentrations, since the volume of perfusion fluid remained constant throughout the experiment.

other tissues. It may, therefore, take some time to reach the liver and this could account for the prolonged effect. With the isolated perfused liver, however, the VPA (500 mg/kg) is only exposed to the liver, leading to quicker excretion of the drug and its metabolites, and may explain why the bile flow reaches its peak within 30 min and then is reduced, whereas in the whole animal the increased flow is maintained for at least 3 h. Octanoate, the straight chain analogue of VPA, has an opposite effect on bile flow, causing a cholestasis instead of a choleresis. This is probably because of the high dose of the fatty acid being administered to the liver. Octanoate will probably be metabolized by hepatocytes in the same way that any straight chain fatty acid would be; there will be no glucuronides of metabolites to be excreted into the bile and hence, no enforced choleresis. In the whole animal, however, octanoate has no effect on bile flow; this may be due to much of the fatty acid being metabolized by other tissues before it reaches the liver.

Effect of VPA on secretory parameters

Rat serum albumin, bovine serum albumin (Kloppel *et al.*, 1986), IgA (Mullock & Hinton, 1981), phospholipids (Barnwell *et al.*, 1984; Lowe *et al.*, 1984) and triacylglycerols, as very low density lipoprotein (Orci *et al.*, 1973; Le Marchand *et al.*, 1973), all are packaged and secreted via secretory vesicles; these are brought to the appropriate pole of the hepatocyte via microtubules. Lysosomal contents are secreted into bile probably by movement of the lysosomes also via the microtubular system (La Russo, 1984; Reuben, 1984; Mullock *et al.*, 1985; Kloppel *et al.*, 1986); this lysosomal secretion (excretion) is measured here by appearance in bile of the marker enzyme acid phosphatase. Several of these biliary secretory parameters appear to be immediately decreased by VPA administration in both the whole animal and in the isolated perfused liver (Figs. 2, 4 and 5). Experiments were also performed in biliary fistula rats using a lower dose of 200 mg of VPA/kg (results not shown). These results indicated that this dose, too, caused widespread inhibition of secretion though mainly to a lesser extent than with the higher dose of VPA. As with bile flow it is noticeable that octanoate has no significant effect on the output of biliary substances in the whole animal (with the minor exception of acid phosphatase output which is increased rather than being inhibited).

Since the secretion of rat serum albumin and triacylglycerols into the perfusate is also decreased, it can be seen that VPA is affecting vesicle transport to both poles of the hepatocyte, i.e. it is not specific to particular parts of the cell.

It is highly improbable that so many different synthetic metabolic pathways (e.g. for proteins and for lipids) could be blocked by VPA and it is unlikely, therefore, that this reduction would be due to an inhibition of their syntheses. Thus it is proposed that the effects of VPA may rather be via a blockage in the transport of these substances. In contrast, bile salt output is not reduced; this is in keeping with its transport across the hepatocyte as discrete molecules rather than in vesicles (Barnwell *et al.*, 1984).

The simultaneous decrease in secretion of these proteins and lipids, which are all secreted by vesicle-mediated processes, therefore suggests that: (i) vesicles, (ii) their interaction with microtubules, or (iii) the microtubules themselves, are in some way affected. This would have the effect of preventing, or slowing down, the vesicles reaching the bile canaliculus (e.g. IgA) or the sinusoid (e.g. rat serum albumin), and the subsequent secretion of their contents. In addition, the decrease in albumin output could also be due, in part, to its binding of VPA in serum or perfusion fluid prior to a receptor-mediated uptake into hepatocytes (Weisiger *et al.*, 1981).

In other studies it has also been shown that colchicine disrupts microtubular systems and reduces the secretion of proteins at the sinusoidal face of the hepatocyte, resulting in an accumulation of secretory vesicles in the cytoplasm (Orci *et al.*, 1973; Le Marchand *et al.*, 1973). Under these conditions some of the vesicles discharge their contents into the bile canaliculus (Barnwell & Coleman, 1983). This is probably a case of misdirected vesicles ('blundersomes'), having no clear-cut pathway to follow, fusing with the bile canalicular membrane due to its close proximity to the Golgi apparatus, and is exemplified by the appearance of fibrinogen in the bile. VPA-treated livers, however, did not show any of this misdirection of vesicles; no fibrinogen, or increase in rat serum albumin, could be detected in bile and hence it may be that the site of action of VPA (or its metabolites) on the microtubular system is different from that of colchicine in that it may act, not by disrupting the microtubules, but rather by interfering with the movement of vesicles along them.

Relationship to VPA toxicity

Since octanoate has an effect in the isolated perfused liver but not in the whole animal, this may suggest that it is normally metabolized by other tissues as well as the liver. Thus, the inhibition of secretion obtained in the

isolated perfused liver may just be due to the large, abnormal concentration of the fatty acid affecting a key step in liver function. VPA, however, not only inhibits secretion in isolated perfused livers but also in whole animals and this may be why it is a hepatotoxic agent whereas octanoate is not.

One of the first noticeable morphological changes in VPA-treated livers has been the accumulation of cytoplasmic fat (Lewis *et al.*, 1982; Kesterson *et al.*, 1984; Powell-Jackson *et al.*, 1984; Gram & Bentsen, 1985; Olson *et al.*, 1986). This may be due not only to the accumulation of esterified VPA or its metabolites but also to lipids which are unable to be secreted due to the interference with vesicular movement which may then lead to fat accumulation. This hepatic steatosis suggests that VPA toxicity may be one of a group of hepatic fat accumulation diseases (see the Introduction). Since an accumulation of triacylglycerols within hepatocytes is a common feature of these diseases, it may be that in all cases there is an interference with vesicular movement, as is observed in the phenomena reported in the present paper. This can therefore explain other side effects of VPA, such as decreased blood fibrinogen concentrations (Dale *et al.*, 1978; Nutt *et al.*, 1978), which leads to clotting problems, and decreased plasma albumin levels, which leads to oedema (Sherlock, 1983).

It is possible that mitochondrial injury could also depress oxidation of fatty acids, leading to a build-up in the cytoplasm of the hepatocyte. This would increase lipid accumulation in the cells. Other studies have shown that VPA interferes with the mitochondrial inner membrane, as seen by disintegrating cristae (Rumbach *et al.*, 1983, 1986; Hayasaka *et al.*, 1986). The loss of this membrane integrity may have many consequences, such as the release of calcium from internal stores; such calcium could be a contributor to the secretory problems caused by VPA either by directly affecting the movement of vesicles along microtubules or indirectly via a second messenger system.

Relationship between VPA and octanoate in the whole animal and in the isolated perfused liver

The observations show that VPA inhibits the secretion of a wide range of substances from both poles of the hepatocyte in both whole animals and isolated perfused livers. However, this is not true of its straight chain analogue octanoate, which although inhibiting secretion in the isolated perfused liver has no effect in the whole animal. The lack of an effect with octanoate in whole animals may be due to its being metabolized or taken up by other tissues before it reaches the liver. If, however, a single large dose of octanoate is given straight to the liver it has a similar effect to an equivalent dose of VPA. The mechanism of the secretory inhibition is not known, but could even be similar in the case of both compounds; possibly they or their metabolites are affecting a key step in the secretory functioning of the hepatocyte. Thus, whereas octanoate is harmless under normal circumstances, VPA inhibits hepatic secretion and this may well be why VPA causes occasional hepatotoxicity whereas octanoate does not.

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