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THE INFLUENCE OF *n*-HEXANE TREATMENT ON THE GLUCURONIC ACID PATHWAY AND ACTIVITY OF SOME DRUG-METABOLOZING ENZYMES IN GUINEA-PIG

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Abstract—Treatment of guinea-pigs with *n*-hexane for 8 days enhanced the *in vitro* glucuronyl conjugation of *p*-nitrophenol. This induction was accompanied by a significantly enhanced microsomal protein content. However, the *in vitro* rates of the *p*-hydroxylation of aniline and the *N*-demethylation of aminopyrine were slightly decreased and unchanged, respectively, during this treatment. An increased urinary excretion of D-glucuronic acid and D-glucaric acid paralleled the induction of UDPglucuronyltransferase. The excretion of glucuronides in the urine was already enhanced within 24 hr after the first dose of *n*-hexane. The conclusion is drawn that *n*-hexane may cause a direct stimulation of the formation of glucuronides, further, on long-term treatment, *n*-hexane may be considered as a specific inducer of the glucuronidation system.

Numerous xenobiotics are able to enhance the activity of the drug-metabolizing enzyme systems in the liver [1]. This response of the liver to drug exposure is often parallelled by a stimulation of the glucuronic acid pathway, which is reflected by an elevated excretion of L-ascorbic acid and D-glucaric acid into the urine. Recently, several authors have suggested therefore, that the determination of D-glucaric acid and L-ascorbic acid in urine may be an indicator of an adaptive acceleration of hepatic drug metabolism [1–5]. Man, other primates and the guinea-pig lack the ability to synthesize L-ascorbic acid. Therefore an enhanced D-glucaric acid excretion might be a useful indicator of an intake of certain compounds foreign to the organism.

The aim of the present study was to establish if *n*-hexane, a volatile organic solvent widely employed in industry [6], affects drug metabolism and the glucuronic acid system in the liver. Although a substance of low toxicity, *n*-hexane can cause weakness and sensory loss in man on chronic exposure [7].

The results show that in the guinea-pig the urinary D-glucaric acid excretion is enhanced after *n*-hexane treatment. The observed alteration has been compared with urinary excretion of other metabolites of the glucuronic acid pathway and the activities of some hepatic enzymes involved in drug metabolism.

METHODS

Animals

Male guinea-pigs, weighing about 190 g, maintained on a Standard Laboratory Cavy Diet L.C. 23-B (Hope Farms), with free access to water, were utilized. *n*-Hexane was administered i.p. in sesame oil at a rate of 3 or 60 mg/kg/day. Control animals received sesame oil in equivalent quantities. Urine samples were collected daily and stored at −20° until examination.

Liver preparations

For the *in vitro* experiments the guinea-pigs were killed by decapitation 24 hr after the last *n*-hexane injection. The livers were immediately removed and cooled in ice. Portions of liver were weighed and finely minced. Homogenates (20%, w/v) were prepared in ice-cold 0·25 M sucrose solution, containing 5×10^{-2} M Tris(hydroxymethyl)aminomethane–HCl (pH 7·4), using a Teflon®–glass Potter–Elvehjem type of homogenizer. Postmitochondrial fractions were prepared from the homogenates by centrifugation (9000 *g* for 20 min). For preparation of the microsomal fractions 9000 *g* supernatants were centrifuged in a Christ Omega II Ultracentrifuge at 105,000 *g* for 1 hr (2°). The microsomal pellets from the latter step were gently resuspended in the homogenization medium with the Potter–Elvehjem apparatus. Suspensions (1 ml) contained microsomes derived from 0·2 g of liver.

Urinary assays

D-Glucaric acid. D-Glucaric acid was measured according to Marsh [8] from the inhibitory effect of D-glucarolactone, to which it is converted by heating at pH 2·0, on β-glucuronidase.

Glucuronides. Dische's carbazole reaction modified by Yuki and Fishman [9] was applied.

Free D-*glucuronic acid.* Dische's carbazole method, modified by Yuki and Fishman [9] for differential analysis was used. The total quantity of free and conjugated glucuronic acid was determined according to Gregory [10].

Enzyme assays

The microsomal *N*-demethylation of aminopyrine and the *p*-hydroxylation of aniline were measured in 9000 *g* supernatants as described by Henderson and Kersten [11].

UDPglucuronyltransferase (EC 2.4.1.17) activity, with *p*-nitrophenol as substrate, was measured in an incubation system consisting of: 0·04–0·06 ml microsomal suspension, 0·05 ml $MgCl_2$ ($3{\cdot}3 \times 10^{-3}$ M final concn), 0·15 ml UDPGA (4×10^{-3} M final concn), 0·1 ml saccharo-1,4-lactone (3×10^{-3} M final concn), 0·2 ml *p*-nitrophenol ($0{\cdot}1$–$0{\cdot}4 \times 10^{-3}$ M final concn) in Tris–HCl buffer (0·05 M, pH 7·4).

The further procedures were done according to Henderson [12] except that for activation of UDPglucuronyltransferase, the microsomal suspensions were treated with the detergent Triton X-100 (0·25% final concn) instead of sonication. The incubation was at 37°C for 15 min. V_{max}-values and K_m of *p*-nitrophenol were calculated by extrapolation of Lineweaver–Burk plots.

Protein assay

Protein was determined according to the method of Lowry *et al.* [13], with bovine serum albumin as reference.

RESULTS

Urinary excretion of D-*glucaric acid,* D-*glucuronic acid and glucuronides.* Figure 1 shows the urinary D-glucaric acid excretion with time during treatment with *n*-hexane (60 mg/kg/day). As can be seen, the amount of D-glucaric acid was enhanced slightly but significantly after an initial lag period of about 5 days. The same phenomenon was observed for the urinary excretion of free D-glucuronic acid. The urinary excretion of glucuronides was stimulated by *n*-hexane treatment since a clearly elevated level of these metabolites in the urine was observed from the first day of treatment (Fig. 1).

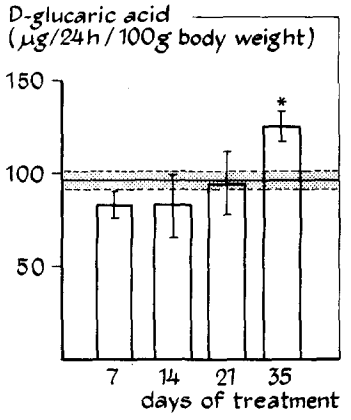

Fig. 2. Effect of chronic treatment with relatively low doses of *n*-hexane (3 mg/kg/day) on the 24-hr urinary excretion of D-glucaric acid. Blocks represent means (±2 S.E.M.) for six animals. The average level of the controls (±2 S.E.M., for six animals, sampling on days 7, 14, 21 and 35) is represented by the horizontal line. (*) indicates: significantly different from the control at $0{\cdot}01 < P < 0{\cdot}02$; for all other values $P > 0{\cdot}05$ (Student's *t*-test).

As is illustrated in Fig. 2, long-term treatment with *n*-hexane for 35 days at a rate of 3 mg/kg/day also induced a statistically significant increase of D-glucaric acid excretion.

N-*Demethylation of aminopyrine and* p-*hydroxylation of aniline.* At three intervals during *n*-hexane treatment (60 mg/kg/day), aniline *p*-hydroxylation and amino-

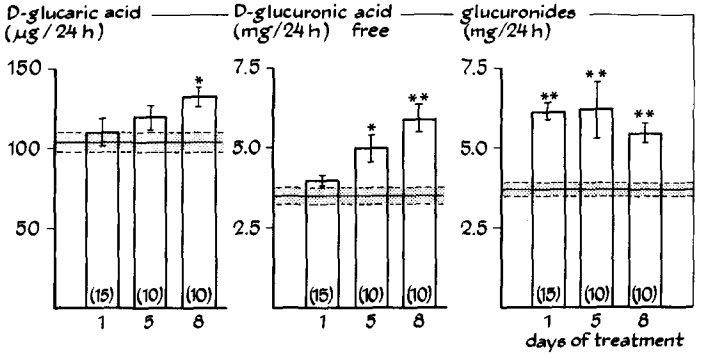

Fig. 1. Effect of *n*-hexane treatment on the 24-hr urinary excretion of D-glucaric acid, free D-glucuronic acid and bound as glucuronide in guinea-pig. Blocks represent means (±2 S.E.M.). The numbers of animals are given in parentheses. The average levels of the controls (±2 S.E.M., for five animals) are represented by the horizontal line. Control sampling occurred on days 1, 5 and 8. The amounts of excreted metabolites are standardized per 100 g body wt. (*) indicates: significantly different from the control at $0{\cdot}02 < P < 0{\cdot}05$; (**) at $0{\cdot}01 < P < 0{\cdot}02$; for all other values $P > 0{\cdot}05$ (Student's *t*-test).

Table 1. Effect of *n*-hexane pretreatment on the *in vitro* activities of *N*-demethylase and *p*-hydroxylase

Duration of treatment (days)	Activity	
	N-Demethylation* of aminopyrine	*p*-Hydroxylation† of aniline
1	32·1 ± 1·0 (5)	3·30 ± 0·13 (5)§
8	28·7 ± 4·1 (5)	3·31 ± 0·04 (5)¶
21	28·2 ± 0·6 (5)	3·44 ± 0·03 (5)‡
Control	29·8 ± 3·0 (5)	4·00 ± 0·18 (5)

* Expressed as μmoles formaldehyde produced/hr/g fresh liver.

† Expressed as μmoles *p*-aminophenol produced/hr/g fresh liver.

The animals received 60 mg *n*-hexane/kg/day i.p in sesame oil. Controls received the same volume of sesame oil only. Mean values are given ± S.E.M.; the values in parentheses designate the number of animals.

Significantly different from the control at: ‡ $0{\cdot}02 < P < 0{\cdot}05$; § $0{\cdot}01 < P < 0{\cdot}02$; ¶ $P < 0{\cdot}01$; for all other values $P > 0{\cdot}05$ (Student's *t*-test).

pyrine *N*-demethylation were measured *in vitro*. From Table 1 it is apparent that the aniline hydroxylation was significantly decreased after 1 day of treatment. This decreased value for the enzyme activity did not change significantly during further treatment with *n*-hexane.

The rate of aminopyrine *N*-demethylation remained at the control level during the treatment period (Table 1). Also a 35 day treatment period with 3 mg *n*-hexane/kg/day did not change the *in vitro* *N*-demethylase activity (Table 2).

UDPglucuronyltransferase. In addition to the microsomal mixed-function oxidase system, the *in vitro* activity of UDPglucuronyltransferase in the liver was studied during the treatment with *n*-hexane. Apparent K_m and V_{max}-values were measured with a fixed relatively low *p*-nitrophenol concentrations ranging from 0·1 to 0·4 mM and a fixed UDPGA concentration of 4 mM. To ensure linearity in the rate of conversion of *p*-nitrophenol with time, preliminary incubations were performed to determine suitable quantities of microsomal suspension so that not more than 15–35 per cent of the *p*-nitrophenol would have reacted with UDPGA during an incubation time of 15 min.

It can be concluded from Fig. 3, that the apparent K_m did not change during *n*-hexane treatment. However, the apparent V_{max} for the *p*-nitrophenol glucuronidation was significantly increased after 8 days to reach values up to 150 per cent of the control at the 21st day. Treatment (35 days) with 3 mg *n*-hexane/kg/day also resulted in an increased V_{max} value (Table 2).

Liver weight and microsomal protein content. As is illustrated in Table 3, liver weights, per unit of body weight, did not alter significantly during the treatment period. The microsomal protein content, however, was

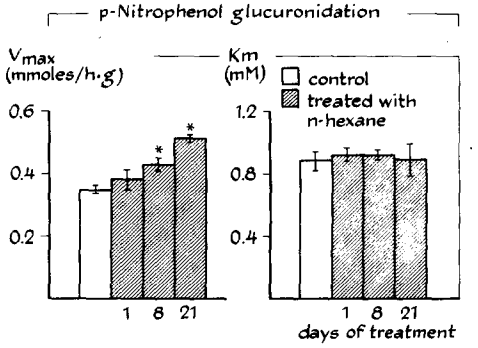

Fig. 3. Influence of *n*-hexane treatment (60 mg/kg/day i.p.) on apparent V_{max} and K_m of *p*-nitrophenol glucuronidation by Triton X-100 activated microsomal preparations. Blocks represent means (± 2 S.E.M.) of 6 separate experiments. V_{max} and K_m are expressed as mmoles/hr/g fresh liver and mM respectively. Control animals were sacrificed on days 1, 8 and 21. (*) indicates: significantly different from the control at $P < 0{\cdot}01$; for all other values $P > 0{\cdot}05$ (Student's *t*-test).

Table 2. Effect of chronic treatment with low doses of *n*-hexane on the *in vitro* activities of hepatic *N*-demethylase and UDPglucuronyltransferase

Treatment	*N*-Demethylation* of aminopyrine	UDPglucuronidation of *p*-nitrophenol	
		Apparent V_{max}†	Apparent K_m (mM)
None (controls)	30·7 ± 2·3 (4)	0·33 ± 0·04 (4)	0·89 ± 0·06
n-Hexane	31·0 ± 0·7 (4)	0·46 ± 0·03 (4)‡	0·89 ± 0·10

Guinea pigs received 3 mg *n*-hexane/kg i.p. in sesame oil for 35 days. Controls received an equivalent volume of sesame oil. Values represent means ± S.E.M. The numbers in parentheses represent number of animals per group.

* Expressed as μmoles formaldehyde produced/hr/g fresh liver.

† Expressed as m-moles *p*-nitrophenol conjugated/hr/g fresh liver. Enzyme preparations were maximally activated by Triton X-100 before incubation.

Significantly different from the control at: ‡ $0{\cdot}02 < P < 0{\cdot}05$; for all other values $P > 0{\cdot}05$ (Student's *t*-test).

Table 3. Effect of *n*-hexane pretreatment on liver weight and microsomal protein content in guinea-pigs

Duration of treatment (days)	Liver wt (g/100 g body wt)	Microsomal protein (mg/g liver)
1	3·18 ± 0·08 (5)	29·0 ± 0·9 (5)
8	3·29 ± 0·08 (5)	31·6 ± 1·1 (5)*
21	3·18 ± 0·09 (5)	33·8 ± 1·2 (5)†
Controls	3·18 ± 0·19 (5)	28·0 ± 1·1 (5)

Guinea-pigs received 60 mg *n*-hexane/kg/day i.p. in sesame oil. Controls received an equivalent volume of sesame oil.

Values represent means ± S.E.M. Values in parentheses represent number of animals per group.

Significantly different from the control at: *$0{\cdot}02 < P < 0{\cdot}05$; †$P < 0{\cdot}01$; for all other values $P > 0{\cdot}05$ (Student's *t*-test).

significantly enhanced after 8 days. Long-term treatment with *n*-hexane at low concentration (3 mg/kg/day) did not measurably change either the liver weights per unit of body weight or the microsomal protein content.

In vitro *action of* n-*hexane on microsomal enzyme activities.* It might be possible that *n*-hexane is still present in the liver preparations tested for *N*-demethylating, *p*-hydroxylating and UDPglucuronidating activities. One can expect, however, that the residual amount of *n*-hexane in the liver is very low at 24 hr after the last injection, since *n*-alkanes in general are well metabolized [18].

In order to establish possible *in vitro* effects of *n*-hexane, this substance was added to preparations from livers of untreated animals (Table 4). *n*-Hexane at a final concn of 0·1 and 0·5 per cent (v/v) had no influence on the activities of *N*-demethylating and *p*-hydroxylating enzymes. Studying the *in vitro* effect of *n*-hexane on UDPglucuronyltransferase the compound was added to untreated and to Triton X-100 pre-activated microsomes. *n*-Hexane strongly activated UDP-glucuronidation. No further activation was observed when *n*-hexane was added to Triton X-100 pre-activated microsomes.

DISCUSSION

Treatment of guinea-pigs with *n*-hexane does not enhance the *p*-hydroxylation of aniline and the *N*-demethylation of aminopyrine. In fact, a slight decrease in *p*-hydroxylase activity was observed after 1 day of treatment.

No remarkable alteration in activity of these oxidative enzymes was observed on addition of *n*-hexane up to a final concentration of 0·5 per cent to microsomal preparations from untreated animals (Table 4). It appeared, however, that this *in vitro* treatment clearly activates *p*-nitrophenol glucuronidation. If *n*-hexane was added to Triton X-100 pre-activated microsomes, no further activation of UDPglucuronyltransferase occurred.

In contrast, treatment of the guinea-pigs with *n*-hexane for several days caused a significant enhancement of the UDPglucuronyltransferase activity of the Triton X-100 pre-activated microsomes. This enhancement was accompanied by a significant increased microsomal protein content.

After chronic treatment with low doses of *n*-hexane (3 mg/kg/day), when the UDPglucuronyltransferase was also significantly enhanced, the microsomal protein content was not measurably altered. Because we did not find any effect on the apparent K_m for the *p*-nitrophenol glucuronidation, it might be excluded that alterations in enzyme–substrate affinities are responsible for the observed increase. From this and the fact that of the drug-metabolizing enzyme systems tested, only UDPglucuronyltransferase shows an increase in activity during *n*-hexane treatment the conclusion might be drawn that *n*-hexane is a specific inducer of the glucuronidation system.

In addition, *n*-hexane also strongly activates *in vivo* the urinary excretion of glucuronides within 24 hr after the first application. This might be in accordance with the *in vitro* activation by *n*-hexane of *p*-nitrophenol glucuronidation (Table 4). However, on the basis of the present data it cannot be exluded that the enhanced excretion of glucuronides in part is due to increased substrate supply to glucuronidation of *n*-hexane metabolites.

As is seen in Fig. 1, the urinary excretion of D-glucaric acid significantly increased during *n*-hexane

Table 4. Effect of *n*-hexane addition to 9000 *g* supernatants from liver homogenates on *N*-demethylase, *p*-hydroxylase and UDPglucuronyltransferase activities

n-Hexane concn % (v/v)	*N*-Demethylation of aminopyrine*	*p*-hydroxylation of aniline*	UDPglucuronidation of *p*-nitrophenol*	
			Untreated	Pre-treated with Triton X-100†
0	31·44	3·47	11·07	96·06
0·1	31·53	3·81	19·32	97·02
0·5	31·54	3·69	32·34	96·54

* Expressed as μmoles converted/hr/g fresh liver.

† Enzyme preparations are maximally activated with Triton X-100 (0·25%) before incubation.

treatment. Even a low daily dose of *n*-hexane (3 mg/kg/day) induces D-glucaric acid excretion after a lag period of at least 21 days (Fig. 2). It has been found by several investigators that the D-glucaric acid synthesis paralleled an induction of drug metabolism due to pretreatment with various compounds. From this the conclusion was drawn that the D-glucaric acid test may be considered suitable to diagnose induction. The results of this investigation give support to this consideration only as far as induction of glucuronidation is concerned.

The mechanism by which drugs stimulate the D-glucaric acid biosynthesis is not yet fully understood. The results of Burns and Evans [15], Conney *et al.* [16], and Evans *et al.* [17] suggest that the primary effect of the stimulating drugs is an increased synthesis of D-glucuronic acid in the liver cell. The increased quantity of D-glucuronic acid is subsequently utilized for the synthesis of its metabolic endproducts, e.g. D-glucaric acid, L-ascorbic acid. Our findings that after *n*-hexane treatment the pattern of the enhanced D-glucuronic acid excretion equals that of the D-glucaric acid excretion, are in agreement with the above concept. However, further investigations are needed to determine at which step in the sequence of enzymic reactions the stimulation is initiated.

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