1 A, B). Activatory effects of ATP of the same magnitude were obtained when plasma membranes were preincubated for 5 min in the presence of activators, Mg^{2+}, and only 0.5 mM ATP (Table 1).

Creatine phosphate, 15 mM in the absence of Mg^{2+}, inhibited the activation of adenylate cyclase by either activator. In the presence of 4 mM $MgCl_2$, we did not observe any inhibitory effect of creatine phosphate. In the next preincubation experiments we used regenerating system and established that as low as 0.1 mM concentration of ATP caused 20 per cent increase of the fluoride or Gpp(NH)p preactivation in the presence of 4 mM Mg^{2+}, (preincubation time 5 min).

The effect of ATP + Mg^{2+} was not diminished by 2-deoxyadenosine, a potent inhibitor of the phosphorylation of liver plasma membrane proteins (unpublished data). Therefore, this ATP effect is unlikely to be linked to phosphorylation.

Due to the action of nucleotide pyrophosphatase and 5′ AMP nucleotidase of plasma membrane, a significant amount of adenosine is formed from ATP during incubation [12]. Adenosine, tested up to 0.5 mM, did not mimic the stimulatory effect of ATP.

The effect of ATP, in principle, could be due to contaminant GTP [4]. GTP, (10 μM) however, reduced the Gpp(NH)p activation by 30 per cent. The possible reason for this inhibition is that GTP is hydrolysed to GDP and the latter also binds to the guanine nucleotide binding protein keeping the cyclase system in the low activity state [13–15]. GTP did not significantly modify the preactivatory effect of fluoride.

In summary, present data indicate that the Mg^{2+}-complexed ATP, besides its substrate role, has a secondary stimulatory effect on the adenylate cyclase activity. This effect is observed at concentrations which are generally used for adenylate cyclase assay. The following mechanisms and artefacts, which could explain this effect of ATP, were ruled out: (1) suspension of the inhibitory effect of creatine phosphate by ATP; (2) stimulation by adenosine or GTP instead of ATP; and (3) the role of phosphorylation. The site of the ATP effect may be the regularory protein or the complex of the catalytic unit and regulatory protein. Since guanine nucleotides and fluoride act through a common regulatory protein [16–18], it is not surprising that Mg^{2+}-complexed ATP influences their effects similarly. The regulatory protein also participates in the hormone stimulation of the cyclase system [19]. Therefore, it is likely that the observed ATP effect is a general phenomenon. Further work on the clarification of this latter point and on the exact mechanism of ATP effect is in progress in our laboratory.

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Abolition of the apparent deficiency of 2-aminophenol glucuronidation in perfused Gunn rat liver by pentan-3-one

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A wide variety of endogenous and exogeneous compounds including drugs, xenobiotics and carcinogens are conjugated with glucuronic acid by microsomal UDP-glucuronosyltransferase (EC 2.4.1.17) prior to excretion and elimination from mammals [1]. Our current knowledge of the regulation of UDP-glucuronyltransferase activity *in vivo* is limited and study of the genetic deficiency of this enzyme provides a good opportunity to examine this problem.

In 1938, Gunn described a mutant strain of Wistar rat which exhibits hereditary hyperbilirubinaemia [2]. UDP-glucuronosyltransferase activity has been shown to be defective in Gunn rat liver, and it has been shown that biochemical lesion in Gunn rat liver results in the complete inability to glucuronidate bilirubin *in vivo* or *in vitro* [3, 4]. This transferase deficiency is also recognised by a very poor ability to glucuronidate 2-aminophenol *in vitro* [5, 6]; however, addition of diethylnitrosamine [7] or alkyl ketones [8, 9] to Gunn rat liver homogenates *in vitro* surprisingly raised the deficient UDP-glucuronosyltransferase activity towards 2-aminophenol up to the activity levels in similarly-treated Wistar rat liver homogenates, such that this deficiency of UDP-glucuronosyltransferase activity was no longer apparent.

It is important to compare the regulation of UDP-glu-

curonyltransferase activity measured *in vitro* with regulation of glucuronidation *in vivo*, in order that artifacts arising during isolation of the transferase are eliminated. Is the glucuronidation of 2-aminophenol by Gunn rat liver deficient *in vivo*? If so, can the deficiency of 2-aminophenol observed in Gunn rat liver *in vivo* be abolished by alkyl ketones?

The only evidence obtained so far has involved the use of liver slice assays. Winsnes and Dutton [10] compared the rate of glucuronidation of 2-aminophenol in slices and homogenates of Gunn and Wistar rat liver. Their results showed that UDP-glucuronosyltransferase activity towards 2-aminophenol in native Gunn rat liver homogenate was only approximately 10 per cent of the activity in unactivated Wistar liver homogenate, whereas glucuronidation of 2-aminophenol by Gunn rat liver slices was approximately 40 per cent of the equivalent measurement with Wistar rat liver. These results suggest that UDP-glucuronosyltransferase activity in Gunn rat liver towards 2-aminophenol may be unstable during isolation and that normal levels of activity may be operative *in vivo*. Indeed, inactivation of the transferase from Gunn rat liver does occur during its isolation and purification [11]. The apparent homogeneous preparations of the enzyme are completely inactive towards 2-aminophenol, although this activity is restored by the addition of diethylnitrosamine [11] or ketones [8].

Unfortunately the metabolism of 2-aminophenol cannot be easily studied *in vivo*, since it is a very toxic compound, due to formation of ferrihaemoglobin in the presence of oxygen [12]. Thus in this paper we have tried to reproduce the situation *in vivo* as far as possible by studying glucuronidation of 2-aminophenol in perfused rat liver. The results show that glucuronidation of 2-aminophenol is deficient in perfused Gunn rat liver and that the defect can be abolished by inclusion of pentan-3-one in the perfusion medium.

Homozygous Gunn rats were from the colony maintained in the Medical Sciences Institute Animal Unit, University of Dundee. This colony was started in 1977 from stock purchased from Prof. B. H. Billing, Royal Free Hospital, University of London. Wistar rats were obtained from the Animal Unit, Institute of Pharmacology and Toxicology, University of Göttingen.

Male Gunn and Wistar rats (350–400 g) were fed *ad lib.* a standard diet containing 20 per cent protein (Altromin, Lage-Lippe, F.R.G.). Liver:body weight ratio of about 4 per cent was not significantly different in both rat strains.

Pentan-3-one (99% pure) and 2-aminophenol (twice resublimed before use) were obtained from B.D.H. Chemical Ltd., Poole, U.K. 2-Aminophenyl glucuronide was from Koch Light Labs., Colnbrook, U.K. D-Saccharic acid 1,4-lactone, 4-nitrophenylglucuronide, 4-nitrophenylsulphate, UDP-glucuronic acid triammonium salt, were from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. Arylsulphatase from *Helix pomatia* was purchased from Boehringer, Mannheim, F.R.G. All other reagents were the purest grade available.

β-Glucuronidase was partially purified up to an ammonium sulphate extract from rat preputial glands by the method of Levvy *et al.* [13]. The specific activity of these preparations was >0.7 μmoles 2-aminophenylglucuronide hydrolysed/min/mg protein. β-Glucuronidase was completely and specifically inhibited by 12 mM D-glucaro 1-4-lactone. The enzyme preparation did not exhibit any arylsulphatase activity towards 4-nitrophenylsulphate as substrate.

UDP-glucuronosyltransferase activity towards 2-aminophenol was measured as previously described [14], and microsomal protein was determined by the method of Bradford [15].

Gunn and Wistar rat livers were perfused with 70 ml of Eagle's minimum essential medium containing 2% (w/v) bovine serum albumin and 0.1% (w/v) heparin, pH 7.4, as described [16, 17], except that erythrocytes were omitted from the perfusion medium. 2-Aminophenol (35 μmoles) and 10-fold molar excess of ascorbic acid (added to prevent auto-oxidation in the perfusion medium) were dissolved in the perfusion medium. Addition of 2-aminophenol, ascorbic acid and pentan-3-one did not alter the pH of the medium. Recirculating perfusion system was used at 35° (to reduce anoxia) and 2 ml samples were removed for analysis after various time intervals up to 60 min. After perfusion the average Gunn liver wet weight was 14.4 ± 1.4 g (N = 15) and the average Wistar liver wet weight was 13.3 ± 1.8 g (N = 10) and thus liver weights from the two rat strains were not significantly different.

The bile duct was not cannulated during perfusion experiments and conjugate excreted in bile was captured in the perfusate.

Aliquots of perfusion medium were assayed for the formation of 2-aminophenylglucuronide by the method of Levvy and Storey [18]. All of the detectable conjugate was completely hydrolysed by incubation with 70 μg rat preputial β-glucuronidase at pH 4.5 and 37° for 30 min. Addition of 12 mM glucaro 1-4-lactone together with β-glucuronidase completely abolished conjugate hydrolysis. Treatment of this conjugate with 250 μg arylsulphatase at 37° for 60 min did not hydrolyse any of the conjugate, indeed 2-aminophenol sulphate formation cannot be detected by the diazotation technique since the sulphate compounds do not interfere under these stringent conditions.

The presumed 2-aminophenylglucuronide in the perfusates was further characterized by thin layer chromatography. Two millilitres of 60 min perfusate was mixed with 1 ml of 0.5 M glycine–HCl buffer pH 1.9. Then the 2-aminophenol conjugates were extracted with 5 ml of *n*-butanol by shaking for 30 min. The separated butanol phase was removed and stored. The aqueous phase was extracted with a further 5 ml of butanol. The butanol extracts were pooled and concentrated to 0.5 ml under reduced pressure. These samples were analysed by t.l.c. on silica gel with ethanol–water–acetic acid (76:19:5).

Visualisation of 2-aminophenol and the major conjugate spots by a spray reagent [19] revealed the presence of two staining spots with mobilities identical to 2-aminophenol glucuronide $R_f = 0.59$ and 2-aminophenol $R_f = 0.74$.

Pairs of Gunn and Wistar rat livers were perfused simultaneously with 2-aminophenol using two identical sets of apparatus in one incubation chamber. The rate of glucuronidation of 2-aminophenol by perfused Gunn rat liver (0.16 μmoles/g wet wt liver/hr) is only 33 per cent of the rate of formation of 2-aminophenylglucuronide by perfused Wistar rat livers (0.48 μmoles/g wet wt liver/hr) (Fig. 1). This result agrees with values (40%) obtained by the liver slice experiments of Winsnes and Dutton [10], but is much higher than the 8 per cent recorded, using slice assays, by Arias [6]. Thus glucuronidation of 2-aminophenol does appear to be deficient in the isolated Gunn rat liver, although not to the same extent as when assayed in UDP-glucuronic acid-supplemented liver homogenates *in vitro* [7, 8]; under these conditions it is virtually absent.

The discrepancy between the extent of the deficiency measured by liver slice assays and UDP-glucuronic acid fortified homogenates [10] might be due to the presence of the 'physiological activator' UDP-*N*-acetylglucosamine [20]. Indeed, if the rate of glucuronide formation by UDP-*N*-acetylglucosamine-stimulated Gunn rat liver microsomes (Table 1) is calculated, using 36 mg microsomal protein/g wet wt liver [21], a value of 0.14 μmoles/g wet wt/hr is obtained, which is very similar to the rate, 0.16 μmoles/g wet wt/hr, observed in perfused Gunn rat liver.

The apparent deficiency of UDP-glucuronyltransferase towards 2-aminophenol can be abolished *in vitro* by treatment of liver homogenates with diethylnitrosamine [7] or alkyl ketones [8]. To see if this specific activation effect could be reproduced *'in vivo'*, pentan-3-one (700 μmoles) was added to the recirculating perfusion medium containing

2-aminophenol at 0, 20 and 40 min of the liver perfusion experiments. A three-fold increase in the rate of glucuronidation of 2-aminophenol by perfused Gunn rat liver up to the rate of 2-aminophenol glucuronidation by perfused Wistar rat liver was observed (Fig. 1). Pentan-3-one did not enhance 2-aminophenol glucuronide formation by perfused Wistar rat liver. It is possible that pentan-3-one is rapidly metabolized by both Wistar and Gunn rat liver cytosolic reductase [22] and does not reach concentrations sufficient to exert its non-specific membrane perturbation which is evident *in vitro*[8]; indeed, daunorubicin is reduced at a rate of 100 μmoles/20 min/rat liver [23] by a ketone reductase. Thus, the specific activator pentan-3-one indeed abolishes the apparent deficiency of 2-aminophenol glucuronidation in Gunn rat liver even in the intact organ.

Levels of transferase activity in Gunn and Wistar rat liver microsomal fractions were also compared after various treatments *in vitro*, to obtain further information about the nature of the enzyme deficiency. Table 1 shows that Gunn and Wistar rat liver microsomal UDP-glucuronosyltransferase activity towards 2-aminophenol are activated to the same extent by UDP-*N*-acetylglucosamine (approx. 4-fold) and by Brij 58 (3- to 5-fold), but only upon addition of pentan-3-one is the apparent transferase deficiency abolished *in vitro*. Treatment of Wistar rat liver microsomes with optimal concentrations of pentan-3-one (or diethylnitrosamine) plus detergent (Lubrol 12A9 or Brij 58) should produce fully activated forms of UDP-glucuronosyltransferase [17]. After such treatment similar UDP-glucuronosyltransferase activity was observed in Gunn and Wistar rat liver microsomes (Table 1). This similarity of what we may here term 'maximum expression' of transferase activity towards 2-aminophenol in treated microsomes again emphasises the existence in both Wistar and Gunn rat of similar transferase protein with potential activity towards 2-aminophenol.

UDP-glucuronosyltransferase can be purified to apparent homogeneity from Gunn rat liver as a functionally defective protein [11], which will exhibit a normal function when pentan-3-one is added to it [8]. Our perfusion experiments indicate that even in intact liver this functional deficiency of Gunn rat liver is abolished by pentan-3-one. All these findings suggest the existence in Gunn rat liver of UDP-glucuronosyltransferase for 2-aminophenol similar to that in Wistar rat liver but in a functionally defective native form.

It is highly instructive to compare these results with those obtained earlier with 1-naphthol as substrate. Glucuronidation of 1-naphthol is similar in perfused liver and in 'native' (unactivated) liver homogenates obtained from both Gunn and Wistar rats, suggesting that 1-naphthol glucuronide formation is not deficient in the Gunn rat [17]. This result is surprising since the UDP-glucuronosyltransferase purified to apparent homogeneity from Wistar rat liver accepts both 1-naphthol and 2-aminophenol as substrates [24, 25] and, thus, UDP-glucuronosyltransferase from Gunn rat liver should also have a defective ability to

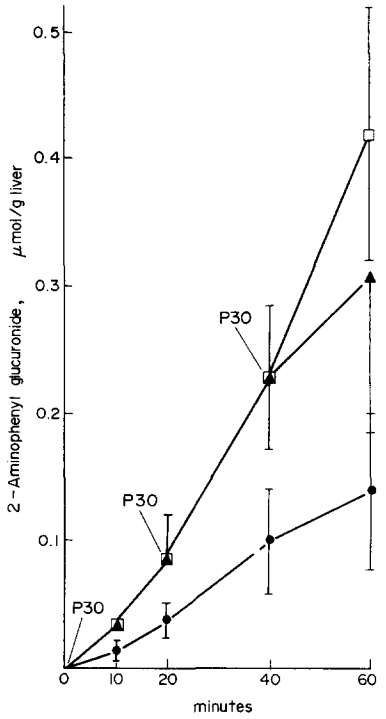

Fig. 1. Appearance of 2-aminophenylglucuronide in the perfusate from Gunn and Wistar rat livers. The mean of four liver perfusions ± S.D. are shown. ●——● Gunn rat liver; ▲——▲ Gunn rat liver + pentan-3-one; □——□ Wistar rat liver ± pentan-3-one. 700 μmoles of pentan-3-one (P3O) dissolved in perfusion medium was added at the time points indicated by the arrows, immediately after sampling of the medium had taken place.

glucuronidate 1-naphthol. However, when this transferase is assayed in detergent-treated homogenates or microsomes, the relative deficiency of activity towards 2-aminophenol is exaggerated (Table 1) and a defective transferase activity towards 1-naphthol is then revealed [11, 17]. This enzyme isolated from Gunn rat liver was also observed to be functionally defective towards both substrates throughout purification [11]; but whereas activity towards 2-aminophenol could be restored to normal levels by treating the purified protein and microsomal fractions with diethylnitrosamine [11] or pentan-3-one [8], that to 1-naphthol could not [17].

Although the nature of the defect of Gunn rat liver UDP-glucuronosyltransferase towards 2-aminophenol remains to be elucidated, the present results demonstrate that pentan-3-one is able to abolish this deficiency in the intact liver. Hence the deficiency observed earlier with microsomes or

Table 1. Effect of membrane perturbants and UDP-*N*-acetylglucosamine on 2-aminophenol UDP-glucuronosyltransferase activity of male Gunn and Wistar rat liver microsomes*

Additions to the assay *in vitro*	UDP-glucuronosyltransferase activity (nmoles glucuronide formed/min/mg protein)	
	Gunn	Wistar
None	0.02 ± 0.007	0.11 ± 0.02
UDP-*N*-acetylglucosamine (3 mM)	0.07 ± 0.02	0.49 ± 0.21
Pentan-3-one (10 mM)	0.27 ± 0.07	0.27 ± 0.07
Brij 58 (0.05% w/v)	0.06 ± 0.01	0.53 ± 0.15
Brij 58 + Pentan-3-one	1.31 ± 0.42	1.44 ± 0.25

* Membrane perturbants were added to enzyme assays at concentrations giving optimal enzyme activation in both rat strains. The mean values ± S.D. of 5 experiments are shown.

UDP-glucuronosyltransferase purified to apparent homogeneity is not merely an artifact due to the isolation procedure but reflects the enzyme defect *in vivo*.

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Inhibition of hepatic lipid biosynthesis by 1-(3-chlorophenyl)-1-methyl-2-phenyl-2-(2-pyridine) ethanol, a hypocholesterolemic agent

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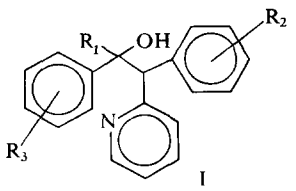
A large number of derivatives of generic structure I [2-(2-pyridine)-1,2-diarylalkanols] were prepared by Burckhalter *et al.* [1] as potential hypocholesterolemic agents having minimal estrogenicity.



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Several of the compounds exhibited significant serum cholesterol-lowering activity in rats with only slight estrogenic effect. Compound 1 (R_1 = CH_3, R_2 = H, and R_3 = *m*-Cl) [1-(3-chlorophenyl)-l-methyl-2-phenyl-2-(2-pyridine) ethanol] was selected from a series of the 133 derivatives for study of its hypocholesterolemic effect in humans. Although the compound was non-toxic and was found to lower serum cholesterol levels in rats, it had no hypocholesterolemic effect in humans or in monkeys [1]. To understand why compound 1 was active in rats, the present investigation was initiated. The results suggest that this compound, or a metabolite(s), lowers serum cholesterol levels by inhibiting hepatic lipid biosynthesis.

Male Sprague–Dawley rats weighing 150–180 g were used for all experiments. Rats were housed two per cage, with Purina Rat Chow and water available *ad lib*. Compound 1 was suspended in 0.5% (w/v) sodium carboxy methyl cellulose (CMC) by mixing in a glass mortar. The suspension was administered to experimental animals (four or five rats) at 25 $mg \cdot kg^{-1} \cdot day^{-1}$ by gastric intubation once a day in the morning for 4 days. Control animals (four or five rats) received equivalent volumes of 0.5% CMC. Animals were weighed daily. On the morning of day 5, [2-^{14}C]acetate (10 μCi/100 g body wt in 0.5 ml saline) was injected intraperitioneally into both control and experimental animals. Exactly 1 hr after the injection of radioactive precursor, blood was collected by cardiac puncture into a test tube containing heparin sodium (100 units). Animals were under light ether anesthesia. The livers were then removed, washed twice with ice-cold saline, and homogenized in