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UDP GLUCURONYLTRANSFERASE AND PHENOLSULFOTRANSFERASE *IN VIVO* AND *IN VITRO*

CONJUGATION OF HARMOL AND HARMALOL

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Abstract—Glucuronidation and sulfation of the phenolic —OH group of harmol and harmalol by the rat in *vivo*, and by rat liver subcellular fractions *in vitro* have been studied. *In vivo* harmol was extensively excreted (59% of the dose in 3 hr) in bile and urine, mostly as harmol-sulfate (70%) but also as harmol-glucuronide (30%). Harmalol was also excreted in bile and urine (50% of the dose in 3 hr), mostly as harmalol-glucuronide (70%), with only a trace of harmalol-sulfate present (less than 3%). *In vitro* kinetic parameters of conjugating activities towards both substrates were determined. Harmol and harmalol were glucuronidated by UDP glucuronyltransferase at comparable rates. Phenolsulfotransferase converted harmol readily to its sulfate-conjugate, whereas harmalol was a very poor substrate of this enzyme. Thus, the excretory pattern of harmol and harmalol can be explained by different rates of conjugation of these substrates by UDP glucuronyltransferase and phenolsulfotransferase, as found *in vitro*.

THE BIOTRANSFORMATION of harmine and harmaline, two hallucinogenic alkaloids, has been investigated by several authors.[1–5] The main pathway of biotransformation consists of (oxidative) demethylation and subsequent conjugation of the product with glucuronate or sulfate (Fig. 1). Thus, in the rat the main metabolite of harmine is the sulfate conjugate of harmol[3] and the main metabolite of harmaline is harmalol-glucuronide.[4] These conjugates are then excreted both in urine and bile. However, because the results of Wong and Sourkes[6] seemed to suggest that both harmol and harmalol are readily glucuronidated *in vitro* by rat liver microsomes, we were intrigued by the finding of the predominant sulfate-conjugation of harmol *in vivo* (after injection of its precursor harmine) and we tried to find an explanation for it.

To avoid complications we used harmol and harmalol to avoid the first biotransformation (Fig. 1). We wanted to clarify whether the presumed preference *in vivo* for glucuronide conjugation of harmalol (after harmaline injection) and for sulfo-conjugation of harmol (after harmine injection)[3,4] could be found with harmol and harmalol themselves and could be traced *in vitro*, to differences of UDP glucuronyltransferase and phenolsulfotransferase activities towards these structurally related compounds. The present results show this to be the case.

MATERIALS AND METHODS

Chemicals. Harmol and harmalol were obtained from Fluka (Buchs, Switzerland) as hydrochloride salts. UDP glucuronate (di-sodium salt) and β-glucuronidase/aryl-

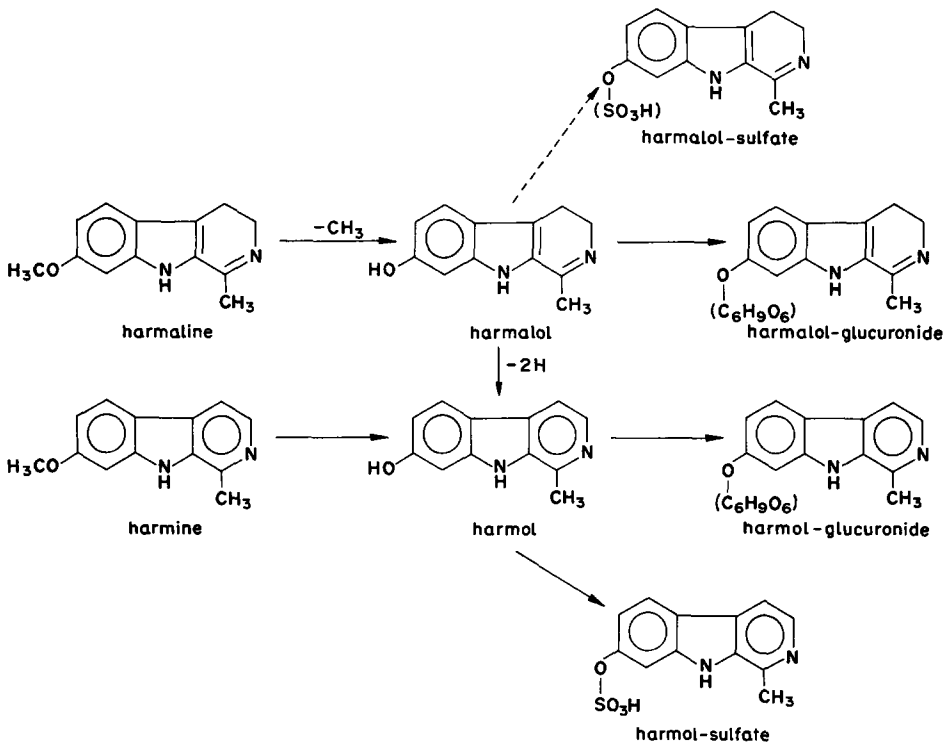

FIG. 1.

sulfatase were obtained from Boehringer (Mannheim, Germany). Saccharo-1,4-lactone was purchased from Calbiochem (U.S.A.).

Rats. Male rats (Wistar, obtained from TNO, Zeist, The Netherlands) weighing 250–350 g were anesthesized with 60 mg/kg pentobarbital (Nembutal®) intraperitoneally. A polyethylene cannula was introduced into the bile duct. The body temperature of the rats, measured rectally, was kept between 37·5° and 38·5° by means of a heating lamp. Bile was collected during periods of 30 min. At the end of the experiment the bladder was exposed and urine was collected directly from the bladder, with a syringe. Harmol and harmalol were injected into the *vena dorsalis penalis* in a volume of 0·25 ml/100 g body wt.

Thin layer chromatography of metabolites of harmol and harmalol. Five microlitres of bile, urine or blood plasma were applied to kieselgel plates (Merck, Darmstadt Germany; kieselgel 60F 254). The plates developed during about 1·5 hr with chloroform:methanol:isopropanol:ammonia 90:10:95:5, (solvent system D from ref. 4), at room temperature and in the dark. When the plates were dry the spots were visualized with u.v. light (350 nm), scraped off and eluted in 3 ml of 0·1 N HCl in the dark overnight at 0–4°. The fluorescence of the resulting solutions was measured with an Eppendorf fluorimeter, primary filter Hg lines 313 and 366 nm and secondary filter 420–3000 nm.

Recovery of the compounds from the t.l.c. plate is shown in Table 1. The percentage recovery was determined by comparing the fluorescence applied to the plate with

TABLE 1. RECOVERY OF HARMOL, HARMALOL AND THEIR CONJUGATES FROM T.L.C.

Compound	Recovery (%)
Harmol	82
Harmol-glucuronide	90
Harmol-sulfate	87
Harmalol	41
Harmalol-glucuronide	90
Harmalol-sulfate	—*

* Could not be determined; see text.

that eluted from it. This was done with solutions which contained either the aglycon or the conjugate; the latter was prepared by incubating the aglycons *in vitro* with UDP glucuronate or 3'-phosphoadenylyl sulphate (PAPS) solutions and a high "concentration" of the respective enzyme, such that 100% conversion of the aglycon to the conjugate resulted, except in the sulfation of harmalol. The recovery of harmalol is only 41 per cent due to a loss of fluorescence during chromatography: if it is eluted immediately after its application, the recovery of fluorescence is about 80 per cent. Harmalol-sulfate could not be determined with this method, because it was not possible to convert all of the substrate harmalol to its sulfate conjugate (see Results).

Fluorescence intensity ratios for the various conjugates, compared to the corresponding aglycons were determined in a similar way. The fluorescence of incubation media in which the aglyons were 100 per cent converted to the conjugates, was compared to the fluorescence of the aglycons which were not treated with enzyme or co-substrate. The results show that the conjugates of harmol have a slightly higher fluorescence than the aglycon, the ratio of the fluorescence intensity of equimolar amounts of harmol: harmol-conjugate being 0·8 (both for the glucuronide and sulfate conjugate). Harmalol-glucuronide had a considerably lower fluorescence than harmalol the ratio being 1·7; this is in agreement with the results of Wong and Sourkes.[6] The ratio for harmalol-sulfate was also taken to be 1·7 but because the sulfate-conjugate of harmalol is synthesized, both *in vivo* and *in vitro*, only to a very limited extent, this ratio has no further implications for the conclusions of the present work.

All values determined in this investigation have been corrected for recovery-percentages and fluorescence ratios.

β-Glucuronidase and arysulfatase hydrolysis. These were performed essentially as described before[7] in 75 mM acetate buffer, pH 5·0 with a mixture of 0·13 U/ml β-glucuronidase and 0·08 U/ml arylsulfatase during incubations of 30 min at 37°. The activity of β-glucuronidase was totally inhibited by the presence of 25 mM saccharo-1,4-lactone. Arylsulfatase activity was not affected by this compound.

UDP glucuronyltransferase activity. UDP glucuronyltransferase activity with harmol and harmalol as substrates was estimated by an adaption of the method of Wong and Sourkes.[8] The incubation medium was as described before:[9] 75 mM Tris–HCl buffer, pH 7·3 containing 5 mM $MgCl_2$. UDP glucuronate concentration was 1·5 mM and routinely 0·15 mM harmol or harmalol was used as acceptor-substrate concentration (all concentrations are final concentrations in the incubation medium). The enzyme source was a rat liver microsomal preparation in 0·154 M KCl as described

before, which was activated by the addition of 0·25% (v/v) Triton X-100 before further dilution with 0·15 M KCl.[10] It has been shown that the addition of this non-ionic detergent activates UDP glucuronyltransferase activity towards a number of substrates.[11–13] The enzyme incubation was performed at 37° for 5–15 min, with about 200 μg microsomal protein/ml of incubation medium. It was terminated by the addition of 0·11 ml 5% (w/v) $ZnSO_4$ per ml of incubation medium. After centrifugation, 30 or 15 μl of the supernatant was applied to a t.l.c. plate as described above. The glucuronides of harmalol and harmol remained at the origin. The production of glucuronide was measured; for kinetic experiments it was measured at three points in time at each substrate concentration.

PAPS biosynthesis. PAPS was prepared according to the method of Van Kempen and Jansen.[14] A 20% rat liver homogenate in 0·154 M KCl was prepared with a Waring Blendor homogenizer. After centrifugation for 1 hr at 44,000 *g* a Sorvall RC2B centrifuge (rotor SS 34), the supernatant was lyophilized and the dry material, which contained the PAPS synthesizing activity, was stored at −20°. For the synthesis of PAPS 40 mg of this lyophilisate was incubated with 8 ml 0·4 M Tris–HCl buffer (pH 7·4), 1 ml 20 mM $MgCl_2$–452 mM K_2SO_4 (in water) and 24·2 mg ATP at 37° for 1 hr. The reaction was terminated by placing the incubation tubes in a boiling water-bath for 1 min. After cooling the precipitate was centrifuged off and the supernatant contained 120–200 μM PAPS.[15]

Phenolsulfotransferase activity. The method was an adaption of the method of Van Kempen and Jansen.[14] The incubation medium consisted of 0·75 ml of the PAPS solution, 0·2 ml of an enzyme suspension, 0·15 ml of the substrate solution (in water) and 0·4 ml of 0·75 M Tris-HCl buffer (pH 7·4) containing 75 mM EDTA (to inhibit further PAPS synthesis) and 94 mM KH_2PO_4 (to inhibit PAPS breakdown). The final concentration of the substrates harmol and harmalol in the incubation medium was 15–150 μM.

After incubation for 5–30 min at 37° the reaction was terminated by placing the tubes in a boiling water bath for 1 min. The precipitate was centrifuged off and 30 μl of the supernatant was applied to t.l.c. plates.

The enzyme suspension was prepared by centrifuging a 20% rat liver homogenate in 0·154 M KCl for 15 min at 600 *g*; the postnuclear supernatant was diluted four times for harmol sulfation but used undiluted for harmalol sulfation.

RESULTS

T.l.c. of metabolites of harmol and harmalol. The R_f values of metabolites of harmol and harmalol were obtained by applying bile, from rats injected intravenously with these compounds, to the t.l.c. plate. The R_f values of harmol, harmalol and their glucuronide and sulfate conjugates are given in Table 2. They are in good agreement with those reported (for solvent system D) by Ho *et al.*[4] The identity of the spots was ascertained, after elution, by hydrolysis with a mixture of β-glucuronidase and arylsulfatase. In the case of the glucuronides, hydrolysis could be totally inhibited by saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase. When sulfate conjugates were treated with the enzyme mixture there was no inhibition of hydrolysis by saccharolactone.

Biliary elimination of harmol and harmalol. When harmol was injected intravenously (20 μmoles/kg; about 4 mg/kg) only the glucuronide and sulfate conjugates

TABLE 2. R_f VALUES OF HARMOL, HARMALOL AND THEIR CONJUGATES

Harmol	0·78
Harmol-sulfate	0·38
Harmol-glucuronide	0·00
Harmalol	0·42
Harmalol-sulfate	0·18
Harmalol-glucuronide	0·00

For details see Methods section on t.l.c

were found in bile (Fig. 2). In the first 30 min period after injection the bile contained equimolar amounts of both conjugates, but in the next 5 periods the concentration of harmol-sulfate (and also the percentage of the dose excreted as sulfate conjugate) was much greater than that of harmol glucuronide. Thus, after 3 hr 20 per cent of the dose had been excreted in bile as sulfate conjugate and 12 per cent as the glucuronide (Table 3).

With harmalol (17·4 μmoles/kg; about 3·5 mg/kg), in bile most of the dose was excreted as the glucuronide conjugate (Fig. 3), 25 per cent in 3 hr (Table 3). In addition 4 per cent (in 3 hr) was excreted as harmol-sulfate which implies that dehydrogenation had taken place. This is in agreement with the findings of Ho *et al.*[4] Very small, but distinctly present, amounts of harmalol-sulfate (less than 1% of the dose in 3 hr) were also found in bile (Table 3).

The concentrations of harmol-sulfate and harmol-glucuronide in bile after harmol injection, and of harmalol-glucuronide after harmalol injection, were initially 0·6–0·7 mM; they declined in subsequent periods in parallel with the decline in the amount of the conjugates excreted in these periods (Figs. 2 and 3). The concentration of harmol-sulfate after harmalol injection was 0·05–0·10 mM in all periods.

Urinary elimination of harmol and harmalol. As can be seen in Table 3 harmol metabolites were extensively excreted in urine when harmol was injected intravenously in rats with cannulated bile ducts. For the greater part harmol appeared in the form of its sulfate-conjugate (22%), but also as its glucuronide (5%); during 3 hours 27 per

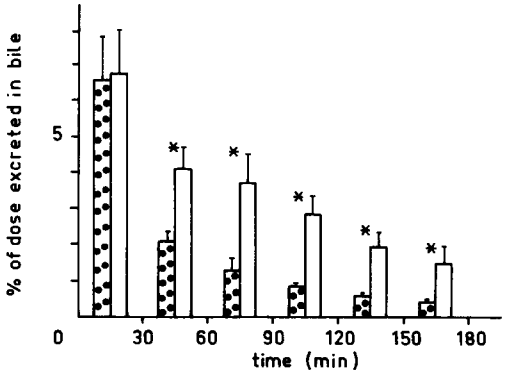

FIG. 2. Biliary elimination of harmol. The dose was 20 μmoles/kg. The shaded bars give the values for the glucuronide conjugate, the white bars those for the sulfate conjugate. Bile was collected for six 30-min periods. The per cent of the dose per period excreted in the form of the respective conjugates is given. The asterisk indicates significant difference ($P < 0.05$) between sulfate and glucuronide. n = 7 and the S.E.M. is shown.

TABLE 3. THE BILIARY AND URINARY EXCRETION OF HARMOL, HARMALOL AND THEIR CONJUGATES

Compound conjugate	Harmol		Harmalol	
	Bile	Urine	Bile	Urine
Harmol	—*	<1	—	—
Harmol-glucuronide	12 ± 2	5 ± 2	—	—
Harmol-sulfate	20 ± 4	22 ± 6	4 ± 2	3 ± 1
Harmalol	—	<1	—	7 ± 2
Harmalol-glucuronide	—	—	25 ± 4	11 ± 2
Harmalol-sulfate	—	—	<1	<1
Recovered in urine and bile (%)	59		50	

In rats with cannulated bile ducts bile and urine were collected during 3 hr after I.V. injection of harmol (20 μmoles/kg) or harmalol (17·4 μmoles/kg). The percentages of the dose recovered in the various fractions are given with their SEM. n = 7.

* – These compounds could not be seen on the t.l.c. plates.

cent of the dose was excreted in urine compared with 32 per cent in bile during the same time. Contrary to the findings of Slotkin and DiStefano[5] who reported that there was no decrease in the urinary elimination of harmine metabolites in rats with cannulated bile ducts (compared with rats with intact bile ducts), we found a significant decrease in the urinary excretion of harmol-sulfate in rats with cannulated bile ducts. In rats with intact bile ducts 36 ± 7 per cent of the dose was found as harmol-sulfate in urine, whereas only 22 ± 6 per cent was found in rats with cannulated bile ducts (n = 6; P = 0·08). This suggests enterohepatic recirculation.

After intravenous administration of harmalol to rats with cannulated bile ducts, harmalol-glucuronide, harmalol and harmol-sulfate were detected in urine. In 3 hr about 21 per cent of the dose was excreted in urine. Harmalol-sulfate was also detectable in urine but only in minute amounts (less than 1%).

Harmol, harmalol and their metabolites in blood. Plasma, obtained from heparinized blood was applied to t.l.c. plates in order to find which metabolites of harmol and harmalol were present in plasma.

Three minutes after injection of harmol the aglycon was no longer detectable in plasma; at the same time a faint spot of harmol-sulfate appeared; the amount in-

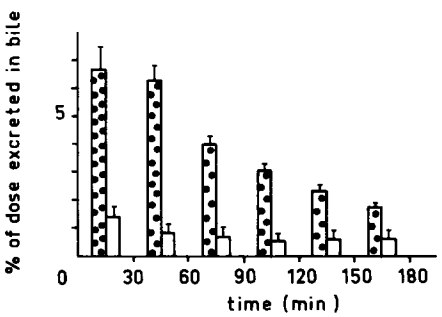

FIG. 3. Biliary elimination of harmalol. The dose was 17·4 μmoles/kg. The shaded bars give the values for harmalol-glucuronide, the white bars for harmol-sulfate. Bile was collected for six 30-min periods. The per cent of the dose per period excreted in the form of the respective conjugates is given. n = 6 and the S.E.M. is shown.

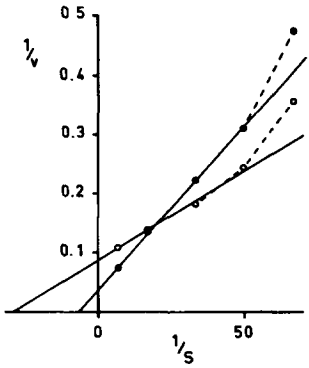

FIG. 4. Lineweaver–Burk plot of UDP glucuronyltransferase with harmalol (○) and harmol (●) as substrate. Substrate concentration is expressed in mM and the velocity (v) as nmoles converted/min/mg of microsomal protein. UDP glucuronate concentration was 1·5 mM. The lowest concentration of harmol gave less reliable results; therefore, the curves have been drawn only through the points of the 4 higher concentrations For further details see Method section.

creased up to 60 min post injection after which time no further determinations were made. A glucuronide spot was not present.

Three minutes after injection of harmalol harmalol-glucuronide was visible on the t.l.c. plate. The aglycon was present in blood plasma up to 60 min post injection. A strongly fluorescing spot of the glucuronide conjugate was present.

Harmol and harmalol as substrate of UDP glucuronyltransferase. Both harmol and harmalol could be glucuronidated *in vitro* with rat liver microsomal UDP glucuronyltransferase, activated by Triton X-100. The K_m values were (at 1·5 mM UDP glucuronate) 150 μM for harmol and 35 μM for harmalol. The respective V_{max} values were 26 nmoles/min/mg of microsomal protein for harmol glucuronidation and 11 nmoles/min/mg of microsomal protein for harmalol glucuronidation (Fig. 4). The

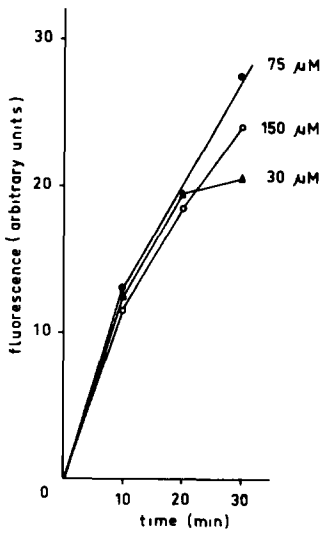

FIG. 5. Sulfation of harmol. The sulfation of harmol at three different substrate concentrations was followed in time. Fluorescence is given in arbitrary units (21 being the fluorescence at which all harmol at 30 μM has been converted). Enzyme source was a postnuclear rat liver supernatant.

K_m values are in reasonable agreement with those found by Wong and Sourkes[6] (69 μM for harmalol and 50 μM for harmol, both at 0·1 mM UDP-glucuronate). They did not determine V_{max} values and did not activate their microsomal enzyme preparation with a detergent.

Harmol and harmalol as substrates of phenolsulfotransferase. There was a great difference in the rate of sulfation of harmol and harmalol by the post-nuclear supernatant in 0·154 M KCl which was used as enzyme source for phenolsulfotransferase. Although after the incubation with 15–150 μM harmalol the sulfate conjugate of harmalol could be seen on the t.l.c. plate, the amount was too small to measure its rate of conversion fluorimetrically. However, harmol was readily sulfated (Fig. 5), the K_m of the substrate being less than 30 μM. We could not determine the K_m because the estimation of initial velocity below 30 μM was not reliable due to the fact that the substrate is exhausted early during the incubation. Thus the downward trend of the curve at 30 μM (Fig. 5) is due to the total conversion of harmol at that concentration, after 30 min. The velocity of conversion at 30–150 μM harmol substrate concentration is about 0·3 μmole/min/g of liver.

DISCUSSION

Although harmol and harmalol are chemically related the physico-chemical properties are rather different, as illustrated by differences in their fluorescence properties.[8] This difference in physico-chemical properties is reflected in their biotransformation. The results of Slotkin *et al.*[2,3,5] and Ho *et al.*[4] suggested that in the rat harmol would be mainly sulfated and harmalol mainly glucuronidated; they, however, injected the precursors of these compounds, harmine and harmaline, which, of course, complicates the interpretation of their results on this point. In our study harmol and harmalol were used and we could thus confirm that harmalol is mainly glucuronidated in the rat and only to a very limited extent sulfated, whereas harmol is preferentially sulfated but also glucuronidated to a considerable extent. In agreement with the results of Wong and Sourkes[6,8] we found that both harmol and harmalol can be glucuronidated by microsomal UDP glucuronyltransferase, at comparable rates. The finding that harmalol, contrary to harmol, is a very poor substrate for phenolsulfotransferase explains why in the *in vivo* experiments harmalol is barely detectable in the form of its sulfate conjugate, whereas considerable amounts of harmol-sulfate are excreted.

It is difficult to predict from our *in vitro* data the ratio *in vivo* of sulfate- and glucuronide-conjugates, because we do not know the actual concentrations of PAPS and UDP glucuronate at the site of synthesis of the conjugates. If we assume[16] that there is about 30 mg of microsomal protein per gram of liver than, from V_{max} values, we can compute a maximum glucuronidating capacity for harmol of about 0·8 μmoles/min/g of liver (at 1·5 mM UDP glucuronate; *in vivo* this concentration is 0·1–0·2 mM, if it is homogenously distributed in all cell fractions[8,17,18] and for harmalol of 0·2 μmoles/min/g of liver. The sulfate conjugating capacity for harmol is about 0·3 μmoles/min/g of liver (at a PAPS concentration of 0·05–0·10 mM; the PAPS concentration *in vivo* in rat liver is not known). For harmalol this must be less than 0·005 μmoles/min/g of liver (estimated for t.l.c. plate).

Nevertheless, the K_m values of phenolsulfotransferase and UDP glucuronyltransferase for harmol (less than 30 μM and 150 μM respectively) present an explanation

for the decrease in the glucuronide/sulfate conjugation ratio in time after the i.v. injection, as observed in Fig. 2. Within a few minutes after the i.v. injection the concentration of unchanged harmol in liver will be highest and it will subsequently decline. When the harmol concentration in liver decreases the conjugation by the transferase with the highest affinity, i.e. phenosulfotransferase, will be favoured relative to glucuronidation (assuming the V_{max} values of both transferases to be independent of harmol concentration). Thus, the decline in glucuronide conjugation relative to sulfate conjugation (Fig. 2) can be expected.

Apart from the difference in biotransformation of harmol and harmalol, there are some further differences in pharmacokinetics between these compounds, although one cannot be sure whether these may be secondary to this difference in biotransformation. Thus, considerably more unchanged harmalol is excreted in urine than unchanged harmol. This correlates well with the prolonged presence of harmalol in the blood observed in the present experiments. This suggests that either harmalol is less readily taken up by the liver than harmol or that much of it is taken up by other tissues, from which it is then slowly released into the blood. Harmol, to the contrary, is rapidly removed from the blood and rapidly excreted both in bile and urine, in the form of its conjugates. The rate limiting step in the elimination of harmol and harmalol from the body is still not known.

REFERENCES

1. A. Villeneuve and T. L Sourkes, *Rev. Can Biol.* **25,** 231 (1966).
2. T. A. Slotkin, V. DiStefano and W. Y. W. Au, *J. Pharmac. exp. Ther.* **173,** 26 (1970).
3. T. Slotkin and V. DiStefano, *Biochem. Pharmac.* **19,** 125 (1970).
4. B. T. Ho, V. Estevez, G. E. Fritchie, L. W. Tansey, J. Idänpään-Heikkilä and W. M. McIsaac, *Biochem. Pharmac.* **20,** 1313 (1971).
5. T. A. Slotkin and V. DiStefano, *J. Pharmac. exp. Ther.* **174,** 456 (1970).
6. K. P. Wong and T. L. Sourkes, *Biochem. J.* **110,** 99 (1968).
7. G. J. Mulder, *Biochem. Pharmac.* **22,** 1751 (1973).
8. K. P. Wong, and T. L Sourkes, *Anal. Biochem.* **21,** 444 (1967).
9. G. J. Mulder, *Biochem. J.* **125,** 9 (1971).
10. G. J. Mulder, *Biochim. biophys. Acta* **289,** 284 (1972).
11. K. K. Lueders and E. L. Kuff, *Archs Biochem. Biophys.* **120,** 198 (1967).
12. A. Winsnes, *Biochim. biophys. Acta,* **191,** 279 (1969).
13. G. J. Mulder, *Biochem. J* **117,** 319 (1970).
14. G. M. J. van Kempen and G. S. I M. Jansen, *Anal. Biochem.* **46,** 438 (1972).
15. G. S. I. M. Jansen and G. M. J. van Kempen, *Anal. Biochem.* **51,** 324 (1973).
16. G. Dallner, *Acta Pathol. Microbiol. Scand. Suppl.* 166 (1963).
17. B. Kuenzel and B Mueller-Oerlinghausen, *N.S. Arch. Pharmakol. exp. Pathol.* **262,** 112 (1969).
18. V. Zhivkov, *Biochem. J.* **120,** 505 (1970).