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Rapid Stimulation of Free Glucuronate Formation by Non-glucuronidable Xenobiotics in Isolated Rat Hepatocytes*

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Vitamin C synthesis in rat liver is enhanced by several xenobiotics, including aminopyrine and chloretone. The effect of these agents has been linked to induction of enzymes potentially involved in the formation of glucuronate, a precursor of vitamin C. Using isolated rat hepatocytes as a model, we show that a series of agents (aminopyrine, antipyrine, chloretone, clotrimazole, metyrapone, proadifen, and barbital) induced in a few minutes an up to 15-fold increase in the formation of glucuronate, which was best observed in the presence of sorbinil, an inhibitor of glucuronate reductase. They also caused an ~2-fold decrease in the concentration of UDP-glucuronate but little if any change in the concentration of UDP-glucose. Depletion of UDP-glucuronate with resorcinol or D-galactosamine markedly decreased the formation of glucuronate both in the presence and in the absence of aminopyrine, confirming the precursor-product relationship between UDP-glucuronate and free glucuronate. Most of the agents did not induce the formation of detectable amounts of glucuronides, indicating that the formation of glucuronate is not due to a glucuronidation-deglucuronidation cycle. With the exception of barbital (which inhibits glucuronate reductase), all of the above mentioned agents also caused an increase in the concentration of ascorbic acid. They had little effect on glutathione concentration, and their effect on glucuronate and vitamin C formation was not mimicked by glutathione-depleting agents such as diamide and buthionine sulfoximine. It is concluded that the stimulation of vitamin C synthesis exerted by some xenobiotics is mediated through a rapid increase in the conversion of UDP-glucuronate to glucuronate, which does not apparently involve a glucuronidation-deglucuronidation cycle.

Vitamin C synthesis, which takes place in the liver of most mammalian species, starts with the formation of free glucuronate from UDP-glucuronate, possibly via glucuronate 1-phosphate as an intermediate (Fig. 1). Reduction and lactonization, probably in this order, lead to the formation of L-gulono-1,4-lactone, which is oxidized to ascorbic acid by L-gulonolactone oxidase, an enzyme associated to the endoplasmic reticulum membrane (reviewed in Ref. 1). This enzyme is absent in several species including humans and guinea pig, for which ascorbic acid is therefore a vitamin. L-gulonate can also be converted in the liver to L-xylulose, through the successive action of L-gulonate 3-dehydrogenase and 3-dehydro-L-gulonate decarboxylase. L-xylulose is then channeled to gluconeogenesis by being converted to xylitol by NADPH-dependent L-xylulose reductase and then to D-xylulose by D-xylulose reductase, a NAD-dependent enzyme. Deficiency in the first of these two enzymes results in a benign condition known as essential pentosuria (reviewed in Ref. 2).

It has been known for several decades that the administration to rats of various drugs such as barbiturates, chloretone, and aminopyrine markedly stimulates the formation and the urinary excretion of vitamin C (3). Aminopyrine was also shown to stimulate the excretion of L-xylulose in pentosuric patients (4). Tracer studies in rats have shown that the formation of radiolabeled ascorbic acid is increased by chloretone when glucose but not when D-glucuronolactone or L-gulonolactone is used as a precursor (5), indicating that stimulation of ascorbate production involves a step upstream of D-glucuronolactone. The mechanism of this effect is, however, still obscure. One potential explanation, involving the formation of glucuronides and their subsequent hydrolysis to free glucuronate, appears unlikely in the case of barbital since this compound is not significantly metabolized (3), but due to the diversity of stimulating compounds, it is not clear whether this conclusion applies to all of them. Induction of UDP-glucose dehydrogenase, UDP-glucuronosyltransferase, and β-glucuronidase has been proposed to participate in the action of some agents (6–9). Vitamin C synthesis has also been shown to be rapidly stimulated in isolated mouse hepatocytes by agents that cause glutathione depletion (menadione, diamide, buthionine sulfoximine), possibly via enhanced glycogenolysis (10). The purpose of the present work was to test whether aminopyrine and other compounds known to stimulate vitamin C and L-xylulose production had a short term effect on the formation of glucuronate in isolated rat hepatocytes, and if such an effect was found, to determine at which step it took place and whether it was related to glutathione depletion.

EXPERIMENTAL PROCEDURES

Materials—Clostridium histolyticum collagenase A and *Escherichia coli* β-glucuronidase were purchased from Roche Applied Science; baker's yeast glutathione reductase, aminopyrine, L-buthionine-(*S*,*R*)-sulfoximine, clotrimazole, diamide, D-galactosamine, menadione, and proadifen were from Sigma; chloretone and 2-vinylpyridine were purchased from Acros; sodium barbital, dimethyl sulfoxide, and resorcinol were from Merck; antipyrine was from Janssen Chimica; metyrapone was from Aldrich. Sorbinil was a kind gift of Pfizer. All other reagents were of analytical grade.

Preparation and Handling of Hepatocytes—Hepatocytes were isolated from fed Wistar rats by a modification (11) of the method of Seglen (12). The cells were resuspended in Krebs-Henseleit bicarbonate buffer at a final concentration of 7–14 mg of protein/ml. Two-ml portions of

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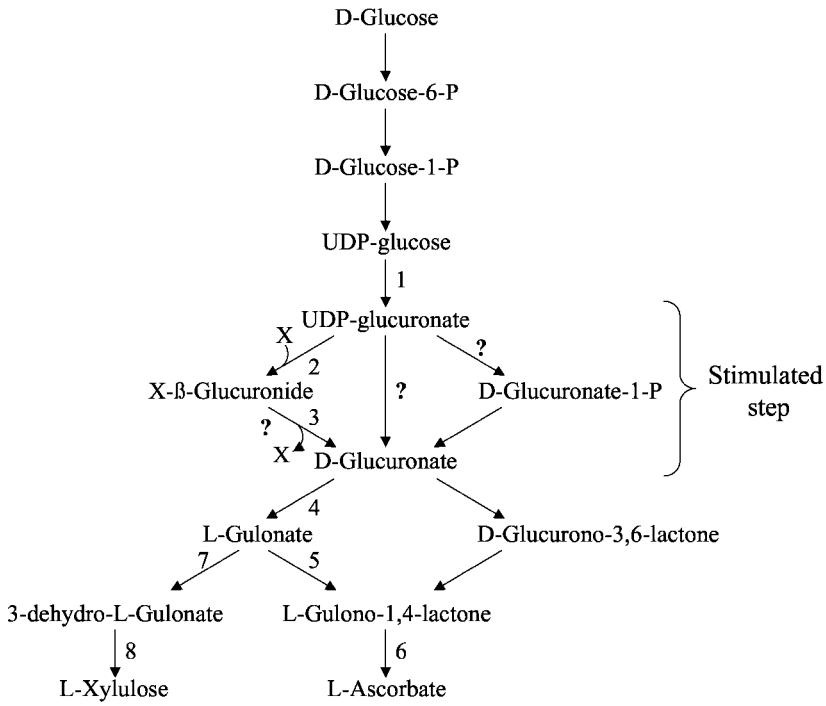

FIG. 1. **Pathway of ascorbic acid and L-xylulose formation.** *1*, UDP-glucose dehydrogenase; *2*, UDP-glucuronosyltransferase; *3*, β-glucuronidase; *4*, glucuronate reductase; *5*, aldonolactonase; *6*, L-gulono-1,4-lactone oxidase; *7*, L-gulonate 3-dehydrogenase; *8*, 3-dehydro-L-gulonate decarboxylase. The *bracket* indicates the step stimulated by aminopyrine and other agents, as shown in the present report.

this suspension were incubated at 37 °C in the presence of 5 mM glucose in 20-ml vials equilibrated with a 95% O_2, 5% CO_2 gas phase. Aminopyrine or other compounds to be tested were added after a 20-min incubation in the presence of Me_2SO or sorbinil. Sorbinil, clotrimazole, chloretone, and menadione were dissolved in Me_2SO and added as 100-fold concentrated solutions; other compounds were dissolved in water. For the measurement of glucuronate, β-glucuronides, UDP-glucuronate, UDP-glucose, and ascorbic acid, the incubations were arrested by mixing a portion of the cell suspension with 0.5 volume of cold 10% (w/v) perchloric acid. For the measurement of intracellular glutathione, the cells were centrifuged through a silicone layer and quenched in 10% (w/v) perchloric acid as described previously (13). For the measurement of glucuronate, β-glucuronides, UDP-glucuronate, and UDP-glucose, the cell extracts were centrifuged, and the supernatants were neutralized and stored at −20 °C until assay. For the measurement of ascorbic acid and glutathione, the acidic supernatants of the cell extracts were stored at −80 °C until assay. Protein was assayed (14) using bovine serum albumin as a standard.

Measurement of Metabolites—Glucuronate was assayed spectrophotometrically using overexpressed and purified[1] *E. coli* glucuronate isomerase and mannonate dehydrogenase, which convert glucuronate successively to fructuronate and mannonate, with concomitant oxidation of NADH to NAD^+ (15, 16). The two enzymes were added successively to a reaction mixture (1 ml) containing 50 mM Hepes, pH 7.1, 0.075 or 0.15 mM NADH, 0.01 mM EDTA, 0.1 mM $MnCl_2$, and up to 200 μl of neutralized perchloric acid extract. The decrease in A_{340} was measured and used to calculate the concentration of glucuronate in the sample. β-Glucuronides were estimated through the difference between the concentration of glucuronate before and after incubation with *E. coli* β-glucuronidase. For this, 200 μl of neutralized perchloric acid extract were incubated for 60 min at 37 °C in the presence of 50 mM Mes,[2] pH 6.5, 100 μg/ml bovine serum albumin, and 0.4 units/ml β-glucuronidase in a final volume of 225 μl.

HPLC assays were used to determine the concentrations of nucleotide sugars and ascorbic acid. UDP-glucuronate and UDP-glucose were separated from each other and from other nucleotides by chromatography of neutralized perchloric acid extracts (100 μl) on a Partisphere Sax column (4.6 × 125 mm; from Whatman) using a gradient of 0.01–0.5 M $NH_4H_2PO_4$ (pH 3.7) at a flow rate of 2 ml/min. A_{254} was measured, the reference wavelength being 350 nm. Concentrations were calculated by comparing the integrated peak surfaces with that of a titrated solution of UMP. Ascorbic acid was determined by chromatographing non-neutralized perchloric acid extracts (100 μl) on an Alltima C_{18} column (4.6 × 250 mm; from Alltech), which was then washed with 50 mM KH_2PO_4 (pH 5.0) for 10 min at a flow rate of 1 ml/min. A_{240} was measured to detect ascorbic acid, the reference wavelength being 350 nm. Ascorbic acid was eluted after ~5 min, and the concentration was calculated by comparing the integrated peak surface with the one of standard ascorbic acid dissolved in 3.3% (w/v) perchloric acid. The column was regenerated by washing for 4 min with a mixture containing 25 mM KH_2PO_4 (pH 5.0), 11% acetonitrile, and 22% methanol and by washing for 4 min with the 50 mM KH_2PO_4 buffer. Results obtained with this method were in excellent agreement with those obtained with the dipyridyl reaction (17), except for samples containing aminopyrine. The latter drug was indeed found to interfere in the colorimetric assay.

Total and oxidized glutathione were assayed by the glutathione reductase assay (18). ATP was assayed as in Ref. 19.

RESULTS

Analysis of the Effect of Aminopyrine—The first question we addressed was whether aminopyrine, one of the agents known to stimulate vitamin C synthesis and pentose formation, would provoke a detectable increase in the concentration of glucuronate in isolated rat hepatocytes. As glucuronate is metabolized to L-gulonate by glucuronate reductase, the incubations were also carried out in the presence of sorbinil, an inhibitor of this enzyme (20). As shown in Fig. 2, aminopyrine increased the concentration of glucuronate in a dose-dependent manner, and this effect was much larger in the presence of sorbinil, which, by itself, also caused an increase in the concentration of glucuronate. Similar experiments, in which perchloric acid extracts were prepared after separation of the cells from the medium by centrifugation, indicated that ≥90% of the glucuronate that had accumulated in the combined presence of aminopyrine and sorbinil was intracellular (results not shown). Since sorbinil had an almost maximal effect at 100 μM, this concentration was chosen in the following experiments.

The time course of the effect of 2 mM aminopyrine is shown in Fig. 3. Following the addition of this agent, the glucuronate concentration increased ~4-fold in 5 min in the absence of sorbinil and remained at this higher steady state value during the rest of the experimental period (Fig. 3*A*). In the presence of the glucuronate reductase inhibitor, the concentration of glucuronate increased to a larger extent and almost steadily during the whole experimental period, although a little faster during the first 5 min (0.40 ± 0.03 μmol/min/g of protein) than

[1] C. L. Linster and E. Van Schaftingen, unpublished results.

[2] The abbreviations used are: Mes, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography.

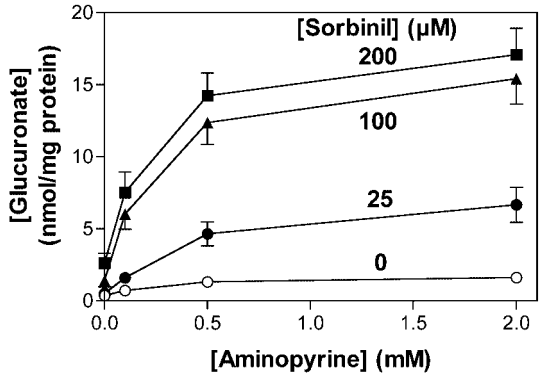

FIG. 2. **Effect of aminopyrine and sorbinil on the concentration of glucuronate in isolated hepatocytes.** Hepatocytes were preincubated for 20 min in the presence of the indicated concentrations of sorbinil (*open circles*, control without sorbinil; *filled circles*, 25 μM sorbinil; *filled triangles*, 100 μM sorbinil; *filled squares*, 200 μM sorbinil). Aminopyrine was then added at the indicated concentrations, and the incubations were arrested 40 min later. Glucuronate was assayed in perchloric acid extracts prepared from the whole cell suspension. Results are means ± S.E. for 4–5 experimental values.

at later times (0.24 ± 0.02 μmol/min/g of protein between 5 and 40 min).

As glucuronate is most likely formed from UDP-glucuronate, we also measured the concentration of this nucleotide (Fig. 3*B*) and of its precursor, UDP-glucose (Fig. 3*C*). Aminopyrine caused a fall in the concentration of UDP-glucuronate, which was unaffected by the presence of sorbinil, whereas it caused a modest and transient increase in the concentration of UDP-glucose, which was again not significantly affected by the presence of sorbinil.

To confirm that UDP-glucuronate was the precursor for the formation of glucuronate, we checked whether resorcinol, a readily glucuronidated phenol derivative (21), and D-galactosamine, which traps uridine nucleotides as UDP-galactosamine and derivatives of it (22), would inhibit the formation of glucuronate induced by aminopyrine in the presence of sorbinil. As shown in Table I, both agents depleted UDP-glucuronate, and in the case of galactosamine, also UDP-glucose. Both of them decreased the basal concentration of glucuronate by a factor of ~3 (resorcinol) and 4.5 (galactosamine) and almost abolished the increase in glucuronate concentration induced by aminopyrine. Resorcinol caused the accumulation of a β-glucuronide, most likely resorcinylglucuronide, to a level that was ~3-fold higher than that of free glucuronate observed in the presence of aminopyrine, consistent with the larger effect of resorcinol rather than the effect of aminopyrine on the UDP-glucuronate pool.

Effect of Other Compounds on the Formation of Free Glucuronate—We also tested the effect of other compounds known to stimulate the formation of ascorbic acid *in vivo*. As shown in Fig. 4*A*, the formation of glucuronate was stimulated by chloretone, antipyrine, and barbital in decreasing order of potency. Stimulation was also observed with pentobarbital and phenylbutazone (both at 1 mM; not shown), a pyrazole derivative similar to aminopyrine. No effect was observed with unsubstituted pyrazole (at 1 mM; not shown).

As aminopyrine is known to be metabolized by cytochrome P450 (23), we checked whether inhibitors of cytochrome P450 could prevent the effect of the pyrazole derivative on glucuronate formation. Quite surprisingly, the three inhibitors that were tested, clotrimazole (24), proadifen, and metyrapone (25), stimulated by themselves the formation of free glucuronate (Fig. 4*B*), and their effect was not additive with that of aminopyrine (not shown). In the case of clotrimazole and proadifen,

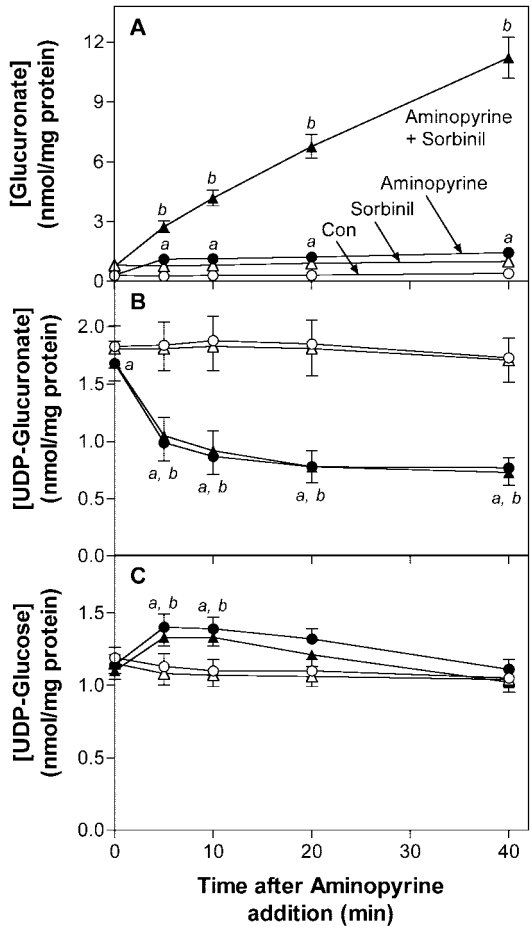

FIG. 3. **Time course of the effect of aminopyrine on the concentrations of glucuronate (*A*), UDP-glucuronate (*B*), and UDP-glucose (*C*) in isolated hepatocytes.** Hepatocytes were preincubated for 20 min with (*open and filled triangles*) or without (*open and filled circles*) 100 μM sorbinil. Aminopyrine was then added at a final concentration of 2 mM (*filled symbols*). Control incubations where aminopyrine was omitted were also performed (*open symbols*). Perchloric acid extracts were prepared on the whole cell suspension at the indicated times for the assay of metabolites. Results are means ± S.E. for 4 experiments. *a* and *b* indicate statistically significant difference (Student's *t* test; $p < 0.01$) of the "aminopyrine" (*filled circles*) and "aminopyrine + sorbinil" (*filled triangles*) conditions *versus* their appropriate control. *Con*, control without sorbinil.

the highest concentration used (0.5 mM) had a lesser effect than lower concentrations, presumably because of toxic effects. This is consistent with the finding that, at a concentration of 0.5 mM, both agents caused a marked decrease in the ATP concentration (down to <10% of the control level; not shown).

Taken together, these results indicate that the effect on glucuronate formation is shared by compounds with quite varied but rather hydrophobic structures. It should also be noted that all agents that were investigated in Fig. 4 exerted a rapid effect. This was indicated by the observation that the glucuronate concentration, reached 10 min after the addition of a maximally active concentration of the drugs, corresponded to 32–37% of the concentration reached after 40 min (results not shown). This value is similar to the one obtained with aminopyrine (37%, Fig. 3*A*).

The effects of some of the agents mentioned above were further investigated in the experiment reported in Fig. 5. As for aminopyrine, the effects of antipyrine, metyrapone, proadifen, clotrimazole, and chloretone on free glucuronate were greatly reinforced by sorbinil (Fig. 5*A*). This was, however, not the case for barbital, presumably because the latter also inhibits glucu-

TABLE I

Prevention of the stimulatory effect of aminopyrine on glucuronate formation by resorcinol and D-galactosamine, two agents causing UDP-glucuronate depletion

Isolated hepatocytes were preincubated for 20 min with or without 1 mM resorcinol or 5 mM D-galactosamine, together with 100 μM sorbinil. Aminopyrine was then added at a final concentration of 2 mM, and the incubation was pursued for 40 min. Glucuronate, β-glucuronides, UDP-glucuronate, and UDP-glucose were assayed in perchloric acid extracts prepared on the whole cell suspension. Results are means ± S.E. for 4 experiments.

Condition	[Glucuronate]	[β-Glucuronides]	[UDP-glucuronate]	[UDP-glucose]
	nmol/mg of protein	*nmol/mg of protein*	*nmol/mg of protein*	*nmol/mg of protein*
Control	1.81 ± 0.50	0.07 ± 0.07	2.32 ± 0.21	1.49 ± 0.11
2 mM aminopyrine	15.38 ± 1.85[a]	0.19 ± 0.16	1.06 ± 0.15[a]	1.49 ± 0.11
1 mM resorcinol	0.65 ± 0.11	43.34 ± 4.12[a]	0.08 ± 0.08[a]	0.99 ± 0.15[a]
2 mM aminopyrine + 1 mM resorcinol	1.20 ± 0.24[b]	44.38 ± 3.20[a,b]	0.04 ± 0.04[a,b]	1.04 ± 0.10[a,b]
5 mM D-galactosamine	0.40 ± 0.12[a]	0.20 ± 0.03	0.07 ± 0.04[a]	0.04 ± 0.02[a]
2 mM aminopyrine + 5 mM D-galactosamine	1.01 ± 0.43[b]	0.10 ± 0.06	0.03 ± 0.03[a,b]	0.03 ± 0.02[a,b]

[a] Significantly different from control by Student's *t* test ($p < 0.05$).
[b] Significantly different from 2 mM aminopyrine.

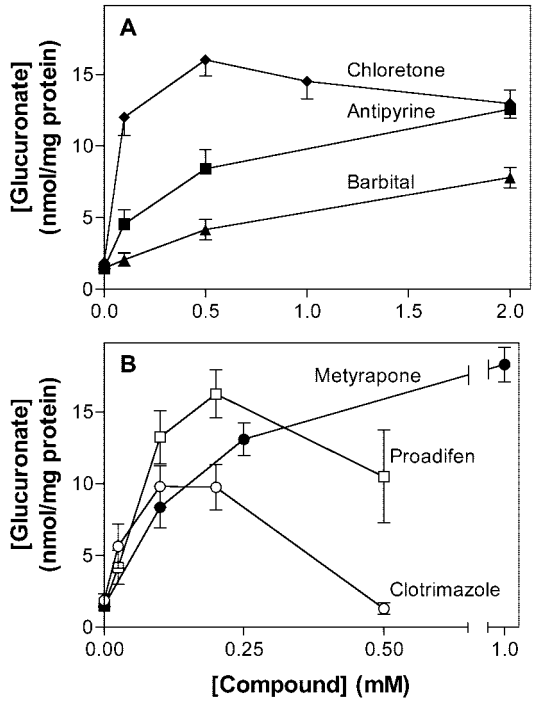

FIG. 4. **Dose dependence of the effect of stimulators of vitamin C formation (*A*) and of inhibitors of cytochrome P450 (*B*) on the formation of free glucuronate in isolated hepatocytes incubated with sorbinil.** Hepatocytes were preincubated for 20 min with 100 μM sorbinil before the addition of the indicated agents (*filled diamonds*, chloretone; *filled squares*, antipyrine; *filled triangles*, barbital; *filled circles*, metyrapone; *open squares*, proadifen; *open circles*, clotrimazole) at the indicated concentrations. The incubations were arrested 40 min later, and glucuronate was measured in perchloric acid extracts prepared on the whole cell suspension. Results are means ± S.E. for 3–4 experimental values.

ronate reductase (26). Similarly to aminopyrine, all agents stimulating the formation of glucuronate caused a decrease in the UDP-glucuronate pool (Fig. 5*C*). With the exception of barbital, all of the agents also caused an increase in the formation of vitamin C, which was markedly (~80%) inhibited by sorbinil (Fig. 5*D*).

As glutathione depletion has been postulated to play a role in the regulation of vitamin C synthesis (10), we also investigated the effect of glutathione-depleting agents on the same parameters. Menadione, buthionine sulfoximine, and diamide caused a significant decrease of intracellular glutathione (Fig. 5*E*) and a significant increase in extracellular GSSG in the case of menadione and diamide (not shown). Despite this, these agents had little or no effect on the formation of free glucuronate or ascorbic acid. By contrast, the agents that most stimulated glucuronate and vitamin C formation had a much lesser or insignificant effect on glutathione levels.

β-Glucuronides were also measured in the experiment shown in Fig. 5 as the difference between the concentration of glucuronate before and after treatment with β-glucuronidase (Fig. 5*B*). As this is a difference assay, it is expected to be more reliable for the conditions in which the free glucuronate concentration is low, *i.e.* where sorbinil has been omitted from the incubation medium. However, there was a fair agreement between the values observed with and without the inhibitor of glucuronate reductase, indicating the reliability of the assay procedure. Among the agents that increased glucuronate formation, only three (antipyrine, proadifen, and menadione) induced the formation of β-glucuronides, which reached concentrations (0.9–1.9 nmol/mg of protein) that were less than 1/20 of the concentration observed in the presence of resorcinol (Table I).

DISCUSSION

The Conversion of UDP-glucuronate to Glucuronate as a Regulated Step in Vitamin C Synthesis—Previous studies using radiolabeled galactose and glucose as precursors have indicated that vitamin C is formed from a UDP-linked sugar, presumably UDP-glucuronate (27). The precise mechanism by which free glucuronate is formed, by way of glucuronate 1-phosphate (28, 29) or a glucuronidated intermediate (8, 30) or even through a direct conversion of UDP-glucuronate to UDP and glucuronate (Fig. 1), is presently unknown. Our study provides two new pieces of evidence indicating the precursor-product relationship between UDP-glucuronate and glucuronate since (*a*) two agents (resorcinol and D-galactosamine) that deplete UDP-glucuronate through quite distinct mechanisms markedly reduce the formation of free glucuronate whether in the absence or in the presence of aminopyrine; (*b*) all agents that stimulate the formation of glucuronate cause an ~50% decrease in the concentration of UDP-glucuronate.

In addition, our results indicate that aminopyrine and other xenobiotics stimulate the formation of vitamin C and L-xylulose by accelerating the conversion of UDP-glucuronate to glucuronate. With the exception of barbital, which inhibits glucuronate reductase (26), all agents stimulated the formation of vitamin C (in the absence of sorbinil) in proportion with their ability to stimulate the formation of glucuronate (in the presence of sorbinil). This is consistent with the concept that what limits the formation of vitamin C is the availability of free glucuronate. Vitamin C formation in the absence of sorbinil amounted to ~30% of the glucuronate that accumulated in the presence of sorbinil, indicating that most of the glucuronate formed from UDP-glucuronate is converted to L-xylulose. The finding that sorbinil, which blocks the downstream conversion

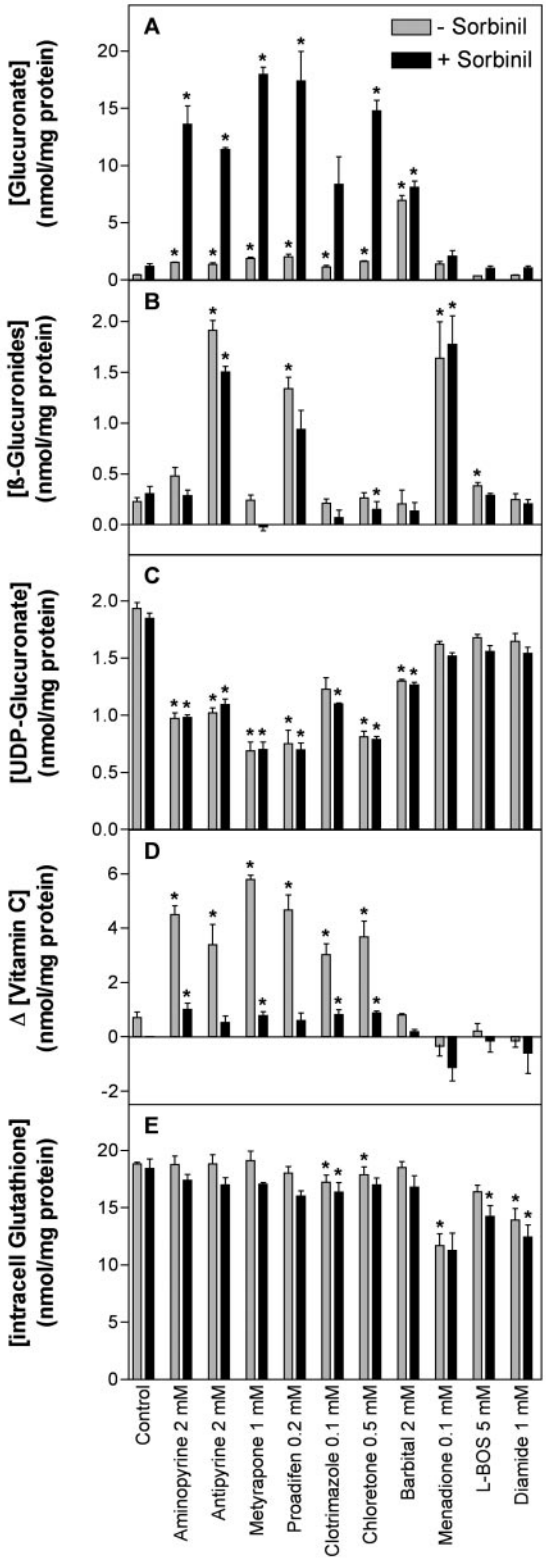

FIG. 5. **Effect of various compounds on the concentrations of glucuronate (*A*), β-glucuronides (*B*), UDP-glucuronate (*C*), and glutathione (*E*) and on the formation of vitamin C (*D*).** Hepatocytes were preincubated for 20 min with (*black bars*) or without (*gray bars*) 100 μM sorbinil before the addition of the indicated agents at the indicated concentrations. The incubations were arrested 20 min later for the assay of glutathione and 40 min later for the assay of glucuronate, β-glucuronides, UDP-glucuronate, and vitamin C. Glucuronate, β-glucuronides, UDP-glucuronate, and vitamin C were measured in perchloric acid extracts prepared from the total cell suspension, whereas total glutathione (GSH + GSSG) was assayed in perchloric acid extracts prepared by centrifugation of the cells through a silicone layer, thus representing total intracellular glutathione. The latter was essentially (>94%) present in its reduced form under all conditions. In the case of vitamin C, the results are presented as the difference between the concentration found in the indicated condition and the concentration found at the same time in the presence of sorbinil only. Under the latter condition, the vitamin C content was 7.61 ± 1.60 nmol/mg of protein. Results are means ± S.E. for 3 experiments. *, significantly different from the appropriate control by Student's *t* test ($p < 0.05$). *intracell*, intracellular; *L-BOS*, L-buthionine-(*S*,*R*)-sulfoximine.

of glucuronate to L-gulonate, did not modify the decrease in UDP-glucuronate induced by aminopyrine and other xenobiotics indicates that free glucuronate, as well as downstream metabolites, exerts no or little effect on glucuronate formation.

The finding that glucuronate remains essentially intracellular, together with the fact that glucuronate added to the extracellular medium is not taken up by hepatocytes,[3] indicates that the hepatocyte plasma membrane does not contain a transporter for glucuronate. This sugar derivative behaves therefore essentially as an intracellular metabolite, in a similar manner as intermediates of other intracellular pathways such as glycolysis and the pentose phosphate pathway. Absence of glucuronate transport in the hepatocyte plasma membrane may explain previous results showing that intraperitoneally administered glucuronate, at variance with 3,6-glucuronolactone, is not converted to vitamin C *in vivo* (5).

Finally, except for the recent study on the effect of glutathione-depleting agents (10), all previous studies had been performed *in vivo*, and it was therefore not clear whether other organs, hormones, or circulating factors were involved in the effect of aminopyrine and other drugs. The finding that the stimulation of glucuronate and vitamin C formation can take place in isolated hepatocytes indicates that it does not require short term hormonal influences or organ-to-organ transfer of metabolites or modified xenobiotics (also, see below).

Involvement of a Short Term Effect—Most of the studies previously investigating the effect of aminopyrine and other xenobiotics on glucuronate and vitamin C formation put the emphasis on the ability of these compounds to induce the synthesis of enzymes such as UDP-glucose dehydrogenase, UDP-glucuronosyltransferase, and β-glucuronidase that participate, or may participate, in the formation of glucuronate (6–9). The present study shows that the stimulation caused by these agents is already maximal at the shortest investigated times (5 or 10 min). In the presence of sorbinil, which blocks further conversion of glucuronate, stimulation of the formation of this metabolite reaches up to 15-fold. The amplitude of this effect indicates that regulation of free glucuronate and vitamin C formation may largely be a short term effect.

A previous study has shown that glutathione-depleting agents stimulate the formation of vitamin C in isolated mouse hepatocytes (10). Of the glutathione-depleting agents that we tested (menadione, buthionine sulfoximine, and diamide), the only one that stimulated glucuronate formation was menadione, and it had a much lesser effect than agents such as aminopyrine, metyrapone, and proadifen that did not significantly affect GSH concentration. In contrast to Braun *et al.* (10), we did not find a stimulatory effect of the glutathione-depleting agents on vitamin C formation. We have no clear explanation for this discrepancy. The use of different vitamin C assays (by HPLC in our case and by a colorimetric assay in the case of Braun *et al.* (10)) provides no explanation since we found identical results with the colorimetric assay as with the HPLC assay, except for an interference of aminopyrine in the colorimetric assay (not shown). Other potential explanations might be linked to a species difference (rat *versus* mouse) or to dietary influences.

[3] C. L. Linster, unpublished results.

The effect of glutathione depletion has been linked to glycogen degradation (10). In this case, the increase in glucuronate formation would be mediated through a push effect, *i.e.* through an increase in the concentrations of glucose 1-phosphate, UDP-glucose, and UDP-glucuronate. However, all agents that stimulated the formation of free glucuronate caused a decrease rather than an increase in the concentration of UDP-glucuronate (Figs. 3*B* and 5*C*) and barely affected the UDP-glucose concentration (Fig. 3*C* and data not shown). Furthermore, glucagon and dibutyrylcyclic AMP failed to affect glucuronate formation in isolated rat hepatocytes.[3] We have therefore no reason to believe that the effect of agents that stimulated glucuronate formation in the present study was linked to enhanced glycogen degradation or to glutathione depletion.

As mentioned in the Introduction, it has been proposed that free glucuronate is formed by hydrolysis of glucuronidated metabolites arising from the stimulating drugs. *In vivo*, this hydrolysis could potentially take place in the intestine following secretion of glucuronidated metabolites in the bile. This possibility can be excluded in the isolated hepatocyte model used in the present study. Furthermore, two pieces of evidence argue against intracellular hydrolysis of glucuronides taking place inside hepatocytes. The first one is that little if any glucuronidated metabolite was formed with the agents that stimulated the formation of free glucuronate, whereas an agent such as resorcinol, which leads to the accumulation of an elevated concentration of its glucuronidated metabolite, did not cause any formation of free glucuronate. The second is that, if a glucuronidated precursor had to accumulate before glucuronate would form, free glucuronate would appear with a lag kinetic, whereas the opposite was observed. Actually, the formation of glucuronate slowed down after an initial rapid period, most likely because of the decrease in the UDP-glucuronate concentration.

Nonetheless, a good argument in favor of a role of UDP-glucuronosyltransferases is the finding of Horio *et al.* (8) that the 3-methylcholanthrene-induced increase in the urinary excretion of ascorbic acid observed *in vivo* is reduced in heterozygous Gunn rats and abolished in homozygous Gunn rats. These rats have a mutation in the gene encoding the UDP-glucuronosyltransferase that is induced by 3-methylcholanthrene (31), explaining the lack of effect of this chemical on the transferase activity. Combined with our own results, which point to a short term effect and to the absence of a glucuronidated intermediate, these data raise the possibility that non-glucuronidable xenobiotics may combine with UDP-glucuronosyltransferase(s) and induce a UDP-glucuronate hydrolase activity, either by acting as pseudosubstrates or through an allosteric effect. Evidence for a UDP-glucuronidase activity of a purified UDP-glucuronosyltransferase has been reported previously (32). It has indeed been shown that the addition of compounds (phenylethyl ether, phenylphenyl ether, *p*-nitrophenylphenyl ether) that bind to the aglycone site of GT_{2P}, a UDP-glucuronosyltransferase purified from pig liver, but are not substrates of this enzyme, led to the formation of free glucuronate at a rate representing up to ~0.03% of the rate of glucuronidation of *p*-nitrophenol. This formation of free glucuronate is stimulated by lysophosphatidylcholine, as is the glucuronidation reaction catalyzed by the same enzyme. Because of the multiplicity of UDP-glucuronosyltransferases, such a mechanism could account for the wide diversity of the compounds that stimulate free glucuronate formation, as well as for their hydrophobicity, a characteristic of the substrates, and presumably also of the pseudosubstrates, of this group of enzymes (33). Although the rate of UDP-glucuronate hydrolysis observed with GT_{2P} may appear quite low, it is likely that other agents acting on other UDP-glucuronosyltransferases induce higher rates of α-glucuronidase activity. Preliminary data obtained on rat liver extracts or microsomes indicate, however, that aminopyrine, chloretone, and metyrapone barely (by less than 20%) affect the formation of glucuronate from UDP-glucuronate, which suggests that the regulation of glucuronate formation from UDP-glucuronate may be quite complex and may involve factors that are lost after cell homogenization.

Further work is obviously needed to determine the identity of the enzyme(s) responsible for the formation of glucuronate. Its (their) ability to respond rapidly to xenobiotics is a property that will certainly be helpful for its (their) identification as well as for the unraveling of the factors that control its (their) activity under physiological conditions.

REFERENCES

1. Smirnoff, N. (2001) *Vitam. Horm.* **61,** 241–266
2. Hiatt, H. H. (2001) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 8th Ed., Vol. I, pp. 1589–1599, McGraw-Hill, New York
3. Burns, J. J., Evans, C., and Trousof, N. (1957) *J. Biol. Chem.* **227,** 785–794
4. Margolis, J. I. (1929) *Am. J. Med. Sci.* **177,** 348–371
5. Burns, J. J., and Evans, C. (1956) *J. Biol. Chem.* **223,** 897–905
6. Hollmann, S., and Touster, O. (1962) *Biochim. Biophys. Acta* **62,** 338–352
7. Horio, F., Kimura, M., and Yoshida, A. (1983) *J. Nutr. Sci. Vitaminol.* **29,** 233–247
8. Horio, F., Shibata, T., Makino, S., Machino, S., Hayashi, Y., Hattori, T., and Yoshida, A. (1993) *J. Nutr.* **123,** 2075–2084
9. Horio, F., and Horie, T. (1997) *Biosci. Biotechnol. Biochem.* **61,** 109–112
10. Braun, L., Csala, M., Poussu, A., Garzó, T., Mandl, J., and Bánhegyi, G. (1996) *FEBS Lett.* **388,** 173–176
11. Bartrons, R., Hue, L., Van Schaftingen, E., and Hers, H.-G. (1983) *Biochem. J.* **214,** 829–837
12. Seglen, P. O. (1973) *Exp. Cell Res.* **82,** 391–398
13. Van Schaftingen, E., Opperdoes, F., and Hers, H.-G. (1987) *Eur. J. Biochem.* **166,** 653–661
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
15. Ashwell, G., Wahba, A. J., and Hickman, J. (1960) *J. Biol. Chem.* **235,** 1559–1565
16. Hickman, J., and Ashwell, G. (1960) *J. Biol. Chem.* **235,** 1566–1570
17. Omaye, S. T., Turnbull, J. D., and Sauberlich, H. E. (1979) *Methods Enzymol.* **62,** 3–11
18. Griffith, O. W. (1990) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Bergmeyer, J., and Grassl, M., eds) 3rd Ed., Vol. VIII, pp. 521–529, Verlag Chemie, Weinheim, Germany
19. Trautschold, I., Lamprecht, W., and Schweitzer, G. (1989) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Bergmeyer, J., and Grassl, M., eds) 3rd Ed., Vol. VII, pp. 346–357, Verlag Chemie, Weinheim, Germany
20. Srivastava, S. K., Petrash, J. M., Sadana, I. J., Ansari, N. H., and Partridge, C. A. (1982) *Curr. Eye Res.* **2,** 407–410
21. Van Schaftingen, E. (1995) *Eur. J. Biochem.* **234,** 301–307
22. Keppler, D. O. R., Rudigier, J. F. M., Bischoff, E., and Decker, K. F. A. (1970) *Eur. J. Biochem.* **17,** 246–253
23. Tanaka, E., and Breimer, D. D. (1997) *J. Clin. Pharm. Ther.* **22,** 237–249
24. Rodrigues, A. D., Gibson, G. G., Ioannides, C., and Parke, D. V. (1987) *Biochem. Pharmacol.* **36,** 4277–4281
25. Lewis, D. F. (2001) *Guide to Cytochromes P450: Structure and Function*, pp. 118–139, Taylor and Francis Inc., New York
26. Sato, K., Inazu, A., Yamaguchi, S., Nakayama, T., Deyashiki, Y., Sawada, H., and Hara, A. (1993) *Arch. Biochem. Biophys.* **307,** 286–294
27. Evans, C., Conney, A. H., Trousof, N., and Burns, J. J. (1960) *Biochim. Biophys. Acta* **41,** 9–14
28. Ginsburg, V., Weissbach, A., and Maxwell, E. S. (1958) *Biochim. Biophys. Acta* **28,** 649–650
29. Puhakainen, E., and Hänninen, O. (1976) *Eur. J. Biochem.* **61,** 165–169
30. Pogell, B. M., and Leloir, L. F. (1961) *J. Biol. Chem.* **236,** 293–298
31. Iyanagi, T., Watanabe, T., and Uchiyama, Y. (1989) *J. Biol. Chem.* **264,** 21302–21307
32. Hochman, Y., and Zakim, D. (1984) *J. Biol. Chem.* **259,** 5521–5525
33. Radominska-Pandya, A., Czernik, P. J., Little, J. M., Battaglia, E., and Mackenzie, P. I. (1999) *Drug Metab. Rev.* **31,** 817–899