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1-Naphthylisothiocyanate-induced permeability of hepatic tight junctions to proteins

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We have studied the early action of 1-naphthylisothiocyanate (ANIT) in relation to its effect on the permeability barrier formed by hepatic tight junctions. Materials having different M_r values [inulin (5000), horseradish peroxidase (HRP) (40000), ovalbumin (also 40000) and pig γ-globulin (IgG) (160000)] were individually pulsed, within 1 min, into perfused rat livers operating under single-pass conditions. In untreated rats, a small peak of HRP and ovalbumin and a comparatively larger peak of inulin were observed in the bile at 7 min. In rats treated with ANIT, with increasing duration of ANIT treatment the inulin peak increased proportionally, whereas the HRP and ovalbumin peaks remained unchanged until after 10 h of ANIT exposure; γ-globulin was not detected in the 7 min bile sample until after 14 h of ANIT treatment. Bile flow in all rats remained approximately the same until after 14 h of ANIT pretreatment, when substantial bile-flow reduction was observed. Phenobarbitone pretreatment increased the effect of ANIT and massively elevated the first HRP peak; it also shortened the time (to 4 h) at which the increase in permeability to this protein was observed. In contrast, the first HRP peak was virtually abolished in rats that had received the mixed-function-oxidase inhibitor SKF 525A. These experiments suggest that (i) ANIT progressively increased the permeability of the junctional barrier before the reduction in bile flow, (ii) the ANIT-increased permeability change seems to be inversely dependent upon the M_r of the infused proteins, and (iii) metabolites of ANIT were involved in the development of the junctional permeability change.

INTRODUCTION

Many different types of proteins have been reported in rat bile (Evans *et al.*, 1976; Kakis & Yousef, 1978; Mullock *et al.*, 1978), and most of these proteins (except the secretory proteins) are derived from the serum (Dive & Heremans, 1974; Dive *et al.*, 1974). These proteins are transferred to bile both by transport in vesicles across hepatocytes and by sieving through tight junctions (Dive & Heremans, 1974; Dive *et al.*, 1974; Thomas, 1980; Thomas *et al.*, 1982). In rats, under normal conditions, vesicle transport is probably the most important mechanism for transport of most of the proteins into bile (Mullock *et al.*, 1978; Hinton *et al.*, 1980; Mullock & Hinton, 1981; Hinton *et al.*, 1984) and includes the receptor-mediated transcytosis of polymeric IgA (Mullock & Hinton, 1981; Orlans *et al.*, 1983). Tight junctions are thought to play an important role in separating and governing the passage of materials between plasma and bile (Bradley & Herz, 1978; Boyer *et al.*, 1979; Jaeschke *et al.*, 1983; Krell *et al.*, 1984; Tavaloni, 1984), but the importance of this paracellular pathway, however, varies from species to species (Hall *et al.*, 1980; John *et al.*, 1983; Orlans *et al.*, 1983; Mullock *et al.*, 1985), and in man this sieving through the tight junctions may be the major route of protein penetration into bile (Mullock *et al.*, 1985).

The paracellular permeability to proteins in rat liver, and its modification, have recently been studied (Lowe *et al.*, 1985). In control rats, infusions containing HRP and inulin were pulsed into isolated rat liver for 1 min under single-pass conditions. Two peaks of appearance of HRP were observed; the first peak occurred at about 7 min and the second peak at 20–25 min. The second peak has been shown to be due to vesicle transport (Lowe *et al.*, 1985) and could be reduced by colchicine, whereas the first peak is relatively insensitive to colchicine. Inulin, which is believed to enter into bile by sieving through tight junctions (Jaeschke *et al.*, 1983), also appeared maximally in bile within the first-peak time scale, and no second peak was observed. The simultaneous first peak of HRP (7 min) appearance, therefore, is probably due to junctional permeability.

In rats pretreated with ANIT, the height of the first peak was elevated 6-fold (Lowe *et al.*, 1985), probably due to change of junctional permeability. ANIT is a compound very effective in causing junctional leakage in rat livers after a single dose (Krell *et al.*, 1982; Jaeschke *et al.*, 1983); these ANIT-treated rats also showed cholestasis after acute dosing, and biliary cirrhosis, as well as cholangitis, after chronic ANIT treatment (McLean & Rees, 1958).

In the present study, rats were pretreated with ANIT for different times, after which the liver was isolated and perfused. Materials of differing M_r were then individually infused into the liver. The extent of ANIT-induced permeability change after the pretreatment periods was studied in relation to the amount of infused materials leaked into bile through the bile/perfusate barrier formed by the hepatic tight junctions. This work centres on the earlier events of junctional-permeability change inflicted by the compound rather than later events concerned with bile-flow reduction and cholangitis. In addition, phenobarbitone (a mixed-function-oxidase

Abbreviations used: ANIT, 1-naphthylisothiocyanate (1-isothiocyanatonaphthalene); HRP, horseradish peroxidase; BSA, bovine serum albumin; PB, phenobarbitone.

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inducer) and SKF 525A (an inhibitor of the mixed-function-oxidase activity) were also used to investigate the effect of changes in the extent of ANIT metabolism upon the permeability changes of tight junctions.

MATERIALS AND METHODS

Materials

Antisera to chicken ovalbumin, pig γ-globulin (IgG) and BSA were purchased from Nordic Immunological Laboratories Ltd., Maidenhead, Berks., U.K. ANIT and HRP were obtained from Sigma Chemical Co., Poole, Dorset, U.K. [^{3}H]Methoxyinulin and [^{14}C]carboxyinulin were obtained from New England Nuclear Corp., Boston, MA, U.S.A. SKF 525A was obtained as a gift from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K. Aspartate aminotransferase assay kits were supplied by Boehringer Corp., Lewes, Sussex, U.K. The plastic cannula tubing (PP10) was purchased from Portex, Hythe, Kent, U.K.

Administration of ANIT

Male Wistar rats weighing between 250 and 300 g were used; they were allowed free access to food and water throughout the study. ANIT was dissolved in olive oil at a concentration of 0.2 g/ml by using an ultrasonic generator. Each rat received a dose of 25 mg/100 g body wt. intragastrically (i.g.) while under light diethyl ether anaesthesia; the same volume of vehicle was administered to control rats.

Liver perfusion

At 0, 2, 4, 6, 8, 10, 12, 14, 15 h after ANIT administration to individual rats, they were anaesthetized with pentobarbital (Sagatal) and the bile duct was then cannulated with PP10 tubing. The liver was then isolated *in situ* as described by Hems *et al.* (1966) and perfused with 150 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 5 mM-glucose, 2 mM-$CaCl_2$, 1% (w/v) BSA, and an amino acid mixture (Barnwell & Coleman, 1983) at a rate of 16–17 ml·min^{-1}. The perfusion medium was continuously gassed with O_2/CO_2 (19:1) and the temperature was maintained at 37 °C within a thermostatically controlled cabinet.

After an initial 60 min of continuous perfusion, the liver was converted to a one-pass perfusion using fresh medium. At the beginning of this 5 min period, 25 mg of HRP and 50 μCi of [^{3}H]inulin (in 1 ml of Krebs–Ringer bicarbonate solution) were co-infused into the perfusion line 15 cm away from the portal-vein cannula. This ensures thorough mixing with the perfusion medium before reaching the liver. Any excess materials not taken up by the liver were washed away during the subsequent 4 min one-pass perfusion. After 5 min one-pass perfusion the liver was converted back to a recirculating perfusion. Five 2 min and ten 5 min bile samples were collected on ice during and after the one-pass infusion respectively. A small blood sample was withdrawn from the rat before liver perfusion and from the perfusate at the end of the experiments. At the end of the experiment the liver was removed, weighed, and homogenized in 50 vol. of Krebs–Ringer bicarbonate solution and kept at −20 °C until required.

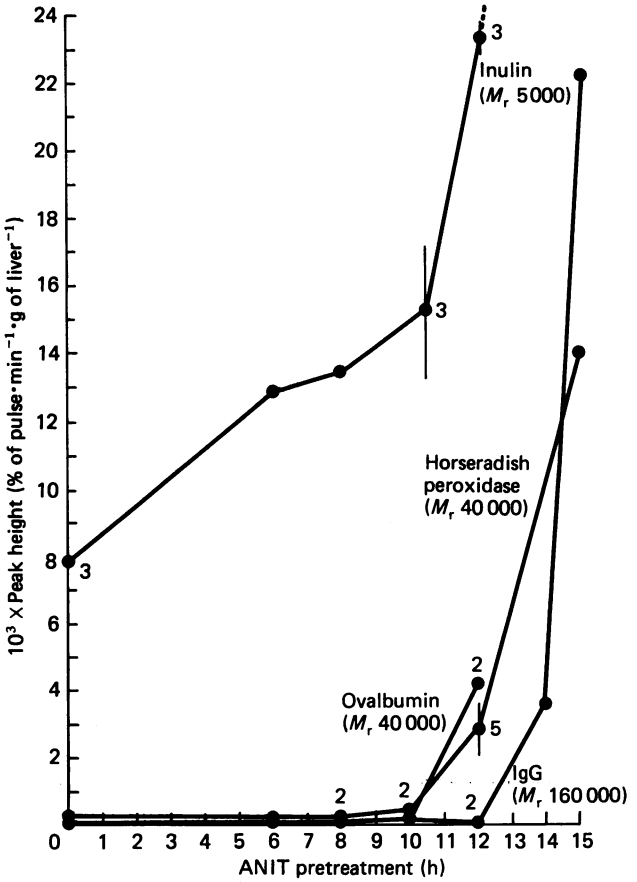

Fig. 1. Biliary output of pulsed molecules of different M_r in relation to extent of ANIT pretreatment

Each point represents the height of the first (7 min) peak of the pulsed material expressed as the percentage of the pulse/min per g of liver. The abcissa represents the duration of ANIT pretreatment before the start of liver perfusion. The number by the side of each point represents the number of observations. The bars represent mean ± S.E.M..

Infusion of ovalbumin and IgG

Before infusing either ovalbumin or IgG solution (170 mg in 1 ml of Krebs–Ringer bicarbonate solution), the liver was converted to a one-pass, BSA-free perfusion medium. This is necessary because these infused protein solutions are comparatively viscous and the BSA-free medium helps disperse the protein more rapidly and evenly before reaching the liver. This procedure can also avoid presenting the liver with the whole bolus of protein, which could cause mechanical damage and obstruction to the liver. After the 1 min infusion, BSA was resupplied to the liver by changing to fresh perfusion medium containing 1% BSA, and the rest of the procedures described above were then followed.

Infusion of differently charged inulins

Non-charged [^{3}H]methoxyinulin (sp. radioactivity 466.4 mCi/g) and negatively charged [^{14}C]carboxyinulin (sp. radioactivity 2.4 mCi/g) were available. [^{3}H]Methoxyinulin was diluted with neutral non-radioactive inulin to obtain the same specific radioactivity as [^{14}C]carboxyinulin. Portions (50 μCi) of each species

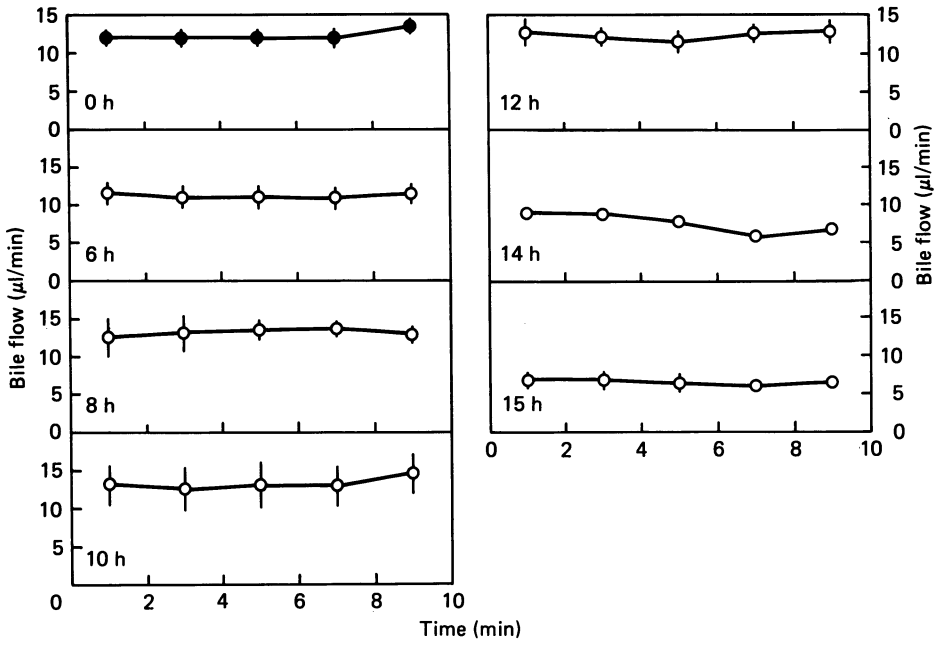

Fig. 2. Bile flow related to length of ANIT pretreatment

Rats were pretreated with ANIT for the length of time indicated to the left of each graph. Each graph represents the mean ±S.E.M. (or range) for all experiments at each ANIT-pretreatment time point (see Fig. 1). The appropriate M_r material was infused at zero time and bile collections were made at 2 min intervals subsequent to this. Numbers of observations: 0 h, 5; 6 h, 3; 8 h, 3; 10 h, 3; 12 h, 7; 14 h, 1; 15 h, 2.

were mixed together and diluted to 1 ml with saline for infusion.

Phenobarbitone (PB) and SKF 525A pretreatment

PB dissolved in saline (20 mg/ml) was given intraperitoneally to rats daily for 3 days at a dose of 60 mg/kg body wt. At 24 h after the last PB injection, ANIT was administered and the livers were isolated at various times after the treatment and perfused as described above.

SKF 525A, in saline, was given to rats intraperitoneally (i.p.) at a dose of 40 mg/kg body wt. The concentration was adjusted so that approx. 1 ml of the drug solution/100 g body wt. was delivered. At 45 min after the injection, ANIT was administered to the rats, 12 h after which the liver was perfused as described above.

Detection of specific proteins in bile

HRP in bile was assayed spectrophotometrically as described by Steinman & Cohn (1972); one unit of enzyme activity was taken to be equivalent to a change of 1*A* unit/min. Ovalbumin and IgG in bile were quantified by radial immunodiffusion (Mancini *et al.*, 1965) with appropriate specific antisera. Suitable standard protein solutions were run on the same plate alongside the samples.

RESULTS

Paracellular permeability to materials of differing M_r with increasing ANIT pretreatment time

The effect of ANIT pretreatment upon the biliary output of a number of proteins of differing M_r is depicted in Fig. 1. Rats were exposed to ANIT for the time shown on the abscissa. Each point on the graph represents the height of the first peak (7 min) of the infused material appearing in bile; for ease of comparison, the results are expressed as the percentage of pulse/min per g of fresh liver. Inulin (M_r 5000) increases proportionally over the initial 10 h ANIT pretreatment period; the increase is most marked from 10 to 12 h. The levels of HRP and ovalbumin (both M_r 40000) remain relatively unchanged until 10 h, after which they greatly increase. IgG (M_r 160000) was not detected in bile until 14 and 15 h. Bile flow for all groups of animals remained approximately the same within the 12 h ANIT exposure time. From 12 h onwards, the fall of bile flow becomes more pronounced, and bile volume is reduced to half in the 15 h experiment (Fig. 2).

The entry rates into bile from the perfusate of negatively charged carboxyinulin and neutral methoxyinulin of the same M_r in animals pretreated for 12 h with either olive oil (control) or with ANIT are compared in Fig. 3. In the control rats, lower percentage recovery of [^{14}C]carboxyinulin than of [^{3}H]methoxyinulin was detected in the same bile samples. The peak of the charged compound is about 0.3 times that of the neutral one. In the ANIT-treated rats, the appearance profile in bile of the two species is relatively similar, and the peak height of the charged compound is about 0.8 times that of the neutral one. There was no prominent second peak (25 min) observed in any of these experiments.

In general, the charge on the molecules may affect their rates of diffusion, since tight junctions appear to restrict passive anionic movement (Bradley & Herz, 1978). This is in keeping with the expectation that diffusion would favour positively charged or neutral molecules rather than negatively charged molecules. From the results, tight junctions after ANIT treatment appear to have lost some of their discrimination between the negatively

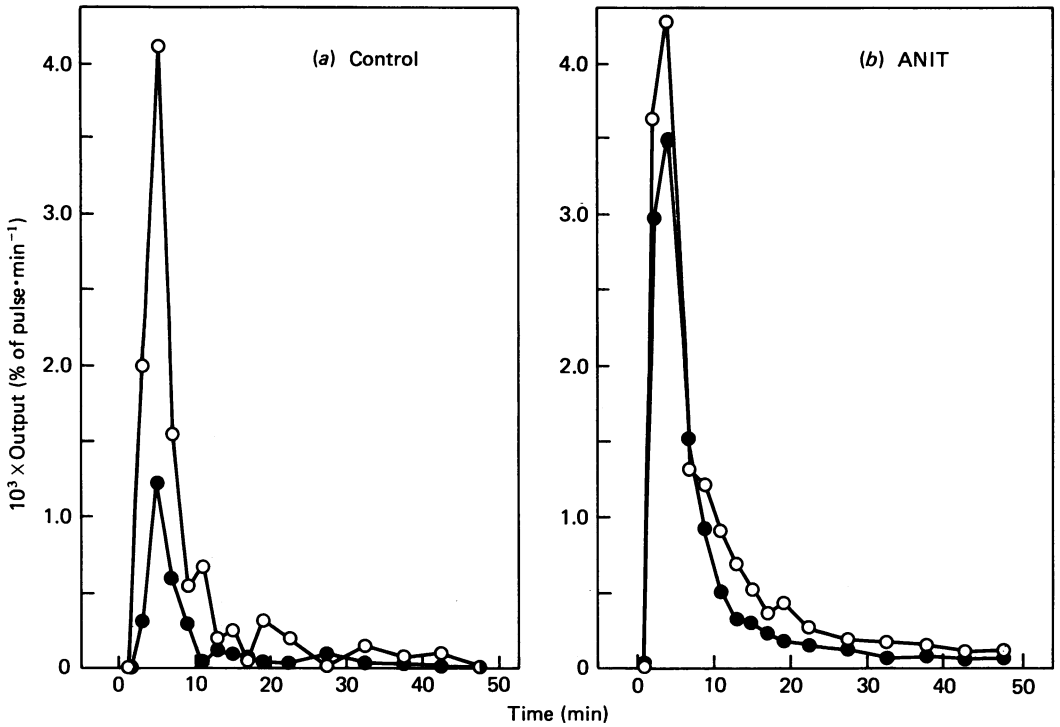

Fig. 3. Biliary output of pulsed inulin (M_r 5000) molecules of different charge

Rats were pretreated for 12 h with either olive oil (control, *a*) or with ANIT (*b*) before liver perfusion. Perfusion was carried out for 1 h before the 1 min co-infusion of neutral [^{3}H]methoxyinulin (○) and negatively charged [^{14}C]carboxyinulin (●). For details, see the Materials and methods section. The results are expressed in terms of the percentage of the infused pulse/min at each point appearing in bile ($n = 1$).

charged and the neutral inulin molecules compared with that observed in the control (0.8 versus 0.3). There is a similar low degree of discrimination in ANIT-treated animals between HRP and the more negatively charged ovalbumin (both of M_r 40000).

Requirement of ANIT metabolism for the increase in paracellular permeability

Rats were pretreated with PB, an inducer of mixed-function oxidase, daily for 3 days and ANIT was then administered for the time indicated in Fig. 4. The results were expressed as units of HRP activity/min and represent the height of the first peak of HRP appearance in bile. Two types of control were performed; one group of controls received PB and olive oil for 8 h and the other received only ANIT for 8 h. The HRP levels in bile for the two controls were too low to appear separately on the graph of the same scale used, and the levels of the two controls were approximately similar. In the PB-pretreated rats, however, 8 h ANIT exposure time massively increased the amount of the HRP peak in bile, to about 350-fold that of either of the controls. After 4 h of ANIT treatment, the increase was also extensive, about 50-fold higher than the controls, and in 2 h-ANIT-pretreated rats a small increase was also observed, but is not significantly different in relation to the number of animals studied.

Other groups of rats were pretreated with SKF 525A, an inhibitor of mixed-function-oxidase activity, 45 min before ANIT administration (indicated on the graph by square symbols; Fig. 5). In this Figure, ● represents the control that had received SKF 525A and olive oil for 12 h. The ○ symbol represents the 12 h ANIT pretreatment only. The HRP peak for the 12 h ANIT treatment was virtually abolished by the effect of SKF 525A, but it is noteworthy that the second peak of both types of ANIT-treated animals remains high compared with the control (●).

Extent of cell damage during the various treatments

The health of the liver in all rats was monitored by measuring the perfusate and serum level of cytosolic enzyme aspartate aminotransferase (see Table 1). In no case was there a large increase after the treatment.

DISCUSSION

Results from the control rats (Fig. 1, 0h ANIT) indicate that tight junctions are relatively more permeable to small molecules (M_r 5000) than to larger molecules such as HRP or ovalbumin (M_r 40000), since a greater proportion of infused inulin was detected in bile. The tight junctions totally excluded IgG molecules (M_r 160000) from penetrating into the bile.

Krell *et al.* (1982) have reported that the ability to concentrate bile acids and the cholephilic dye sulphobromophthalein was gradually lost between 4 and 7 h after ANIT administration. These findings agree with the present observations, namely that the biliary inulin level increases steadily over the early 8 h ANIT pretreatment period, and indicate that the permeability of the bile/plasma barrier has started to increase within this early 8 h ANIT pretreatment period and appears to allow smaller molecules such as inulin to sieve through

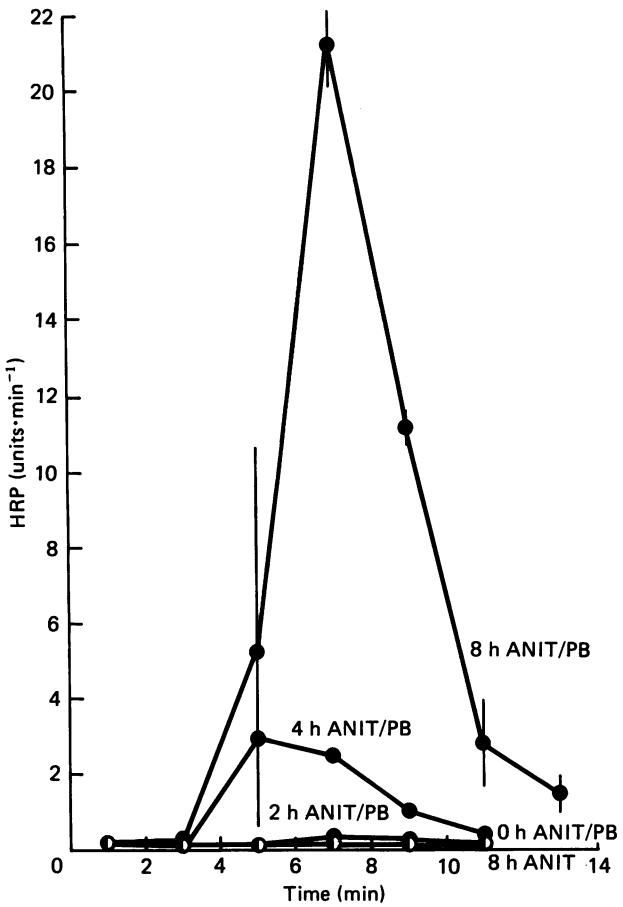

Fig. 4. Biliary output of pulsed HRP in rats treated with ANIT subsequent to phenobarbitone induction

Rats were treated with phenobarbitone for 3 days (see the Materials and methods section). ●, Rats pretreated with PB. ○, No PB treatment. They were then treated with ANIT for the time indicated. All values are expressed as the amount of HRP in bile/min subsequent to the 1 min HRP pulse. Results are means ± S.E.M. ($n = 3$) or variability ($n = 2$) (bars).

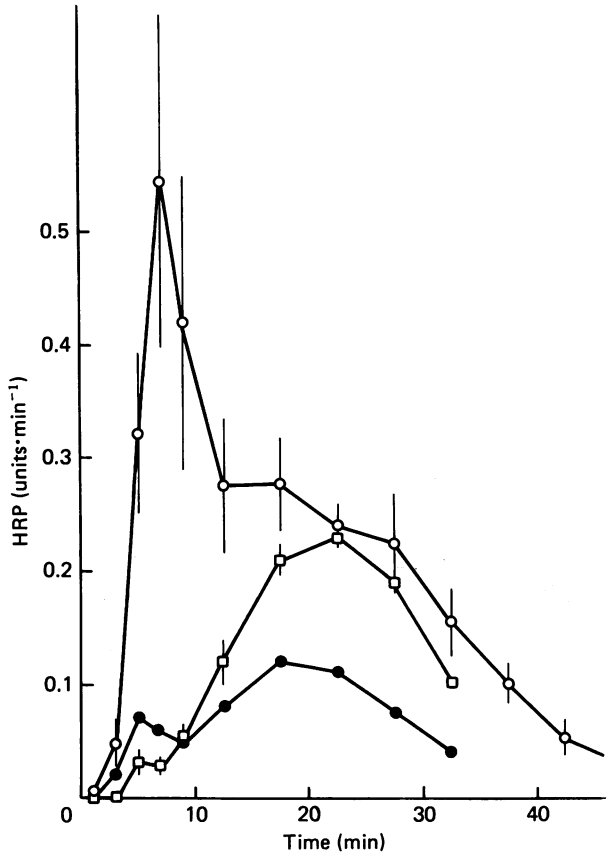

Fig. 5. Biliary output of pulsed HRP in rats treated with ANIT and SKF 525A

All curves show HRP output subsequent to the 1 min HRP pulse. SKF 525A was given 45 min before 12 h ANIT administration (see the Materials and methods section). Symbols: ●, SKF 525A/olive oil ($n = 1$); □, SKF 525A/ANIT ($n = 3$); ○, ANIT alone ($n = 5$). Results are means ± S.E.M. (bars).

more easily than larger molecules. Between 10 and 12 h the permeability to molecules of size about 40 kDa started to increase, but the tight junctions still retain the ability to exclude molecules of molecular size 160 kDa (IgG); increased permeability to this molecule did not develop until a later stage (14 and 15 h).

In the infusion experiments utilizing proteins of differing M_r, 12 h ANIT pretreatment seems to be the critical time when the tight junctions become strikingly leaky. This coincides with the time when an ANIT-induced hyperbilirubinaemia develops in intact animals has been reported (Indacochea-Redmond & Plaa, 1971; Drew & Priestly, 1976). The cause of this hyperbilirubinaemia may, in part, be due to an ANIT-stimulated bilirubin synthesis (Roberts & Plaa, 1966), but, more importantly, may involve the progressive breakdown of the junctional barrier between blood and bile, since the elevated serum bilirubin, the form secreted into bile by the liver, is largely conjugated (Plaa, 1969). Hence the development of hyperbilirubinaemia and subsequent intrahepatic cholestasis probably relate to the inability of the tight junctions to prevent the leakage of materials from bile to serum or vice versa.

Table 1. Plasma aspartate aminotransferase levels

Blood was taken into heparin before commencement of liver perfusion, and plasma was prepared by centrifugation. Animals had been pretreated with the appropriate regimen before the removal of the sample. Values represent the amount in the plasma (volume) estimated as compared with the total amount in the liver obtained after liver homogenization at the end of the experiment. Values represent means ± S.E.M. where $n = 3$ (or range, where $n = 2$).

Treatment	Liver activity (% of total)
Olive oil	0.062 ± 0.010 (2)
ANIT (12 h)	0.120 ± 0.012 (3)
PB (3 days) + ANIT (8 h)	0.058 ± 0.013 (3)
SKF 525A + ANIT (12 h)	0.143 ± 0.010 (2)

Tight junctions can be envisaged as a molecular sieve excluding molecules according to their sizes. ANIT seems to stretch the sieve and make the 'perforations'

progressively bigger, such that the sieve becomes sufficiently large to allow infused material of a particular size to pass through. The sudden increased penetration of individual infused molecular species into bile after a specific period of ANIT pretreatment time supports this premise. The extent of ANIT-induced permeability change is inversely dependent on M_r; recent findings that the relative amounts of individual human plasma proteins in bile compared with plasma bear an inverse relationship to the M_r further suggests that their entry route into bile is due to sieving through M_r-determining tight junctions (Mullock *et al.*, 1985).

The enhancement of the ANIT effect by PB on HRP penetration and the reduction of the time sequence of the ANIT effect point to an involvement of ANIT metabolites in the development of the junctional damage leading to increased permeability to proteins. In the non-PB-pretreated rats, the relatively slow development of the leaky junction is likely to be due both to the slow rate of supply of active metabolite(s) by the liver and to the absorption rate of ANIT from the gut. ANIT, after being taken up by the enzymically active liver induced by PB, is rapidly biotransformed into active species, and the increasing rapidity of response suggests that metabolite supply rather than gut absorption is the more important factor. SKF 525A, which inhibits ANIT metabolism, virtually abolishes the effect of ANIT. These experiments, with an independent experimental approach, extend the suggestion by Roberts & Plaa (1965) that ANIT may first be metabolized by the liver into the one (or more) active metabolite(s) that is (are) responsible for the effect.

Furthermore, the health of the livers was monitored by measuring aspartate aminotransferase release. The increase in junctional permeability does not correlate with the changes in serum aspartate aminotransferase concentration (see Table 1), indicating that the increased permeability to protein cannot be attributed to increased general liver cell damage.

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