

THE METABOLISM OF 2-ALLYL-2-ISOPROPYLACETAMIDE *IN VIVO* AND IN THE ISOLATED PERFUSED RAT LIVER

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Abstract—The metabolism of allylisopropylacetamide (AIA) was studied in normal and phenobarbitone (PB)-pretreated intact male rats and in rats with biliary fistula. Because of the side effects of AIA in the intact rat, the hepatic metabolism of AIA was further investigated in the isolated rat liver perfused with defibrinated rat blood firstly, to confirm the *in vivo* results and secondly, to further characterize some of the processes involved in the biliary excretion of drugs.

At least four days were required to eliminate a single porphyrinogenic dose of 400 mg [2-¹⁴C] AIA per kg body wt from the intact rat, 70 per cent of the administered radioactivity appeared in the urine, as AIA and three metabolites A, B, and C, and 10 per cent in the faeces. AIA, 2-isopropyl-4,5-dihydroxypentanamide (AIA-glycol) and 2-isopropyl-4,5-dihydroxypentanoic acid- γ -lactone (AIA-lactone) were identified in ether extracts of glucuronidase-sulphatase-hydrolysed urine. AIA and two other metabolites, D and E, were excreted in bile. The metabolism of AIA by the perfused liver appeared quantitatively similar to that in fistula rats judged by the biliary excretion and by the decline in microsomal cytochrome P-450 after AIA. PB-pretreatment of the rats increased the per cent dose excreted per hour in the bile 2 to 3-fold, enhancing the initial excretion rate of metabolite D from 4 to 5-fold and that of AIA almost 2-fold.

The decrease of microsomal P-450 after AIA administration to PB-pretreated rats, previously considered to be biphasic, has been shown to have an additional component with half-life of 4 min. The rapid decline in hepatic P-450 after AIA in PB-pretreated rats correlated with an increased biliary excretion of one particular metabolite of AIA. A pharmacokinetic analysis of the biliary excretion data based on a two-compartment model shows that the rate-limiting step in the biliary excretion of both AIA and metabolite D can be adequately represented as a first-order linear reaction.

Chemicals such as 2-allyl-2-isopropylacetamide (AIA), 3,5(bis)ethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl pyridine (DDC), polycyclic hydrocarbons and certain steroids have been extensively used in animal studies to produce changes in haem metabolism, particularly of the rate-limiting enzyme in haem synthesis δ -aminolaevulinic acid synthetase (ALA-S; EC 2.3.1.3.7). AIA appears not to act by specific enzyme inhibition of any steps of the haem biosynthetic pathway, as has been demonstrated for DDC. Isotope studies [1] have demonstrated an increased destruction of the haem moiety of cytochrome P-450 after AIA, forming green pigments. The effects of AIA have been shown to be related to its metabolism by the liver and it is believed that an "active metabolite", derived from the allyl group may be responsible for the breakdown in P-450 [1, 2]. Such an active metabolite is probably too unstable to appear outside the liver but a study of the nature of metabolites of AIA would provide further evidence for relationships between the effects of AIA and the metabolism and excretion of AIA itself.

The primary consequences of some of the porphyrinogenic compounds, particularly AIA, are complicated by numerous toxic and nutritional effects which occur soon after administration in intact rats [3] (Smith, personal observations). We, therefore, utilized

a system in which rat livers were perfused with defibrinated rat blood, enabling investigation of the primary adaptive responses of the liver to AIA, uncomplicated by extrahepatic factors. With this enzyme, a detailed study of the biliary excretion of AIA and its metabolites together with the effect of sodium phenobarbitone (PB) on this excretion was undertaken. The metabolism of AIA and changes in P-450 concentration are compared with those obtained from rats with a bile fistula. We report here on the data which was analyzed by a pharmacokinetic model. Direct evidence has been obtained that in rats, pretreated with PB to induce cytochrome P-450, AIA administration causes a fall in P-450 which is accompanied by a different AIA metabolism from that seen without PB-pretreatment.

MATERIALS AND METHODS

Chemicals. AIA, unlabelled and [2-¹⁴C]labelled were supplied by Roche Products, Ltd., Welwyn Garden City. The radioactive purity of [2-¹⁴C] AIA (sp. act. 13 μ Ci/mg) was determined by thin-layer chromatography (t.l.c.) on Silica gel G (D5 CAMAG) in two solvent systems. A single peak was determined in ethanol:glacial acetic acid (10:1, v/v) and in ethanol:acetone:glacial acetic acid (5:5:1 by volume) with R_F values of 0.8 and 0.7, respectively.

Animals. All rats were males, from the King's College Hospital Medical School Colony (Wistar).

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The metabolism and excretion of AIA

Intact rats. Rats (205–216 g) were housed in individual metabolic cages, the room temperature was maintained between 20–25° with a light-dark cycle of 12 hr. When indicated, rats were injected i.p. with PB in 0.5 ml 0.15 M saline; they received 50 mg PB per kg body wt at 11.00 hr and 17.00 hr. On Day 2, a single injection of 80 mg PB per kg weight was given at 11.00 hr; food was then withheld until after injection of 400 mg [2-¹⁴C] AIA per kg body wt at 11.00 hr on Day 3. About 4.00 ml AIA solution (20 mg AIA/ml 0.15 M NaCl; sp. act. 0.16 μ Ci/mg) was injected s.c. in the loose skin at the back of the neck.

Urine and faeces were collected every 24 hr for 7 days except on Day 3 when urine was collected 0–6, 6–12 and 12–24 hr after AIA injection. Urinary δ -aminolaevulinate (ALA) and porphobilinogen (PBG) excretion were usually determined, immediately or after storage at –20° for not longer than 24 hr, by the method of Mauzerall and Granick [4]. On Day 7, the rats were killed by exsanguination from the dorsal aorta while under light ether anaesthesia.

Duplicate samples of liver, kidney, heart, brain, epididymal fat pad, subcutaneous fat, pancreas, spleen, small intestine (not contents), lung and salivary gland were wrapped in filter paper (Whatman No. 1), total weight less than 500 mg, and stored in Packard vials at 4° for 24 hr before combustion for radioactivity determination. Radioactivity was also determined in duplicate blood samples.

Bile fistula rats. These rats weighed 200–220 g and were housed in restraining cages (Bowman, Dartford). Room temperature was maintained at about 25° to avoid hypothermia. The bile duct was cannulated under ether anaesthesia; the duodenum was gently exposed and displaced to enable cannulation of the bile duct with Portex polyethylene tubing (o.d. 0.8 mm, bore 0.4 mm) with at least 0.5 cm in the duct. The procedure was completed within 10–15 min. Cannulation was carried out in fed rats, 24-hr-fasted rats and in rats pretreated with PB as described above. Bile was collected every 15 min until injection of either unlabelled or of [¹⁴C] AIA (sp. act. 0.1 μ Ci/mg) as described for the intact rats; usually 1 hr after recovery from the ether anaesthesia. Bile collection continued for three 5-min intervals and subsequently every 15 min. Total radioactivity was determined in blood samples from the dorsal aorta and a sample of liver was taken for total [¹⁴C] determination.

Perfusion. The perfusion apparatus consisted of a closed, recirculating system housed in a perspex cabinet maintained at 37–39° based on that of Miller, Bly, Watson and Bale [5], as modified by Abraham and Dawson [6]. Satisfactory perfusion under hydrostatic pressure was carried out through the portal vein alone for up to 5 hr with dialysed, defibrinated homologous rat blood [7], previously collected from the dorsal aorta under ether anaesthesia from fed 300–450 g rats; the liver donors weighed 180–220 g. During perfusion, pressure in the portal vein cannula was monitored using a strain gauge pressure transducer (Bell and Howell, England). Perfusion flow was continuously recorded using a drop counter which enabled rapid detection of changes in perfusion flow;

this is especially important at the beginning of perfusion. Bile was collected until the end of perfusion, at the same times as with the bile fistula rats.

The perfusion conditions were portal pressure 8–12 mm Hg (1.1–1.6 kPa), flow rate 0.8–1.5 ml min⁻¹ g liver⁻¹, initial pH 7.4, pO₂ 80–140 mm Hg (11–18.7 kPa), pCO₂ 30–40 mm Hg (4.0–5.3 kPa). The bile flow rate was initially up to 3.0 μ l min⁻¹ g liver⁻¹ decreasing to 0.8–2.0 μ l min⁻¹ g liver⁻¹; liver wet wt:dry wt ratio 3.0–3.5; blood lactate/pyruvate ratio 8–25; haemolysis rate during perfusion was 2–3% per hr.

The perfused livers were either from 24-hr-fasted rats or from rats pretreated with PB as above and fasted after the third injection of PB; perfusion was performed on Day 3. After 0.5 hr of perfusion AIA (sp. act. 0.2 μ Ci/mg), in amounts specified in the Results section and dissolved in 2.0 ml of 0.15 M saline, was added to the perfusion fluid reservoir where complete mixing took place within 2 min. In experiments in which the disappearance of AIA and appearance of AIA metabolites in the perfusion fluid were investigated, 2.0 ml samples were removed at every minute for the first 10 min and then after every 15–30 min for estimation of total radioactivity and that in fractions obtained by ether extraction and subsequent t.l.c. The total radioactivity was also measured in bile and in fractions obtained from these samples by t.l.c. Perfusion lasted 3–4 hr after addition of AIA to the perfusion fluid.

Cytochrome P-450. One-half hr after starting perfusion of livers from PB-treated rats, the papilliform lobe was ligated with Mersilk, 5.5 gauge, and placed within 5 sec in 0.15 M Tris-KCl buffer and microsomal pellets were prepared without delay. After a further 2 hr of perfusion the left lobe was removed and similarly treated. Microsomal suspensions prepared from these samples in 150 mM KCl were diluted with an equal volume of 0.1 M phosphate buffer, pH 7.0, to 200–400 mg protein/ml. The P-450 content was determined from the CO reduced vs reduced difference spectrum, using an extinction coefficient for E_{450–490} of 91 cm⁻¹ mM⁻¹ [8]. The protein concentrations were determined by the method of Lowry *et al.* [9] using bovine serum albumin (fraction V, Sigma) as the standard.

Perfusions were started at 11.00 hr and after 0.5 hr the papilliform lobe was removed immediately before the addition of AIA to the perfusion medium. The total dose of AIA dissolved in 2 ml 0.15 M saline, was equivalent to the range 100–400 mg per kg body wt of liver donor. In different perfusions the left lobe was removed 0.5, 1.0 and 2.0 hr after AIA had been added at an initial average concentration of 0.35 mg AIA/ml. To investigate possible interlobular differences of P-450 in intact rats, phenobarbitone-pretreated rats from the same group as the liver donors, were killed by cervical dislocation and the left and papilliform lobes were removed for P-450 determination.

The time course of the effect of AIA on P-450 were studied in intact rats and in three separate series of rats, pretreated with PB. Rats were killed 0, 0.25, 0.5, 0.75, 1, 1.5 and 3.25 hr after AIA injection. The effect of glucose was also studied in a similar series of pretreated rats. Glucose (15 g per kg body wt) dissolved

in about 2 ml water, was given by gastric intubation before injection of PB and AIA on days 2 and 3 respectively.

Radioactivity measurements. Rat bile (5–10 μ l) from fistula rats and perfused livers was dissolved in 0.5 ml hyamine hydroxide, 1.0 ml methanol and 10.0 ml toluene phosphor. Blood (0.05 ml) was dissolved in 0.5 ml "Soluene 100" (Packard tissue solubilizer) and decolourised with 0.15 ml of 30% w/v hydrogen peroxide; after 10 min 0.05 ml glacial acetic acid was added to neutralize the Soluene base and then 10.0 ml of toluene phosphor. Urine (0.05–0.5 ml) from intact rats was dissolved in 0.5 ml "Soluene 100" in a Packard vial to which 10.0 ml toluene phosphor was added. These solubilized samples were stored in the dark at 4° and were counted at least twice within 1–2 days. All tissues and faeces were combusted to $^{14}\text{CO}_2$ which was trapped in ethanolamine in a Packard Tri-Carb sample oxidizer (Model 305).

The radioactivity was counted in Toluene phosphor (4.0 g PPO and 0.1 g POPOP dissolved in 1 l of toluene) or in dioxan phosphor (120.0 naphthalene, 8.0 g PPO, 0.4 g POPOP, 200.0 ml methanol, 40.0 ml ethylene glycol made up to 2 l with dioxan) utilizing a Packard Tri-Carb 3000 scintillation spectrometer. Counting was continued until a S.D. of at least 1% (20,000 counts) was achieved.

Extraction of AIA and its metabolites

Urine. Urine (0.5–1.0 ml) was extracted with ether before and after hydrolysis at pH 5.0–5.2 with a β -glucuronidase-sulphatase preparation ("ketodase": 5,000 I.U./ml) using 0.2 ml ketodase per ml urine and incubated at 37° for 24 hr. For acid hydrolysis, 0.01 ml concentrated sulphuric acid per ml urine was employed. Ether extractions were carried out successively at pH 9 and pH 7 (followed by amyl alcohol extraction). Ether extracts at each pH were pooled and brought to dryness in nitrogen at 40°. Recovery of radioactivity from urine of [^{14}C] AIA-treated rats showed that although extraction of AIA was complete at pH 9, that of the metabolites was not. Extraction of the metabolites was subsequently completed at pH 7. The efficacy of extraction of radioactivity as determined from the difference in ^{14}C contents of the aqueous layer before and after extraction was 50–80 per cent but by counting aliquots of the evaporated solvent 20–40 per cent, presumably due to the loss of volatile metabolites.

Thin-layer chromatography of rat urine. The residue of the ether extracts was dissolved in ether-ethanol mixture (1:1, v/v) and measured aliquots were used for the determination of total radioactivity and for analysis by t.l.c. with multiple development in ether ($\times 3$). The separate components were detected as yellow spots after exposure of the t.l.c. plates to iodine vapour and by scanning for radioactive areas using the Packard Radiochromatogram Scanner (Model 7201). The spots were compared with simultaneously chromatographed standards of nonradioactive AIA, 2-isopropyl-4,5-dihydroxypentanamide (AIA-glycol), 2-isopropyl-4,5-dihydroxy pentanoic acid- γ -lactone (AIA-lactone) and 2-allyl-2-isopropyl acetic acid. The radioactive areas of Silica gel were scraped quantitatively from the plates into Packard vials and eluted with 10.0 ml of dioxan phosphor. A loss of only 3

per cent was incurred for authentic [^{14}C] AIA (7,000–70,000 dpm).

Thin-layer chromatography of bile. The bile samples were acidified with 0.5 vol of 1.0 N sulphuric acid to convert any epoxide present to the more stable diol; then 10–20 μ l were quantitatively transferred to the Silica gel plate (0.3-mm thick) with methanol.

Identification of AIA and its metabolites in bile. The radiochromatogram scan of bile chromatograms developed in chloroform:glacial acetic acid (10:1, v/v) revealed two peaks R_F 0 and 0.43. The latter was shown to be AIA by chromatography with authentic AIA in several different solvent systems and by gas-liquid chromatography (g.l.c.)–mass spectrometry (MS). AIA was in this way shown to be present in bile both from perfused livers and in fistula animals after all treatments. The R_F of allylisopropylacetic acid in this system was 0.8 and was not detected in bile. A preliminary development with carbon tetrachloride caused no elution of radioactivity and improved the resolution of subsequent elution in ethanol:acetone:glacial acetic acid (5:5:1 by vol) which gave three radioactive peaks D (R_F 0.35), E (R_F 0.55) and F (R_F 0.85); no further fractions became apparent on subsequent continuous elution. Compound F was AIA. Compound D, nearest the origin, was not identified but was shown neither to be allylisopropylacetic acid, AIA-glycol nor AIA-lactone but there was insufficient quantity for combined g.l.c.–MS. Compound E showed some similarities with AIA glycol lactone but was not conclusively identified by g.l.c.–MS. AIA epoxide was not expected since bile samples were acidified before chromatography with the specific object of converting these to the more stable glycol.

Thin-layer chromatography of ether extracts of rat blood. Blood was extracted at pH 7 by 4×2 vol of ether. The ether extracts were divided into four, the total radioactivity was determined in two and the other two were evaporated and stored at -20° before multiple development in ether, as described above for urine, was carried out.

Synthesis of 2-isopropyl-4,5-dihydroxy pentanamide (AIA-glycol) and 2-isopropyl-4,5-dihydroxy pentanoic acid- γ -lactone (AIA-lactone)

AIA was converted to the corresponding glycol by oxidation with permanganate and the lactone was synthesized from the glycol according to the method of Doedens [10]. For this work 4 g of AIA, dissolved in 320 ml of 0.1 N NaOH (0–10°) was slowly added to 175 ml potassium permanganate (1.88 g/100 ml). Methanol (20 ml) was added followed by acidification with 3 g of ammonium chloride. The filtrate was (a) evaporated to dryness at 50° under vacuum and the dry residue extracted three times with 400 ml of acetone; evaporation of these combined extracts yielded an oily material from which AIA-glycol was crystallized, with difficulty, from methanol by isopropyl ether, or (b) acidified to pH 3–4 with HCl, evaporated to about 40 ml under vacuum and heated for 1 hr at 90–95°; the cooled solution was extracted three times with two volumes of diethyl ether and after drying with sodium sulphate evaporation yielded AIA-lactone, a pale yellow oil.

Identification of AIA and its metabolites. Gas-liquid chromatography and mass spectrometry was carried

out in collaboration with Dr. S. Al-Sarraj at Chelsea College of Science, London, using 2-metre \times 1.5-cm (internal diameter) column of Chromasorb G (80–200 mesh) with carbowax liquid phase (7%) and flame ionization detection. With N_2 carrier gas (30 ml/min) and a column temperature of 167° the retention time of authentic AIA was 7.6 min and that of allylisopropylacetic acid was 3.9 min. This work was continued in collaboration with David Wright at the Physico-Chemical Measurements Unit, Harwell and mass spectra of the authentic AIA, allylisopropylacetic acid, 2-isopropyl-4,5-dihydroxy pentanamide (AIA-glycol) and 2-isopropyl-4,5-dihydroxy pentanoic acid- γ -lactone (AIA-lactone) were obtained.

The identification of AIA-glycol and AIA-lactone was completed by comparison of the i.r. spectrum (KBr discs) with that of the authentic compound. The OH absorptions (ν_{\max}) were found at 3400 and 1050 cm^{-1} . The CH stretching vibrations at 2800–3000 cm^{-1} showed significant contributions from the methyl groups, and there was evidence for isopropyl groups at 1370 and 1390 cm^{-1} . The prominent carbonyl signal at 1760 (br) cm^{-1} was consistent with the presence of a tetra hydrofuran-2-one ring of a γ -lactone, with associated C—O stretching near 1200 cm^{-1} .

For the glycol derivative of AIA (2-isopropyl-4,5-dihydroxypentanamide) the intense band at 3200–3500 cm^{-1} showed the expected splitting related to the OH and NH_2 . The CH stretching bands were unchanged and the bands for the isopropyl groups were present. The differences between these two compounds were found in the carbonyl region, the glycol showing prominent absorptions at 1610 and 1660 cm^{-1} , consistent with the aminocarbonyl group $—CONH_2$. Identification of AIA and its metabolites in the biological samples was then confirmed by comparison with the results of these authentic compounds.

RESULTS

Induction of experimental porphyria was confirmed by the urinary excretion of ALA and PBG which were increased 8 to 15-fold in the 48 hr after the standard dose of 400 mg AIA per kg body wt.

Excretion of $[2-^{14}C]$ AIA and its metabolites. In the 96 hr after $[2-^{14}C]$ AIA means of 68 and 11 per cent of administered radioactivity were excreted in the urine and faeces respectively, mostly within the first 48 hr. Faecal excretion was minimal after 48 hr although urine excretion was about 23 per cent during the following 48 hr (Fig. 1). The total radioactivity recovered in the organs together with that excreted during the 120 hr after AIA amounted to 70–91 per cent of the injected radioactivity; blood and organs accounting for about 1 per cent each, most being in the liver and kidney (Fig. 2). Any differences in the distribution of total radioactivity in the urine, faeces or organs resulting from PB-pretreatment were not apparent at this time.

The effect of hydrolysis of rat urine containing $[2-^{14}C]$ AIA and its metabolites. Ether extracted almost twice as much radioactivity from enzyme-hydrolyzed urine compared with unhydrolyzed urine; less radioactivity was extracted into ether after acid hy-

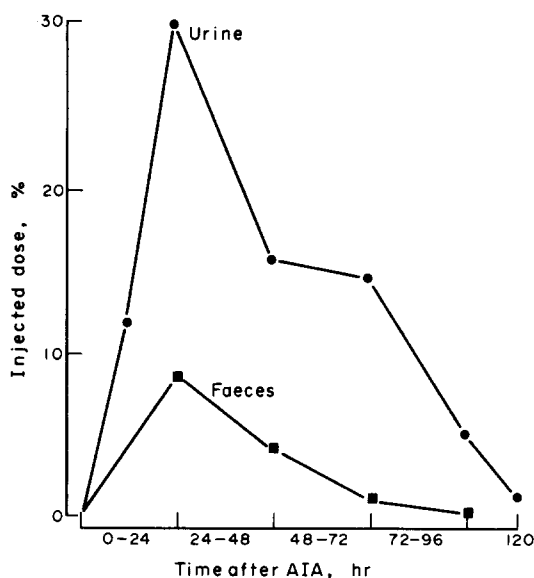


Fig. 1. Typical excretion patterns of urinary and faecal radioactivity by rats after $[2-^{14}C]$ allylisopropylacetamide (AIA). Four rats received 400 mg AIA per kg body wt s.c. at time 0. Two rats were pretreated with PB (see text) before AIA injection. The pattern of excreted radioactivity was similar in all these animals; PB-pretreatment produced no significant changes. The results in the figure are from a rat which received AIA alone.

drolysis than after enzyme hydrolysis. The ether-soluble radioactivity from the 0–48 hr enzyme-hydrolyzed urine amounted to 50–80 per cent of the total radioactivity in the urine.

Identification of AIA and metabolites. Multiple development of the ether extracts of enzyme-hydrolyzed urine revealed four radioactive peaks AIA and the metabolites, A, B, and C. About 4 per cent of the administered $[2-^{14}C]$ AIA was excreted unchanged

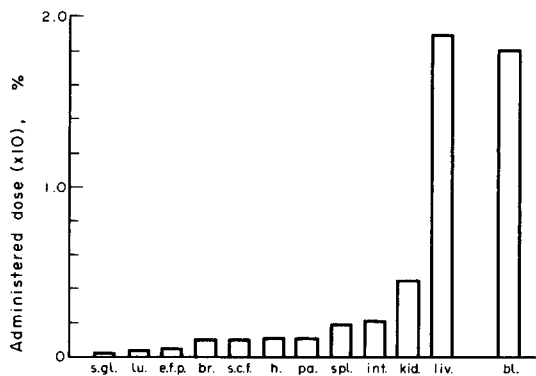


Fig. 2. The distribution of radioactivity in tissues and blood of the rat four days after a subcutaneous injection of $[2-^{14}C]$ allylisopropylacetamide. Four rats received 400 mg AIA per kg body wt s.c.; two rats were pretreated with PB (see text) before AIA injection. This histogram represents the distribution of radioactivity remaining in the tissues and blood (bl.) four days after injection with radioactive AIA. There was little difference between the two groups. The tissues were salivary glands (s. gl.), lung (lu.), epididymal fat pad (ef.p.), brain (br.), subcutaneous fat (s.c.f.), heart (h.), pancreas (pa.), spleen (spl.), small intestine (int.), kidney (kid.), liver (liv.).

* The value for blood has been reduced by half for scale.

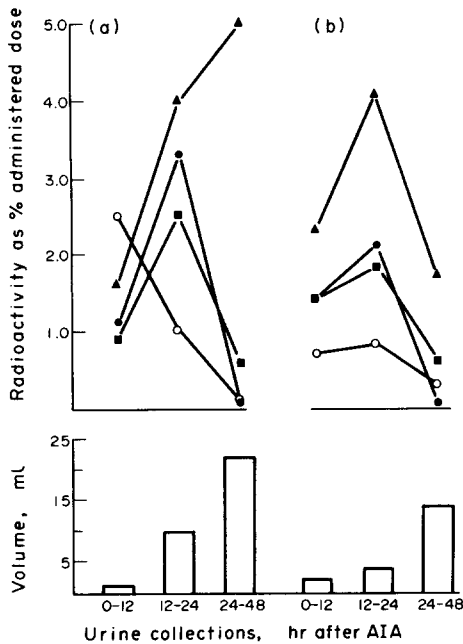


Fig. 3. Urinary excretion of allylisopropylacetamide and its metabolites: the effect of phenobarbitone on the pattern of excretion 0–48 hr after $[^{14}\text{C}]$ allylisopropylacetamide. After 'ketodase' hydrolysis of aliquots of the urine collections AIA (●) and three metabolites, AIA-glycol (■), AIA-lactone (▲) and metabolite C (○), see text, were extracted into ether and separated by t.l.c. (a) One rat which received 400 mg AIA per kg body wt. (b) Representative excretion from two rats, PB-pretreated before being given AIA.

0–48 hr after injection of AIA. The presence of AIA was confirmed using g.l.c.-MS; AIA has a molecular ion with m/e of 141, loss of the methyl group, allyl group and isopropyl group produced peaks with m/e of 126, 100 and 98 respectively.

Metabolite A, which was eluted more rapidly than AIA, was considered to be 2-isopropyl-4,5-dihydroxypentanoic acid- γ -lactone by comparison with authentic material developed simultaneously. Similarly, metabolite B was shown to be 2-isopropyl-4,5-dihydroxypentanamide. The presence of these two compounds in the ether extracts was confirmed using g.l.c.-MS; because of the close correspondence between their mass spectra i.r. spectroscopy was used to distinguish between them. Metabolite C was present in the smallest amount but was not identified.

Urinary excretion of AIA, 2-isopropyl-4,5-dihydroxypentanamide (AIA-glycol) and 2-isopropyl-4,5-dihydroxypentanoic acid- γ -lactone (AIA-lactone). Zero to 12 hr after AIA, excretion of metabolite C was higher than in subsequent collections and was about 50 per cent higher than that of AIA-lactone which in turn was almost twice that of AIA and AIA-glycol, which were present in equal amounts (Fig. 3). Excretion of AIA and AIA-lactone had increased by 12–24 hr, after which AIA excretion was minimal. Twenty-four–48 h after AIA, the lactone was the major excretion product. PB-pretreatment increased the excretion of AIA-lactone in the 0–12 hr collection and AIA and AIA-glycol were again present in equal proportion; excretion of metabolite C was low.

In the 48 hr after injection of $[^{14}\text{C}]$ AIA to one rat which had not received PB, 4.6 per cent of AIA, 10.6 per cent of AIA-lactone and 4.0 per cent of AIA-glycol were recovered from ether extracts of "ketodase"-hydrolyzed urine. In two PB-pretreated rats 3.6 and 4.4 per cent was recovered as AIA, 8.1 and 5.7 per cent as the lactone and 3.8 and 5.9 per cent as the glycol. The overall recovery of urine radioactivity during the 48 hr after AIA was 23.1 per cent in the rat which received AIA alone and 18.1 and 18.2 per cent in the two PB-pretreated rats. The relative proportions of AIA and its metabolites excreted in the faeces was not investigated.

Fistula rats. AIA alone produced no great changes in the flow of bile unless the rats had been pretreated with PB when 2-fold increases occurred rapidly during the first 2 hr (Fig. 4). Biliary flow returned to normal by 3 hr in all animals. In two bile fistula rats not PB-pretreated, one fasted and one fed, the excreted radioactivity was slightly higher in the second hour after AIA. Hourly excretion in all animals then decreased to 1–2 per cent of the dose. In two PB-pretreated rats the highest excretion of radioactivity usually occurred in the first hour after AIA; PB increased this from 2.5 to 5.5 per cent of the administered dose. In both fed and fasted rats, biliary AIA and its two metabolites were present in

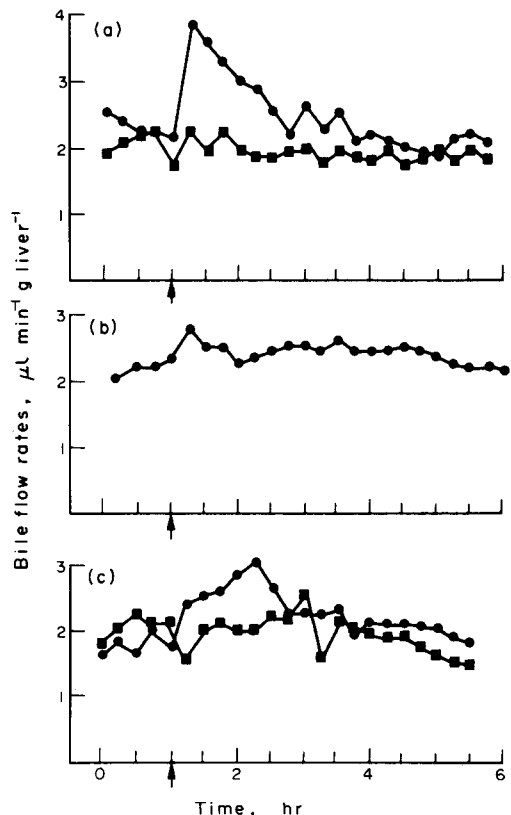


Fig. 4. The effect of a single s.c. injection of allylisopropylacetamide on bile flow rates in bile fistula rats. After cannulation of the bile duct, bile was collected every 15 min. AIA was injected s.c. at a dose of 400 mg per kg body wt at the time indicated by the arrow. The controls (■) were injected s.c. with an equal volume of saline. (a) The rats were pretreated with PB (see text) before administration of AIA. (b) 24-hr-fasted rat. (c) Fed rats.

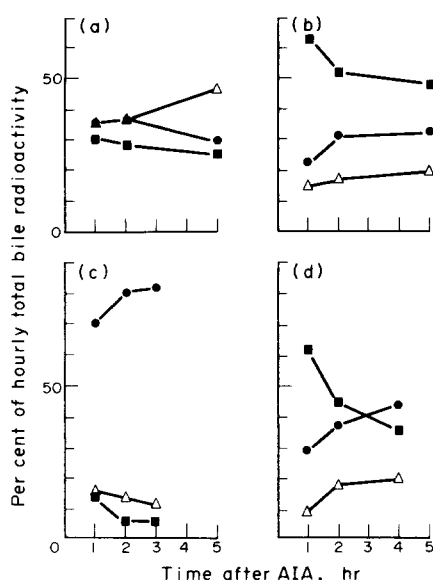


Fig. 5. The effect of phenobarbitone-pretreatment on the proportions of AIA and its metabolites excreted in the bile from fistula rats and the perfused rat liver. Unchanged AIA (●) and two metabolites, D (■) and E (△) were identified in bile after separation by t.l.c. Bile was collected (see text) (1) from perfused livers after addition of AIA to the perfusion medium at an initial concentration of 0.35 mg/ml (c), or (2) from bile fistula rats after s.c. injection of 400 mg AIA per kg body wt (a). Bile was collected as described above, but the rats were pretreated with PB (see text) before administration of AIA (d and b respectively).

about equal amounts over the first 5 hr after AIA injection in contrast to the rapid elimination, principally of metabolite D, and the gradually increasing concentration of AIA and metabolite E by the PB-pretreated rats (Fig. 5).

Figure 6 shows the initial excretion rates of radioactivity which agree with the sums of the individual initial excretion rates of AIA and its metabolites (Table 1) which were linear for at least 1-hr after the administration of AIA. The total radioactivity amounted to 3.6 and 2.7 per cent dose per hr in the fed and fasted rats respectively and was increased to 6 per cent by PB-pretreatment. Although the initial excretion rate of AIA was almost doubled after phenobarbitone, that of metabolite D was increased 4 to 5-fold, while that of metabolite E was hardly affected. In the fed animal, not given PB, the initial excretion of metabolite E was higher than that of AIA and metabolite D. In spite of these differences the maximum biliary excretion of AIA in the fasted rat occurred 30–45 min after injection and was 13 nmole min^{-1} g liver $^{-1}$, subsequently decreasing to 4 nmole min^{-1} g liver $^{-1}$. These were slightly, but not significantly, higher in a fed rat and were only slightly increased by PB-pretreatment, the maximum to 14 and 18 nmole min^{-1} g liver $^{-1}$ which decreased to 5 and 6 nmole min^{-1} g liver $^{-1}$ 4–5 hr after AIA in two rats.

Perfused liver. In the perfused liver, increases in bile flow after the addition of AIA to the perfusate were difficult to distinguish because this experimental period coincided with the rapid phase of bile secretion normally encountered in the course of perfusion. Using the liver from a fasted rat most excretion of

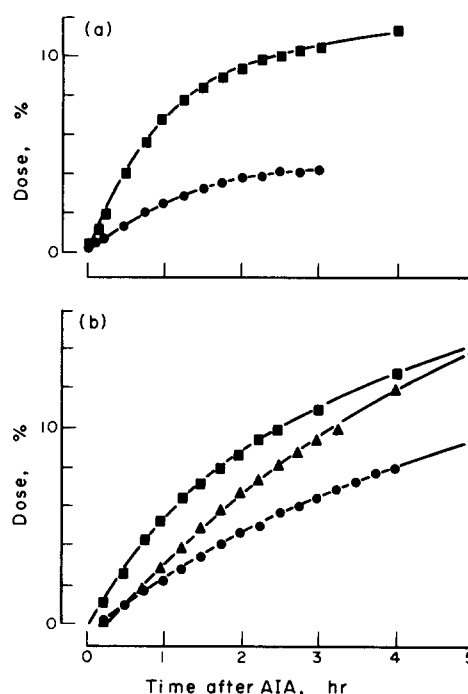


Fig. 6. Cumulative biliary excretion of radioactivity after the administration of $[2-^{14}\text{C}]$ allylisopropylacetamide in fistula rats and in the perfused rat liver. (A) Bile was collected from perfused livers after the addition of $[2-^{14}\text{C}]$ AIA to the perfusion medium at an initial concentration of 0.35 mg/ml. The liver donors were a 24-hr-fasted rat (●) or a rat fasted after standard PB-pretreatment (■) (see text). (B) Bile was collected from bile fistula rats after s.c. injection of 400 mg AIA per kg body wt. The rats were 24-hr-fasted (●), PB-pretreated, fasted (■) or fed (▲).

radioactivity occurred in the first hour, as in the fistula rats, and this was increased by PB-pretreatment of the liver donor (Fig. 6). The initial excretion of AIA was 2–3-fold higher in the perfused liver than

Table 1. Initial linear biliary excretion rates of AIA and two metabolites by bile fistula rats and perfused rat livers

	Initial excretion rates (% injected dose/30 min)		
	AIA	Metabolite D	Metabolite E
Bile fistula rats			
Fed	0.30	0.50	0.70
24-hr-fasted	0.40	0.40	0.40
PB-pretreated, 24-hr-fasted	0.70	1.80	0.30
Perfused liver			
24-hr-fasted	0.80	*0.30	0.20
PB-pretreated, 24-hr-fasted	1.00	*2.00	0.30

Bile was collected from fed, 24-hr-fasted and PB-pretreated, 24-hr-fasted fistula rats after a s.c. injection of 400 mg $[2-^{14}\text{C}]$ AIA per kg body wt. The perfused livers were from either a 24-hr-fasted rat or a PB-pretreated, fasted as above; after 0.5 hr perfusion $[2-^{14}\text{C}]$ AIA at an initial concentration of 0.35 mg/ml was added to the perfusion medium. Determination of AIA and its metabolites excreted in the bile was carried out after separation by t.l.c.

* Linear excretion rates for 0–30 min after AIA; all others were linear for at least 0–60 min after AIA.

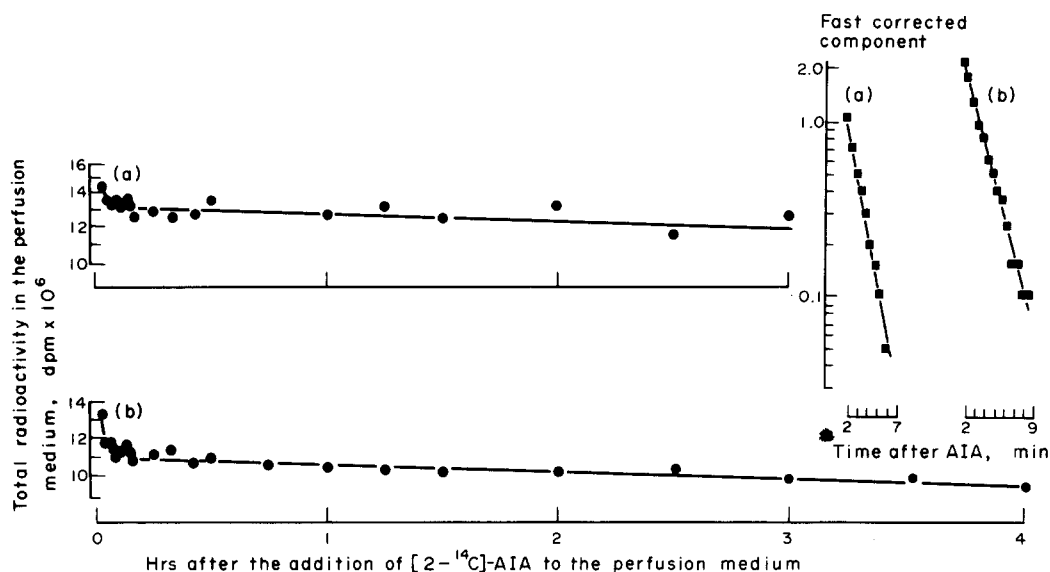


Fig. 7. Semilogarithmic plot of the decrease of radioactivity from the blood after the addition of $[2-^{14}\text{C}]$ allylisopropylacetamide to the perfusion medium. This figure illustrates the graphically-fitted curve of the decrease of total radioactivity from the blood (O) using the 'peeling technique' of Matthews [39]. Two exponential components were resolved: one of short half-life (inset, see text) and one of long half-life (after the addition of $[2-^{14}\text{C}]$ AIA at an initial concentration of 0.35 mg/ml to the perfusion circuit). (a) 24-hr-fasted liver donor. (b) PB-pretreated, 24-hr-fasted liver donor (see text).

that found in the fistula rats and this was enhanced by phenobarbitone, although not so much as the excretion of metabolite D. The *in vitro* initial excretion rates of metabolite D were almost identical with those in the fistula rats (Table 1). The initial excretion rate of metabolite E was decreased in the perfused liver compared with a fasted fistula rat but was the same in both after PB-pretreatment. In the perfused liver from a fasted rat the maximum excretion rate of AIA was $9 \text{ nmole min}^{-1} \text{ g liver}^{-1}$ at 5–10 min after AIA, decreasing to $2 \text{ nmole min}^{-1} \text{ g liver}^{-1}$ at 2–3 hr. These rates were slightly but not significantly increased by phenobarbitone to 11 and $3 \text{ nmole min}^{-1} \text{ g liver}^{-1}$.

Pharmacokinetic analysis

Radioactivity in the perfusate. In the preliminary perfusion in which the biliary excretion of AIA and its metabolites was not investigated the circulating radioactivity present as metabolites of AIA in extracts of blood increased steadily to reach 6 per cent of the total at 2 hr. However, this figure represents a minimal proportion of the total circulating radioactivity after 2 hr of perfusion. The presence of volatile radioactive metabolites of AIA was indicated by a recovery of only 40–50 per cent during t.l.c. of samples taken 1.5 and 2.0 hr after AIA, instead of the usual recovery of 80–100 per cent. The decrease of radioactivity in the blood during perfusion with $[^{14}\text{C}]$ AIA of livers from two fasted rats, one PB-pretreated, were analysed as two exponential components (Fig. 7). The initial fall of AIA radioactivity in the perfusion medium was rapid, the rate constant being 0.6 min^{-1} ($t_{1/2} = 1.2 \text{ min}$) in the fasted liver and 0.5 min^{-1} ($t_{1/2} = 1.4 \text{ min}$) after PB-pretreatment. Subsequently this had decreased to 0.053 hr^{-1} and 0.063 hr^{-1} in the two livers respectively. The validity of this analysis was confirmed by the close agreement

of the sum of the constants β_1 (1.03 and $2.18 \text{ dpm} \times 10^6$) and β_2 (13.2 and $11.0 \text{ dpm} \times 10^6$) and the dose which was 99.9 per cent for the fasted liver and 101.8 per cent for the PB-treated donor. The total radioactivity accounted for, including the total ^{14}C remaining in the perfusate, ranged from 91–101 per cent of the administered dose.

Biliary excretion. The biliary excretion data from the perfusions was analysed in the simplified two compartment model described in the Appendix. Figure 8 illustrates the resolution of a typical semilog plot of the radioactivity present in each bile collection as AIA or its metabolites, similar plots were derived for the experimental data from three fistula rats (Fig. 9) and two perfused livers. The results of this pharmacokinetic analysis have been restricted to the rate constant $k_{12(\text{fast})}$ and $k_{12(\text{slow})}$ and k_2 because this analysis was undertaken primarily to compare the kinetic characteristics of liver metabolism and biliary excretion of the isolated perfused rat liver with those *in vivo* in the fistula rat.

Table 2 summarizes the kinetic results of the biliary excretion of AIA and its metabolites which generally agreed well with the proposed model. The value for k_2 , the rate constant for the rate-limiting step in the transfer from liver to bile for AIA, was unaffected by PB in the perfused livers but was decreased by half in the fistula rats. The rate constant for metabolite D was faster than that of AIA and was also consistently decreased by PB. The value for metabolite E in the fasted fistula rat and perfused liver was similar to that of AIA but the effect of PB could not be satisfactorily assessed with this model as the experimental data declined mono-exponentially from Q_{max} .

Effect of AIA on hepatic microsomal P-450 *in vivo*. In fed rats not given phenobarbitone, 1 hr after AIA the concentration of P-450 had decreased exponentially to $0.75 \text{ nmole P-450/mg protein}$ (Fig. 10D). This

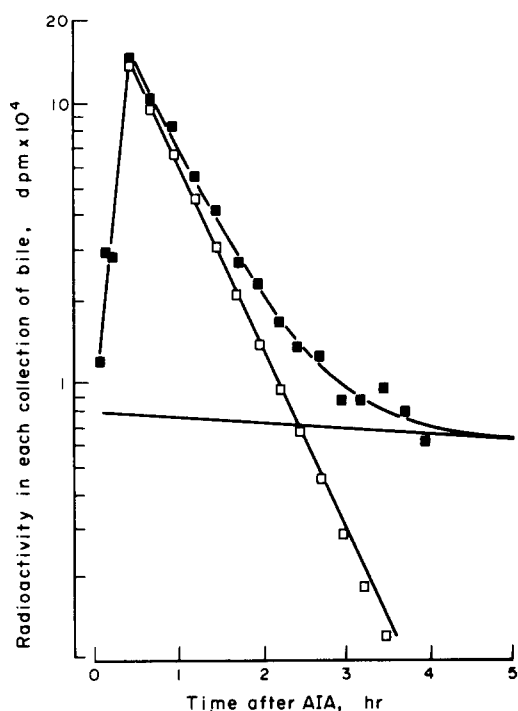


Fig. 8. Resolution of the biliary excretion of metabolite D by the perfused rat liver. Using the pharmacokinetic analysis described in detail in the Appendix these results are typical for the resolution into two exponential components of the biliary excretion of both AIA and its metabolites by the perfused liver (and fistula rats). This experimental data (■) was taken from the perfusion of the liver from a PB-pretreated, 24-hr-fasted rat and the slow component was derived from the disappearance of radioactivity from the perfusion medium (see text) (See also Fig. 9).

falls in the range 0.6–0.8 nmole P-450/mg protein found 1 hr after AIA in fasted rats treated with phenobarbitone. The rate curves of P-450 decrease were almost parallel in three separate groups of rats pretreated with phenobarbitone and a typical experiment is illustrated in Fig. 10B. The decrease in P-450 after AIA in intact phenobarbitone-pretreated rats is the resultant of two exponential components determined graphically using “peeling techniques” of Matthews [12] producing a slow phase of half-life 3.3 hr and a corrected fast phase of half-life 4 min (Fig. 11). In a single series of rats treated identically but given glucose the pattern of exponential decline of P-450 appeared to be not greatly affected by glucose treatment (Fig. 10C).

The effect of AIA on microsomal P-450 in the perfused liver. In control perfused livers, there was no significant decrease in P-450 concentration over 2.5 hr (Table 3). In preliminary experiments with nine phenobarbitone-pretreated rats there was a per cent decrease in the concentration of P-450 of 41–54 after exposure for 1.0 hr to AIA, the initial concentration of which ranged from 0.3 to 3.0 mg/ml. There was no direct correlation between fall of P-450 concentration and AIA concentration in perfusion fluid (see Fig. 12). However, the effect of AIA on P-450 levels was much reduced and more variable at blood concentrations lower than 0.3 mg/ml; in three perfusions there were decreases of 4, 15 and 31 per cent after

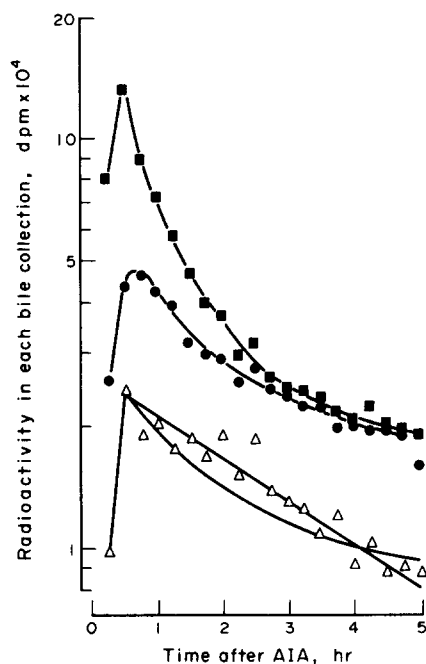


Fig. 9. The biliary excretion of AIA and its metabolites by a bile fistula rat, pretreated with phenobarbitone. The pattern of biliary excretion of AIA (●), metabolite D (■) and metabolite E (△) in a bile fistula rat which received 400 mg AIA per kg body weight s.c. at time 0. This rat had received the standard PB-pretreatment and 24 hr fast (see text) before injection of AIA. (c.f. Fig. 8).

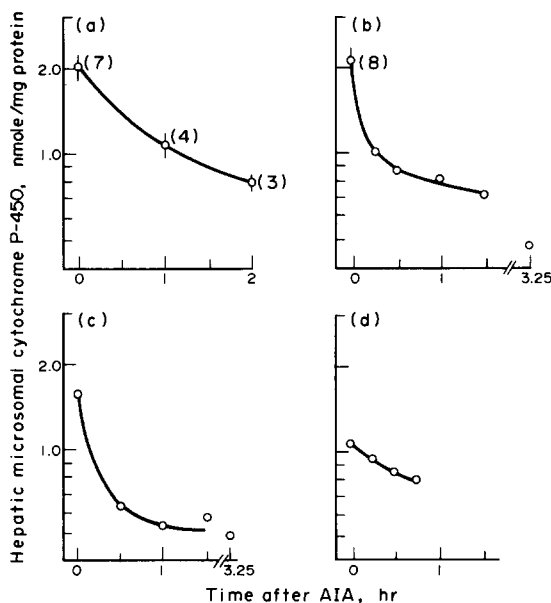


Fig. 10. Decrease in hepatic microsomal cytochrome P-450 after allylisopropylacetamide in intact rats and in the perfused rat liver. (a) Perfused liver from 24-hr-fasted, phenobarbitone-pretreated rats. (b) 24-hr-fasted, phenobarbitone-pretreated rats. (c) 24-hr-fasted, phenobarbitone-pretreated rats given glucose by gastric intubation. (d) Fed rats. Details of the dose of AIA, phenobarbitone and glucose are given in the experimental section. All experimental points are the average of at least two observations, the number of which is given in parenthesis and the standard deviation is indicated.

Table 2. The rate constant (k_2) of the rate-limiting step in the biliary excretion of [2- 14 C] AIA

		k_2 (min^{-1})	$k_{1,2(\text{slow})}$ (hr^{-1})
AIA	Bile fistula rats		
	24-hr-fasted	0.037	0.093
	PB-pretreated, 24-hr-fasted	0.019, 0.019	0.069, 0.062
	Perfused livers		
Metabolite D	24-hr-fasted	0.023	0.053
	PB-pretreated, 24-hr-fasted	0.023	0.063
	Perfused livers		
	24-hr-fasted	0.041	0.053
Metabolite E	PB-pretreated, 24-hr-fasted	0.026	0.063
	Bile fistula rats		
	24-hr-fasted	0.035	0.095
	PB-pretreated, 24-hr-fasted	0.03	0.059
Metabolite E	Perfused livers		
	24-hr-fasted	0.021	—
	PB-pretreated, 24-hr-fasted	0.001 (mono-exponential)	—

The two-compartment model for the biliary excretion of AIA and its metabolites by the perfused liver is described in detail in the Appendix where the relevance of applying the model to the data from bile fistula rats is also discussed. The excretion of AIA and its metabolites rose rapidly to Q_{max} and the rate constant ($k_{1,2(\text{slow})}$) of the slow component of the bi-exponential decline from Q_{max} is included in the above table as well as the rate constant (k_2) derived by subtraction of the slow component from the original excretion curve.

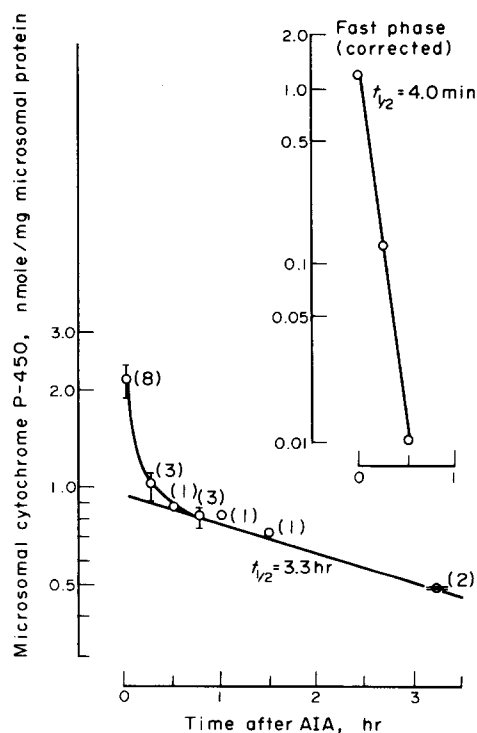


Fig. 11. The decrease of hepatic microsomal cytochrome P-450 after administration of allylisopropylacetamide to phenobarbitone-pretreated rats. This graph was derived by superimposing two time-courses from two separate groups of rats. The number of rats for each time point is indicated in parenthesis and the range is shown. The fast corrected component ($t_{1/2}$ 4.0 min) was derived by subtraction of the final linear part of the time-course ($t_{1/2}$ 3.3 hr) extrapolated to time 0 from the experimental points on the original curve.

the addition of 0.2 mg AIA/ml perfusion fluid. A single perfusion with the highest perfusate concentration of 3.0 mg AIA/ml produced a decrease in P-450, after 1.0 hr, of 50 per cent (0.71 nmole P-450/mg protein) well within the range found for one-tenth of this circulating concentration of AIA. The effect of AIA on P-450 concentration in the perfused liver was also determined after 0.5 and 2.0 hr exposure to 0.35 mg AIA/ml. There was a 23 per cent decrease by 0.5 hr and in three experiments decreases of 54, 61 and 66 per cent by 2.0 hr. The results of perfusions carried out at the same concentration of AIA and already included in the preliminary experiments described above were used to construct the time curve of the decrease in P-450 in Fig. 9A. The concentration of P-450 at $t = 0.5$ hr is not included in the time-course

Table 3. The effect of perfusion on microsomal cytochrome P-450 in livers of phenobarbitone-pretreated rats

	Cytochrome P-450 nmole/mg protein, (nmole/g liver) mean \pm S.D. (n)
Unperfused	
(a) Papilliform lobe	2.02 \pm 0.32 (8) (75.0 \pm 8.40)
(b) Left lobe	2.24 \pm 0.24 (8) (76.7 \pm 7.41)
Perfused	
(c) Papilliform lobe (after 0.5 hr perfusion)	1.90 \pm 0.35 (11) (56.5 \pm 14.6)
(d) Left lobe (after 2.5 hr perfusion)	1.93 \pm 0.53 (7) (66.6 \pm 16.1)

Tests of significance a:b $P > 0.1$; c:d $P > 0.8$; b:c $P > 0.02$; b:d $P > 0.1$.

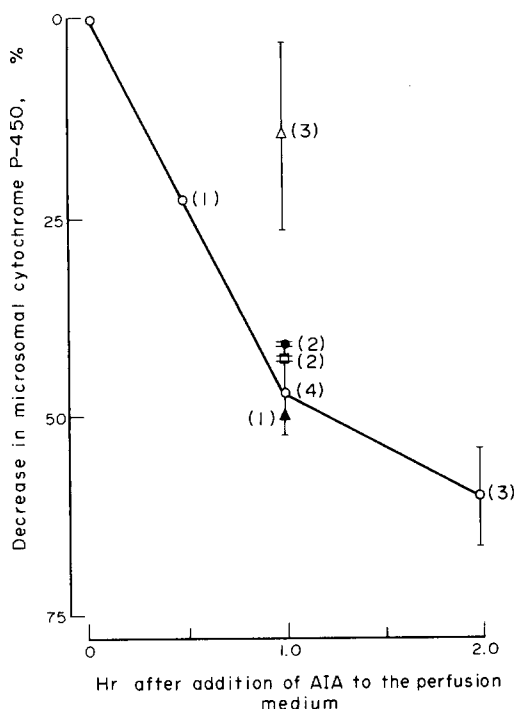


Fig. 12. The decrease in microsomal cytochrome P-450 after the addition of allylisopropylacetamide to the perfusion medium. The liver donors were pretreated with phenobarbitone as described in the text. Initial perfusion medium concentration of AIA mg/ml (dose equivalent mg/kg body wt of liver donor) Δ , 0.16-0.2 (100); \circ , 0.33-0.39 (200); \square , 0.5-0.59 (300); \bullet , 0.73 (400); \blacktriangle , 3.0. The results are expressed as the mean and the range is indicated together with the number of perfusions in parenthesis.

because it was low although the rate of decline of P-450 during this perfusion was parallel with the four terminated at 1 hr.

DISCUSSION

Comparison of the biliary and urinary excretion of radioactivity of AIA and its metabolites has shown that the metabolism of AIA is complex and supports the existence of an enterohepatic circulation. Doedens [10, 11] suggested that because of the inactivity *in vivo* of the saturated 2-propyl-2-isopropylacetamide the porphyrinogenic activity of AIA might be associated with the allyl group. Doedens considered that the AIA-lactone and its glucuronide were the major urinary metabolites excreted together with AIA and AIA-glycol. Administration of AIA-epoxide, AIA-glycol or AIA-lactone caused no increased PBG excretion suggesting that these metabolites were unimportant in the porphyrinogenic activity of AIA and indicating the need for further work on the metabolism of a porphyrinogenic dose of AIA.

From the present work, up to 80 per cent of the administered [^{14}C] AIA was accounted for in the urine and the urinary excretion of AIA, AIA-glycol and AIA-lactone were confirmed. However, the losses determined from measurements of radioactivity at the various steps of purification resulted in a much lower overall recovery, suggesting the loss of unidentified

volatile metabolites. A considerable proportion of this radioactivity was present in the urine as glucuronide and possibly other conjugates. The glycol might be present as a sulphate conjugate and AIA might conjugate with glutathione, presumably after epoxide formation.

The urinary and faecal excretion of radioactivity by intact rats after [^{14}C] AIA was not greatly affected by pretreatment with PB which can induce a variety of drug-metabolizing enzymes but the possibility that PB can change the extent of glucuronidation of AIA or a metabolite of AIA has not been investigated. Since the effect of AIA on P-450 and ALA-S are related to its metabolism [1, 13], direct evidence of changes in AIA metabolism resulting from PB-pretreatment was sought in the biliary excretion of AIA and its metabolites.

Factors affecting biliary excretion. The relationship between hepatic metabolism and subsequent biliary excretion is complex. PB, for example, increases biliary flow [14] and this can increase biliary excretion of simultaneously administered drugs; biliary excretion is impaired in cholestasis but there is no direct relationship between changes in bile flow rates and in biliary elimination. The significance of the rapid choleretic effect of AIA, maintained for 2 hr after injection of PB-pretreated fistula rats and its relevance to the processes involved in the biliary secretion of AIA and its metabolites is difficult to assess. In the fistula rats maximum excretion of AIA and its metabolites occurred when there was greatest biliary flow. However, the rates of biliary excretion of AIA and both metabolites in the fistula rats were almost identical with those in the perfused livers from fasted and PB-pretreated, fasted donors. In the perfused livers the concentration of AIA and its metabolites increased 4- to 5-fold without large sustained increases in bile volume, after PB-pretreatment of the liver donor. This indicated that in the perfused liver the fraction of bile flow, which is independent of bile acid secretion [15], enabled biliary excretion of PB-pretreated and fasted donors at rates similar to those in fistula rats but without increased bile flow. Since a bile acid-independent excretion of 70 μl bile per kg body wt has been suggested in rats [16] the 2-fold biliary flow in the fistula rats, already enhanced by the PB-pretreatment, may be due to increased secretion of the bile acid-dependent fraction of total flow.

The biliary excretion of AIA and its metabolites, analyzed according to the two-compartment model, and the initial excretion rates showed good agreement between the perfused liver and the bile fistula rats. This model was used in an attempt to compare the rate constants for the various transfer steps. PB-pretreatment resulted in a 50 per cent decrease in the rate of transfer from liver to bile of metabolite D in both the perfused liver and in the fistula rats and for AIA in the fistula rats. If the rate of metabolism of AIA to metabolite D was the rate-limiting step in its biliary secretion in normal rats, as suggested for 3-MC* by Levine [17], PB-pretreatment, resulting in the induction of drug-metabolizing enzymes, might be expected to increase the rate of transfer from liver to bile of metabolite A. The faster transfer of metabolites from liver to bile is consistent with increased polarity and subsequently more rapid elimination

* 3-Methyl cholanthrene.

from the liver. Alternatively, because of conflicting evidence as to whether metabolism is rate-limiting in biliary excretion [18–21], the rate constant, k_2 , in this work may be due to rate-limiting transport into the cytosol or into the bile. The occurrence of conjugated urinary metabolites of AIA in intact rats and the low proportion of metabolized AIA present in the perfusate, indicate that biliary secretion of AIA and its metabolites, although small, may be a significant route of excretion for the complete elimination of AIA from the intact animal.

Abnormal breakdown of P-450 after AIA. AIA caused a decrease in microsomal P-450 levels in the perfused liver of PB-pretreated rats. The destruction of microsomal P-450 was confirmed in intact rats, similarly pretreated. There was a smaller, but significant, decrease in normal, fed rats. The turnover of microsomal P-450 haem with radioactive ALA has been shown to be biphasic, with an initial half-life of 8–10 hr and a second slower phase of half-life 24–48 hr [22–24]. Corresponding half-lives of 11 and 50 hr have been reported after phenobarbitone [25]. Evidence is presented for at least three components of microsomal P-450 breakdown after AIA. The value for the second phase after AIA in PB-pretreated rats agrees with that reported by Meyer and Marver after chronic treatment with AIA to PB-pretreated rats. The relationship between the very rapid initial decline reported here and the rapid production of malonaldehyde from microsomal lipid peroxidation with evolution of CO [26] remains to be elucidated. It may be related to an hitherto undetected very early phase of the initial component thought to contribute in normal animals to one of the early non-erythropoietic peak(s) of labelled bilirubin occurring within a few minutes of [^{14}C] ALA injection [27]. Landaw [28] suggested two, three or more components of the early-labelled peak of [^{14}C] CO occurring within 1–2 hr after injection of [^{14}C] ALA. After AIA the early-labelled bilirubin was not increased in contrast to carbon monoxide production from the methene bridge carbon atoms of the protoporphyrin nucleus, emphasizing the abnormal breakdown of the haem after AIA [29, 30].

The decrease in P-450 after AIA, originally thought to represent loss of haem solely from the microsomal cytochromes, is now considered to reflect the presence of a small hepatic haem fraction with a short biological half life and this fraction is increased by PB [37, 1]. Hepatic P-450 concentration also decreases after exposure to hepatotoxic compounds such as carbon tetrachloride [32] or carbon disulphide [33]. Cobaltous chloride also decreases P-450 concentration by inhibiting its synthesis at the ferrochelatase step [34]. The loss of haem after AIA *in vivo* results from destruction of existing haem of the microsomal fraction and cytosol [35, 17, 36] rather than to an inhibition of synthesis. The breakdown products produce a characteristic green colouration of the microsomes [1, 25].

In the perfused liver, although the decrease in P-450 was quantitatively similar to that found in intact rats, the initial decline was slower. These differences may be contingent on factors such as the concentration of AIA in the perfusate, extent of protein binding of AIA and the influence of these on the uptake of AIA by the perfused liver.

A non-linear dose-response between the s.c. injection of 20–200 mg AIA per kg body wt and the decrease of microsomal P-450 1 hr after injection has been shown in PB-pretreated rats [25]. A dose-response was not clearly apparent in the perfused liver except that the decrease in microsomal P-450 was variable at the lowest concentrations of AIA and a decrease of 50 per cent at 1 hr consistently occurred for 0.3–3.0 mg AIA/ml; (0.2–0.7 mg AIA/ml corresponded to 100–400 mg AIA per kg body wt).

Factors affecting the metabolism of AIA in vivo. The observed biliary excretion patterns provide direct evidence that PB-pretreatment enhanced the hepatic metabolism of AIA and that this increased metabolism was accompanied by an increased disappearance of hepatic microsomal cytochrome P-450 induced by the PB-pretreatment. The production of metabolite D in normal rats and after PB can be correlated with the decrease in P-450. Should studies on the decay of radioactively-labelled haem confirm the presence of a component with a half-life of 4.0 min., this half-life is of an order compatible with the concept of substrate binding (AIA) to monooxygenase P-450 during metabolism. Reactive metabolites, covalently bound at their site of action [37], usually have a short biological half-life, and they may not be excreted by the liver. A reactive metabolite of AIA responsible for P-450 loss might be metabolized to a stable derivative such as metabolite D which may be a glutathione conjugate of AIA epoxide. Such a conjugate might be hydrolyzed before or during t.l.c. to AIA-glycol, followed by lactone formation, but these two compounds could not be unequivocally detected in the bile.

The decrease of P-450 after AIA is lessened when P-450 has been induced by 3-MC (Unsold and De Matteis, personal communication) which may be a result of changes in the metabolism of AIA. The decrease in P-450 after secobarbital is also low in control and 3-MC-treated rats [2]. There is some evidence of epoxide metabolites of both AIA and secobarbital and the protective effect of 3-MC could be due to (1) differences in rates of routes of metabolism influencing the rate of production of the epoxide, (2) induction of epoxide hydrolase by 3-MC influencing the biological "half-life" of the epoxide [38], (3) availability of conjugating moieties, such as glutathione, influencing the half-life of the epoxide as shown for bromobenzene [37], or (4) a specific effect on one of the P-450 "cytochromes".

Lability of a specific P-450 cytochrome after induction by PB, due to binding or proximity to AIA, AIA epoxide or other "active metabolite" during metabolism with subsequent effects on AIA metabolism, rather than to a decrease in the "regulatory haem pool" could explain the lack of induction of ALA-S [12] (Smith, personal observation) by AIA in PB-pretreated rats despite the rapid decrease in P-450 concentration. However, a decrease in P-450 is not always associated with epoxide formation, e.g. bromobenzene [39], but is often related to ALA-S induction [1]. In chick embryo liver cell cultures compounds such as AIA are not readily metabolized but are potent inducers of ALA-S [40, 41]. The lack of induction of ALA-S by AIA in intact rats after PB-pretreatment was considered by Kaufman, Swan-

son and Marver [12] to be due to the increased rate of metabolism of AIA by the liver leading to low levels of AIA inadequate for ALA-S induction. The half-life of AIA in the blood was decreased from 5 hr to 0.5 hr after PB and the distribution of radioactivity in the hepatic cell fractions indicated a rapid hepatic elimination of AIA.

We recovered about 20 per cent of the administration AIA in the liver and excreted in the bile within 5 hr after AIA administration. Little radioactivity (about 4 per cent of the administered AIA in two PB-pretreated fistula rats) was excreted in the urine during this time. During perfusion the total radioactivity in the liver reached an average of 7.5 per cent dose (3–4 hr after AIA) and in the fistula rats it averaged 6 per cent and was unaffected by PB-pretreatment. Although the relative amounts in the liver as AIA and its metabolites were not determined, this hepatic radioactivity was equivalent to about 3 nmole AIA/mg liver (calculated from the specific activity of AIA). The initial rapid uptake of AIA with an average $t_{1/2}$ of 1.3 min by perfused livers from fasted and PB-pretreated, fasted rats was almost identical with the rapid hepatic uptake of benzpyrene, $t_{1/2}$ of 1.7 min, after i.v. injection in intact rats [47].

Excretion patterns have often been used to classify human porphyrias and have enabled some comparisons to be made with the experimental porphyrias. Modern techniques have demonstrated enzyme deficiencies in two types of human porphyria. In DDC-induced porphyria there is inhibition of ferrochelatase, with a dose-response in terms of porphyrin accumulation; but the action of other porphyrinogenic agents such as AIA, HCB and griseofulvin, where excretion patterns and induction of ALA-S have been investigated in great detail in several species, is still unknown. The effect of PB-pretreatment on the pattern of biliary excretion and the *in vitro* destruction of microsomal P-450 show the perfused liver to be a useful system for investigations (especially during the initial first 2 hr) of the mode of action AIA in relation to its metabolism.

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APPENDIX

A two-compartment model of the biliary excretion of AIA and its metabolites in the isolated perfused rat liver

[2-¹⁴C]Allylisopropylacetamide (AIA) was given as a bolus injection into the blood at $t = 0$. The decrease of

radioactivity from the blood was followed throughout the perfusion and the radioactivity present as AIA or its two metabolites was determined in consecutive bile collections.

The two-compartmental analysis has been applied separately to AIA and to its metabolites (Fig. 13).

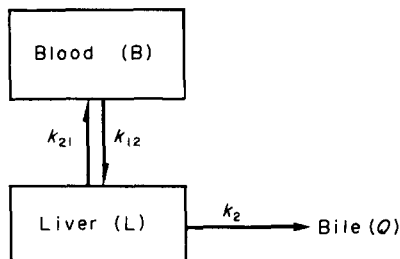


Fig. 13.

B , L and Q are the amounts at time, t , of radioactivity per administered dose of AIA or metabolite in blood, liver and bile compartments respectively. These can be converted to the concentrations by dividing by the volumes of the compartments; k_{12} , k_{21} and k_2 are first-order rate constants which are related to the graphically determined half life ($t_{1/2}$) by the equation $k = 0.693/t_{1/2}$.

Rate of removal from the blood,

$$-dB/dt = k_{12}B - k_{21}L \quad (1)$$

Rate of appearance in the liver,

$$dL/dt = k_{12}B - (k_2 + k_{21})L \quad (2)$$

Rate of excretion in the bile,

$$dQ/dt = k_2L \quad (3)$$

Assumptions for the model

(1) The radioactivity present as AIA or its metabolites in the various compartments, defined as B , L and Q changed exponentially.

(2) L was bi-exponential and is expressed viz

$$L = A_1e^{-\alpha_1 t} + A_2e^{-\alpha_2 t},$$

where A_1 and A_2 are constants, the intercepts at $t = 0$ of the semilog plots of the two components with gradients $-\alpha_1$ and $-\alpha_2$ respectively.

(3) Protein binding was negligible but there is no experimental evidence that this is so.

(4) (a) *For the first five minutes of perfusion.* Passage of AIA from the blood to the liver was assumed to be by diffusion in the direction of a concentration gradient. Formation of metabolites was assumed to be negligible during this time and thus it was considered that no appreciable mass flow of AIA metabolites from liver to blood occurred.

(b) *From five minutes of perfusion until the end of the perfusion.* Unmetabolised AIA can diffuse (i) from the blood into the liver, or (ii) from the liver back into the blood depending on the extent of uptake of AIA, or (iii) from liver to bile. During perfusion the blood concentration of AIA was always greater than in the liver, and the concentration of gradient (or net material flow) for AIA was now from liver to bile. In one perfusion the relative proportions of AIA and its metabolites were determined and by 2 hr 94 per cent of the circulating radioactivity was still present as unmetabolised AIA. The net material transfer of AIA and its metabolites from the liver was probably predominantly into the bile under the experimental conditions.

The analysis of the radioactivity excreted in the bile (Q) was considered before and after the maximum (Q_{\max}) and related to two separate phases of the blood disappearance curve. The disappearance of ^{14}C from the blood was bi-exponential during perfusion. By comparison with the rate

of excretion of ^{14}C in the bile it was considered that for the first five minutes the blood disappearance of ^{14}C was predominantly mono-exponential with a short half-life and thereafter for the rest of the perfusion the component with a longer half-life predominated.

For the model when $t = 0$, $B = \text{AIA administered}$, $L = 0$ and $Q = 0$

$$-dB/dt = k_{12}B \quad (5)$$

$$dL/dt = k_{12}B - k_2L \quad (6)$$

For 0–5 min, $k_{12} = k_{12(\text{fast})}$

From 5 min to end of perfusion $k_{12} = k_{12(\text{slow})}$,

$$B = \beta e^{-k_{12}t} \quad (7)$$

$$L = A_1(e^{-k_{12}t} - e^{-k_2t}),$$

where

$$A_1 = k_{12}/(k_2 - k_{12}) \quad (8)$$

and

$$Q = A_1e^{-k_2t} - (k_2A_1/k_{12})$$

$$e^{-k_{12}t} + A_1(k_2 - 1)/k_{12}$$

$$= A_1e^{-k_2t} - (k_2A_1/k_{12})e^{-k_{12}t} + 1. \quad (9)$$

Since B , L and Q have been expressed as dpm/unit dose the total amount of radioactivity in each compartment can be calculated by multiplying B , L and Q by the dose. Thus when $t \rightarrow \infty$, $Q \rightarrow 1$. Total excreted in bile as $t \rightarrow \infty = 1 \times \text{injection dose}$. The observed time course of Q is characteristic of a system illustrated in Fig. 14 and which can be described by the set of differential equations. During the early time course there is a net material flow of AIA and any metabolites formed from the liver to bile. As the concentration in the liver increases so the net transfer from liver back to blood can increase and this would contribute to the observed decrease in the level of Q .

Q_{\max} occurred at about 30 min of perfusion with AIA and thereafter the steep decline was bi-exponential, due predominantly to the transfer from liver to bile with rate constant k_2 , modified by the flow along the pathway characterised by the rate constant $k_{12(\text{slow})}$. From then until the end of perfusion, equations (7), (8) and (9) apply, the true values of k_2 being calculated from the gradient of the fast component of the bi-exponential decline obtained graphically to subtraction of the slow component from the initial experimental curve. The component with the long half-life ($t_{1/2}$) is due to the slow uptake of AIA from the blood, i.e. the component with rate constant $k_{12(\text{slow})}$ will be made slower depending on the magnitude of the component with the rate constant k_{21} . The slow component was difficult to assess from the biliary data of the perfused livers because these perfusions were ended only 3 and 4 hr after the administration of AIA. The biliary excretion data from the perfused livers were therefore extrapolated using the gradient for the slow component derived from the

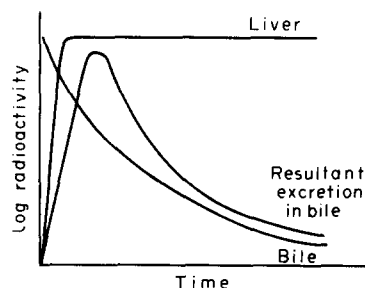


Fig. 14. Theoretical time course of radioactivity as AIA or a metabolite in liver and bile.

blood data, this gave a satisfactory assessment of the value of k_{12} since, using the data from the bile fistula rats, halving the $t_{1/2}$ of the slow component ($k_{12(\text{slow})}$) caused a decrease of only 4 per cent in k_2 .

In the fistula rats the rate constants for the disappearance of AIA in the blood will be affected by slow absorption from the s.c. injection site, extrahepatic uptake of AIA and excretion in the urine. These influences will decrease

the rate at which Q_{max} was attained, decrease the value of Q_{max} and influence the gradient of the component from which k_2 was derived. The biliary excretion of AIA and its metabolites by a fed fistula rat rose slowly to a low Q_{max} which decreased slowly compared with the excretion by PB-pretreated rats which agreed well with the model (Fig. 8.).