Effects of 3 synthetic peptides analogous to neurohypophyseal hormones (obtained in the analytically pure state from Protein Research Foundation, Osaka) on the excitability of 2 identifiable giant neurones (the PON, periodically oscillating neurone; and the TAN, tonically autoactive neurone) of *Achatina fulica* Férussac (bath application)

Substance	Amino acid sequence	Concentration (kg/l)	Effect on PON	Concentration (kg/l)	Effect on TAN
Deamino-dicarba-oxytocin (D-D-oxytocin)	⌊→Tyr-Ile-Gln-Asn-Asu-Pro-Leu-Gly-NH_2	10^{-4} [a]	E	2×10^{-4}	(—)
Deamino-dicarba-Arg-vasotocin (D-D-Arg-vasotocin)	⌊→Tyr-Ile-Gln-Asn-Asu-Pro-Arg-Gly-NH_2	10^{-4} [a]	E	2×10^{-4}	(—)
Deamino-dicarba-Arg-vasopressin (D-D-Arg-vasopressin)	⌊→Tyr-Phe-Gln-Asn-Asu-Pro-Arg-Gly-NH_2	2×10^{-4}	(—)	2×10^{-4}	(—)

E, excitatory effect. (—), no effect. [a], critical concentration to produce the effect. Asu, α-amino suberic acid.

On the other hand, the microdrop application of D-D-Arg-vasopressin in the same amount did not affect the PON biopotential (Figure 2, C). We conclude from these results that the PON excitation caused by the first 2 peptides is due to the membrane depolarization of this neurone.

In Figure 3, two I–V curves (a, measured in the physiological state, and b, measured 3 min after 2×10^{-4} kg/l D-D-oxytocin application) have been superimposed. In the presence of D-D-oxytocin at this concentration, the PON neuromembrane was remarkably depolarized. In Figure 3A, the two I–V curves have been superimposed using the initial polarization level (just before the transmembrane current application) as the common standard. It seems that the PON neuromembrane resistance may be decreased by D-D-oxytocin. On the other hand, in Figure 3B, the same two I–V curves have been superimposed using the firing level of the neurone as the common standard. The two I–V curves are concordant in the wide range of membrane polarization level. We conclude that the I–V curve measured in the presence of D-D-oxytocin at 2×10^{-4} kg/l is almost identical with that in the physiological state (Figure 3B) and that the depolarization of the neuromembrane, produced by D-D-oxytocin, only appears to cause some decrease of membrane resistance, since the I–V curve of the molluscan giant neurone is not linear[9,10] (Figure 3A).

By bath application the 3 peptides analogous to neurohypophyseal hormones with respect to the excitability of the TAN (tonically autoactive neurone) were examined. These peptides at 2×10^{-4} kg/l had no effect on TAN excitability.

BARKER et al.[11] reported that the neurohypophyseal hormonal peptides, including Arg-vasopressin, oxytocin and Arg-vasotocin, caused membrane depolarization of slowly oscillating giant neurones of *Otala lactea* (Cell 11[12]) and of *Aplysia californica* (R 15[13]), and augmented their bursting pace-maker potential (BPP) activities. Our experimental results with the PON of *Achatina fulica* using synthetic peptides (deamino-dicarba-) analogous to neurohypophyseal hormones were somewhat different than those of GAINER et al., while assuming[4] the PON to correspond to Cell 11 of *Otala lactea*. The critical concentration of D-D-oxytocin and D-D-Arg-vasotocin to produce an excitatory effect on the PON is much higher than that of Arg-vasopressin, oxytocin etc. reported by GAINER et al.

The PON neuromembrane was not sensitive to D-D-Arg-vasopressin at 2×10^{-4} kg/l. The amino acid sequence of D-D-Arg-vasotocin differs only at the second amino acid from that of D-D-Arg-vasopressin. Our results show that the PON neuromembrane can discriminate this structural difference between the two substances. We can say that D-D-oxytocin and D-D-Arg-vasotocin showed a specific excitatory effect on the PON neuromembrane.

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Metabolism of a Biliary Metabolite of Phenacetin and Other Acetanilides by the Intestinal Microflora

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Summary. In vivo, rat intestinal micro-organisms mediate the metabolic hydrolysis of the biliary metabolite (*N-acetyl-p-aminophenyl glucuronide*) *of phenacetin and related compounds.*

In the course of studies on the significance of the intestinal microflora in the metabolism of phenacetin and related compounds in the rat[3,4], evidence has been obtained that *N*-acetyl-*p*-aminophenyl glucuronide is excreted in part in bile and is subsequently metabolized by the intestinal microflora.

Methods and materials. Details of the synthesis of [^{14}C]labelled compounds, chromatographic and radiochemical methods employed are given in our previous publications[3,4]. The biliary metabolite was characterized by co-chromatography with authentic reference compounds[4], autoradiography and by specific chemical and enzyme-degradative procedures[4,5]. Microfloral incubations were carried out by our usual procedure[3]. Conjugates or bile extracts containing conjugates were sterilised by membrane filtration, using sterile Swinnex filter units fitted with white plain filters (Millipore S. A., France).

Male and female Wistar derived rats were dosed with test substances (50 mg/rat, p.o.) as suspensions in water (to which bile salts had been added) and were subsequently maintained in 'Metabowl' metabolism cages[4]. Urine, faeces and CO_2-trapping fluid were collected during experiments, through which periods the animals had free access to food and water. Some male rats were also pretreated with neomycin sulphate (30 mg/day, p.o. to each of 5 rats) or with a (5:7) aureomycin hydrochloride-phthalylsulphathiazole mixture (120 mg/day, p.o. to each of 2 rats).

Four female rats, which had been bred under germ-free conditions and from which the normal intestinal flora had been shown by bacteriological testing to be absent, were kept in metabolism cages, situated in sterile plastic jacket isolators[6,7] and given γ-irradiated Dixons PRM diet $\pm$ test substances and sterile water.

Biliary secretions were sampled, following cannulation of the common bile duct of rats maintained in specially designed restraining cages[8].

Results. Although the presence of *N*-acetyl-*p*-aminophenol was clearly demonstrated in the faeces of normal rats after dosage with phenacetin, paracetamol, methacetin or acetanilide, it was not detected in the faeces of similarly dosed animals which had received antibiotics orally or which were germ-free. Nevertheless, this compound was found in faecal extracts from previously antibiotic-treated rats in which the gastrointestinal flora had subsequently been allowed to develop, and from germ-free rats following conventionalization.

The biliary excretion of radioactivity following the administration of radiocarbon-labelled acetanilide derivatives is given in the table, and in each case, the only biliary metabolite detected was *N*-acetyl-*p*-aminophenyl glucuronide. Moreover, incubation of bile extracts, derived from animals dosed with these compounds and from other rats given (50 mg, p.o.) *p*-phenetidine hydrochloride, *p*-anisidine or Benorylate (4-acetamidophenyl-2-acetoxybenzoate), with mixed inocula of rat caecal microorganisms under anaerobic conditions resulted in the formation of *N*-acetyl-*p*-aminophenol. This aglycone was also formed when an authentic sample of *N*-acetyl-*p*-aminophenyl glucuronide (provided by Dr. J. Shibasaki, Nagasaki University, Japan) was similarly incubated.

The microflora was also shown to be capable of effecting hydrolysis of two related conjugates not normally present in bile namely *N*-acetyl-*p*-aminophenylsulphate and *p*-aminophenyl glucuronide which when incubated under identical conditions readily gave rise to their corresponding aglycones.

Discussion. The results obtained during experiments, in which germ-free rats and antibiotic-treated rats were used, indicate that the formation of the faecal metabolite, *N*-acetyl-*p*-aminophenol, in vivo was dependent upon the activities of the gut microflora. Moreover, our present studies show that *N*-acetyl-*p*-aminophenol glucuronide is voided in the bile of rats, and that this occurs to the extent of approximately 10–20% dose. The latter is consistent with an earlier report[9] that a significant level of biliary excretion (i.e. >10% dose) will occur when a compound voided in the bile of rats has some degree of polarity and a minimum molecular weight of 325 $\pm$ 50. Moreover, the difference in the proportion of the dose voided in the bile (10–20% dose) and faeces[4] (about 1% dose) of rats given [acetyl-^{14}C]phenacetin or [acetyl-^{14}C] paracetamol would seem to indicate some enterohepatic circulation of *N*-acetyl-*p*-aminophenyl glucuronide and/or its microbial product.

However, it is worth pointing out that other studies[10,11] with germ-free rats have indicated that β-glucuronidase associated with the gut may not be entirely of microbial origin. Moreover, the turnover of mucosal cells and indeed the activity of any β-glucuronidase[11] in the gut lumen would be influenced by the pH of intestinal contents and also by the diet of the animals. Unfortunately we cannot assess exactly the significance of tissue β-glucuronidase under our experimental conditions but had this been great, we would have expected to have found at least traces of *N*-acetyl-*p*-aminophenol in the faecal extracts derived from germ-free and antibiotic-treated rats. Although these extracts were monitored for this compound, it was not detected therein.

Evidence of microbial involvement in the degradation of *N*-acetyl-*p*-aminophenyl glucuronide was obtained when samples of the authentic compound or bile extracts containing it were incubated with rat caecal microorganisms thereby releasing the aglycone, *N*-acetyl-*p*-aminophenol. Although the microfloral hydrolysis of other glucuronides has been well documented[12], only a few instances of the microfloral degradation of ethereal sulphates have been reported[12]. However, we have demonstrated that *N*-acetyl-*p*-aminophenyl sulphate (a major urinary metabolite of phenacetin and related compounds)[4] is readily hydrolysed in vitro by incubates derived from the rat intestine.

We conclude from these studies that the intestinal microflora of the rat contributes to the metabolism of phenacetin and related compounds in the intact animal by effecting the degradation of the biliary metabolite, *N*-acetyl-*p*-aminophenyl glucuronide to *N*-acetyl-*p*-aminophenol which may facilitate reabsorption from the gut of the latter compound.

Biliary excretion of radioactivity by normal (untreated) male and female rats in the 0–24 h period, following oral administration of radiocarbon-labelled acetanilide derivatives (50 mg; 0.3–1.05 μCi)

Compound dosed	^{14}C Excretion[a] (% dose) in bile of a) male rats	b) female rats
[Acetyl-^{14}C]Phenacetin	7.9 (6.5–10)[4]	11 (9.0–13)[3]
[Acetyl-^{14}C]Paracetamol	14 (12–16)[3]	22 (14–28)[3]
[Acetyl-^{14}C]Acetanilide	15 (12–17)[3]	6.9, 7.8
[Acetyl-^{14}C]Methacetin	6.8 (6.0–8.0)[3]	15 (12–17)[3]

[a] Mean values are given, with the ranges in parentheses and the number of animal used indicated by the superscript. Where less than 3 animals were examined, individual results are given.
Bile collected over 24–72 h was also monitored but found to contain a negligible proportion ($\leq$1%) of the dose.

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