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NON-SPECIFIC INHIBITION OF SOME RAT LIVER MEMBRANE-BOUND ENZYMES BY AN ADENYLATE CYCLASE INHIBITOR, RMI 12330 A

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Abstract—RMI 12330 A [*N*-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride] inhibits choleratoxin-induced intestinal hypersecretion, presumably *via* an inhibition of the mucosal enzyme adenylate cyclase. We previously reported the inhibition by RMI 12330 A of the adenylate cyclase activity from rat liver plasma membrane preparations. We firstly extend here these results, showing the inhibition by RMI 12330 A of adenylate cyclase activity from spleen, brain, heart and kidney. Furthermore, in plasma membrane preparations, this agent inhibited the activities of Mg^{2+}-ATPase and leucyl-β-naphthylamidase but had no effect upon 5'nucleotidase. In non-activated rat liver microsomes, the activities of *p*-nitrophenol and bilirubin UDP-glucuronosyltransferases, and of glucose-6-phosphatase, were enhanced by low concentrations of RMI 12330 A but inhibited by higher concentrations. This biphasic effect disappeared when digitonin-activated or Emulgen 911-solubilized microsomal membranes were used. Thus, RMI 12330 A does not behave as a specific inhibitor of adenylate cyclase, since it also perturbs other membrane-associated enzyme activities.

Specific enzyme inhibitors or activators can serve as useful probes for the investigation of multicomponent enzyme systems. Compounds similar to the well known inhibitor of the Na^+, K^+-dependent ATPase, ouabain, have been sought for the elucidation of the molecular structure and the mechanism of action of the adenylate cyclase system [ATP: pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. Over the past few years, a new series of organic cycloalkyl molecules has been investigated [1]. The most promising compound, RMI 12330 A [*N*-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride] has been reported to inhibit the adenylate cyclase from rabbit intestine [2] and rat liver plasma membrane [3]. We will show here that RMI 12330 A alters also other enzyme activities from rat liver plasma membrane and microsomes.

MATERIALS AND METHODS

Materials

RMI 12330 A was the kind gift of Dr. H. R. Rohr and Dr. N. L. Wiech, from Merrell-National Laboratories, Cincinnati, U.S.A. Its structure is shown in Fig. 1. It was dissolved in ethanol and diluted with 50 mM Tris–HCl buffer, pH 7.6, except for the *p*-nitrophenol uridine diphosphate-glucuronosyltransferase activity [UDP-GTA; UDP-glucuronate β-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17] assays where it was diluted with 0.25 M phosphate buffer, pH 7.1. The final ethanol concentration never exceeded 3 per cent (v/v) of the total incubation medium and was equally present in the corresponding controls.

Porcine crystalline glucagon (Novo Laboratories, Bagsvaerd, Denmark), phenylmethyl sulfonyl fluoride, creatine phosphate (Calbiochem, LA, U.S.A.), dithiothreitol, bovine serum albumin, L-leucyl-β-naphthylamide HCl, Lubrol PX, UDP-glucuronic acid, ammonium salt, and other nucleotides (Sigma, St Louis, U.S.A.), creatine kinase (Boehringer, Mannheim, Germany), bilirubin (British Drug Houses Ltd., Poole, U.K.), ethyl anthranilate (Eastman Kodak, Rochester, U.S.A.), *p*-nitrophenol (Koch-Light Laboratories Ltd., Colnbrook, U.K.) were used without further purification. Emulgen 911 was a gift of Kao-Atlas Ltd (Tokyo, Japan). [α-^{32}P]ATP (21.5 Ci/m-mole) was obtained from New England Nuclear Corporation (Boston, U.S.A.), cyclic [8-^{3}H]AMP (13 Ci/m-mole) was obtained from the CEA (Saclay, France). Sodium fluoride and all other chemicals were of analytical grade from Merck (Darmstadt, Germany).

Methods

Liver plasma membrane preparation. Liver plasma membranes from female Wistar rats (90–120 g body weight, fed *ad lib.*), were prepared according to the procedure devised by Neville [4] up to step 11. The purified membrane preparations which were suspended in 1 mM $NaHCO_3$ could be stored up to 6 weeks in liquid nitrogen without any loss of adenylate cyclase activity [5]. Similar experimental results were obtained with several different batches of liver membranes.

Particulate fractions from other organs. Particulate fractions from spleen, brain, heart and kidney were prepared as follows. A 1-gram fraction from each tis-

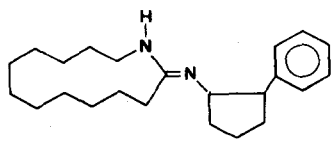

Fig. 1. Structure of the RMI 12330 A.

sue in 11 ml of 1 mM $NaHCO_3$ was homogenized with a loose-fitting Dounce homogenizer. After two washing steps, performed by centrifuging at 2000 *g* for 10 min at 4°, the pellets enriched with plasma membranes were resuspended in 3 vols of 1 mM $NaHCO_3$ solution. The membrane preparations were stored in liquid nitrogen until use.

Rat liver microsomal preparation. Rat liver microsomes were prepared as previously described [6]. The resulting pellet was resuspended and diluted with 0.25 M sucrose, or in 0.1 M Tris–acetate buffer, pH 7.5, with or without 2% (w/v) of digitonin, for the *in vitro* assays.

Solubilized UDP-glucuronosyltransferase preparation. The solubilized fraction from rat liver microsomes was prepared basically according to the procedure of Del Villar *et al.* [7] the microsomal preparation, at a concentration of about 17 mg protein/ml, was resuspended and dispersed in 0.1 M Tris–acetate buffer, pH 7.5, containing Emulgen 911 (0.2 mg/mg protein), 25% (v/v) glycerol and 0.1 mM dithiothreitol. The preparation was stirred for 30 min at 0° under N_2 and then centrifuged at 105,000 *g* for 4 hr. The assays were performed using the supernatant fraction, which then contained about 5 mg protein/ml.

Adenylate cyclase assay. Adenylate cyclase activity was performed under the usual conditions [8]: 60 μl of 50 mM Tris–HCl pH 7.6 containing 0.5 mM ATP, 10 mM NaF, 1 mM EDTA, 3 mM $MgCl_2$ and 400 μg membrane proteins/ml. Incubation was initiated by addition of the enzyme preparation and was performed for 10 min in a shaking water bath at 33°. Reactions were terminated by the procedure of Ramachandran [9], or more recently by a modification of the procedure of White [10]. Samples were added to 0.2 ml of 0.5 N HCl, boiled for 6 min, buffered with 0.2 ml of 1.5 M imidazole and finally applied to alumina columns. Cyclic AMP was then eluted with 3 ml of 10 mM imidazole pH 7.6. Results are expressed as nmoles cyclic AMP formed in 10 min per mg protein at 33°. The results, obtained from triplicate determinations, agreed within ±5 per cent.

ATPase activity. Total ATPase (ATP phosphohydrolase, EC 3.6.1.3) was assayed, using the conditions described by Emmelot and Bos [11] and as previously reported [12]. The medium contained 66 mM NaCl, 33 mM KCl, 5 mM $MgCl_2$, 25 mM Tris–HCl, pH 7.2, 5 mM ATP (sodium or Tris salt) and 40 μg of membrane proteins in a volume of 1 ml. The reaction, initiated by the addition of membranes, was run at 37° for 10 min, and was stopped by adding ice-cold trichloroacetic acid (5% (v/v) final concentration). Protein was removed by centrifugation and inorganic phosphate was estimated in the supernatant by the method of Fiske and Subbarow. Results are expressed as μmoles of inorganic phosphate liberated in 10 min per mg protein. Each enzymic assay was done in duplicate and agreed within ±3 per cent.

Leucyl-β-naphthylamidase activity. This enzyme (α-aminoacyl peptide hydrolase, EC 3.4.11.1) was assayed according to the method of Lauter *et al.* [13] in 500 μl of 100 mM phosphate buffer, pH 7.4, containing 0.1 mM L-leucyl-β-naphthylamide HCl. The assay was initiated with membrane proteins (58 μg/ml of incubation medium) and stopped after 15 min at 37°, with 2 ml of 0.1 M borate buffer, pH 10.7. The β-naphthylamine liberated was determined with an Eppendorf spectrofluorometer.

5'Nucleotidase activity. The 5'nucleotidase activity (5'ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed according to Lauter *et al.* [13]. It was performed in 1 ml of 25 mM Tris–HCl buffer pH 7.2, 5 mM AMP, 10 mM $MgCl_2$ and 90 μg/ml of membrane proteins. After addition of 1 ml of 10% (v/v) trichloroacetic acid the incubation mixture was centrifuged and the inorganic phosphate liberated estimated by the method of Fiske and Subbarow.

UDP-glucuronosyltransferase activities (UDP-GTA)

Bilirubin UDP-GTA. This was measured according to a micro-modification [14] of the method of Van Roy and Heirwegh [6] and Black *et al.* [15] i.e. a system using diazotized ethyl anthranilate and in which the excess of unconjugated bilirubin does not react [16]. The final concentrations in the assay mixture were: 65 mM triethanolamine buffer, pH 7.4, 7.8 mM $MgCl_2$, 0.27 mM bilirubin, 2.7 mM UDP-glucuronic acid, 0.12 mM albumin, and, when present, 0.3% digitonin. Three preparations of protein were used in the assays: digitonin-activated and non-activated microsomal proteins (3.3 mg/ml) suspended in 0.1 M Tris–acetate buffer, pH 7.5, and Emulgen 911-solubilized proteins (1.6 mg/ml) in a final volume of 160 μl. The reaction was initiated by addition of bilirubin solution and continued at 37° for 15 min.

p-Nitrophenol UDP-GTA. This was assayed according to the method of Zakim and Vessey [17] with slight modifications: the final concentrations were 10 mM UDP-glucuronic acid, 0.4 mM *p*-nitrophenol, 0.1 M phosphate buffer, pH 7.1, and 0.2% digitonin when present. Three different protein preparations were used in the assays: digitonin-activated microsomal suspension in sucrose (1 mg/ml); non-activated microsomal proteins in sucrose (2 mg/ml); and Emulgen 911-solubilized proteins (1.6 mg/ml) in a final volume of 100 μl. The assay was started by addition of UDP-glucuronic acid solution, and incubated at 37° for 10 min.

Glucose-6-phosphatase. The activity of glucose-6-phosphatase (D glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was measured according to Harper [18] with the following modifications: incubations were carried out at 37° in a total volume of 0.3 ml in 0.1 M citrate buffer, pH 6.5 containing 1 mg/ml of microsomal protein. The final concentration of glucose-6-phosphate was 0.04 M. The reaction was initiated by addition of glucose-6-phosphate solution, and incubated for 10 min. It was stopped by addition of 2.0 ml of 10% (v/v) trichloroacetic acid. An aliquot of the supernatant was used for the measurement of inorganic phosphate by the method of Fiske and Subbarow. In all experiments, proteins were measured according to Lowry's procedure, using bovine serum albumin as standard.

RESULTS

Effect of RMI 12330 A upon the rat liver plasma membrane enzymes. We have previously reported [3] an inhibition of the basal and stimulated liver adenylate cyclase activities by concentrations of RMI 12330 A ranging from 10 μM to 5 mM. The irreversible

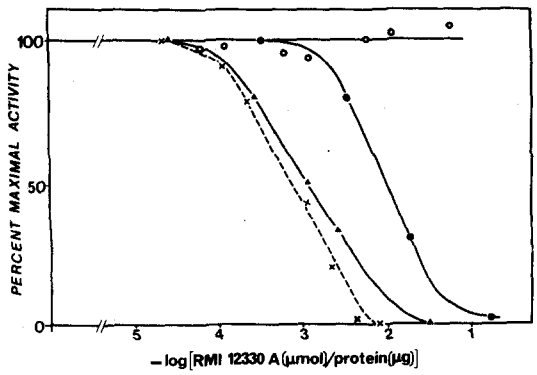

Fig. 2. Action of the RMI 12330 A upon various enzymes of the liver plasma membrane. Assays of cyclase (×), ATPase (▲) leucyl-β-naphthylamidase (●) and 5'nucleotidase (○) were performed as described in Methods. The reactions were initiated by addition of the enzyme preparation to the incubation medium together with the indicated amount of RMI 12330 A with no preincubation. The range of concentration of the drug was from 10 nM to 10 mM. The effect of the drug is expressed as the percentage of the specific activity with no inhibitor added which was: 2.2 nmoles (adenylate cyclase), 10 μmoles (ATPase), 170 nmoles (leucyl-β-naphthylamidase) and 500 nmoles (5'-nucleotidase)/10 min/mg protein.

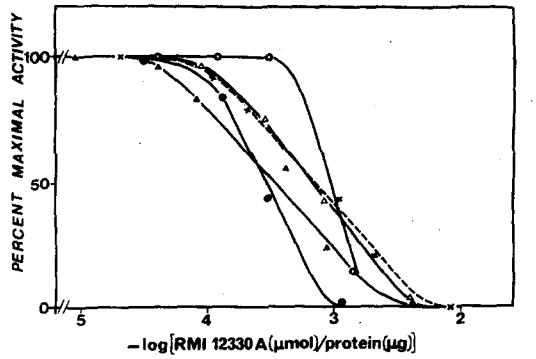

Fig. 3. Inhibition of the adenylate cyclase of particulate fractions of different rat tissues. The particulate fractions of different tissues: spleen (△), brain (○), heart (●) and kidney (▲) were prepared as described under Methods. Adenylate cyclase was assayed in presence of varying amounts of RMI 12330 A (from 10 nM to 5 mM). The concentrations of protein per assay were: 1400, 3800, 3800 and 1330 μg/ml for spleen, brain, heart and kidney respectively. The results obtained with plasma membrane from rat liver (×), assayed under the same conditions, are given as comparison. The effect of the drug is expressed as the percentage of the activity with no inhibitor added. These activities were: 2.2, 0.6, 0.75, 0.5 and 1.2 nmoles cyclic AMP/10 min/mg protein for liver, spleen, brain, heart and kidney respectively.

binding of this agent to plasma membrane [3] varies greatly with the amount of protein present in the incubation medium, thus the most satisfactory way to express the experimental results was as a function of the ratio of the inhibitor concentration to membrane protein content, that is, μmoles RMI 12330 A/μg protein.

Figure 2 shows the effects of increasing amounts of RMI 12330 A, upon the activities of the NaF-stimulated adenylate cyclase, ATPase, 5'nucleotidase and leucyl-β-naphthylamidase activities from rat liver plasma membranes. The inhibition of ATPase and adenylate cyclase was similar and appeared to be well related to the concentration of the inhibitor with half maximal inhibition occurring at 10^{-3} μmoles RMI 12330 A/μg protein. A 10-fold greater ratio of RMI 12330 A/protein was required for the same percentage of inhibition in the case of leucyl-β-naphthylamidase. The 5'nucleotidase activity was not affected at any of the inhibitor concentrations used.

Interaction of RMI 12330 A with cyclase system in other tissues. The effect of RMI 12330 A was tested on adenylate cyclase from particulate fractions from spleen, brain, heart and kidney. In all cases, the inhibition began at ratio RMI 12330 A–protein of 10^{-4}–10^{-5} μmoles/μg, and was complete for a ratio of 10^{-3}–10^{-2} μmoles/μg (Fig. 3). However, the dose–response curve of the action of the inhibitor upon the adenylate cyclase system from rat brain exhibited a definite shift to the right. This might be due to the high level in the brain preparation of lipids, which could have interacted with the RMI 12330 A, a highly hydrophobic compound. A similar shift of the curve with respect to the brain cyclase was observed in a previous study from this laboratory using hexachlorophene [19], and may be due to the same reason.

Effect of RMI 12330 A upon the rat liver microsomal UDP-glucuronosyltransferase activity. Figure 4 shows

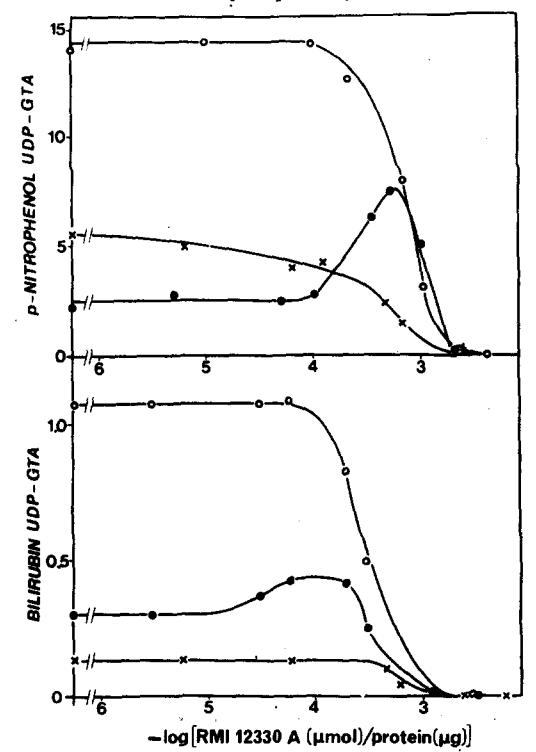

Fig. 4. Effect of increasing amounts of RMI 12330 A upon the UDP-glucuronosyltransferase activity towards bilirubin and *p*-nitrophenol as substrates, in digitonin-activated (○), non-activated (●), and Emulgen 911-solubilized (×) rat liver microsomal preparations. The specific activity (expressed as nmoles of substrate conjugated per min per mg protein) is plotted against the ratio of inhibitor content over microsomal protein. RMI 12330 A was dissolved in ethanol, buffered as described in the text and diluted from 0 to 4 mM so that all the assays in each experiment contained the same amount of ethanol. The assays were performed as described in the text.

the biphasic effect of RMI 12330 A upon the activities of *p*-nitrophenol and bilirubin UDP-GT in rat liver microsomal preparations with no detergent added. At ratios of $5 \cdot 10^{-4}$ and $1 \cdot 10^{-4}$ μmoles RMI 12330 A/μg protein, there was a 300 and 140 per cent maximal stimulation of the activities of *p*-nitrophenol and bilirubin UDP-GT respectively. At drug–protein ratios greater than $2 \cdot 10^{-3}$ μmoles/μg, complete inhibition was obtained. Spectral studies showed that no shift occurred when bilirubin was mixed together with a RMI 12330 A preparation, thus eliminating the possible formation of any inhibitor-substrate complex. Nor did RMI 12330 A inhibit the diazotation of bilirubin under our experimental conditions, that is when RMI 12330 A was added at the end of the enzymic assay before coupling with diazotized ethylanthranilate. When digitonin-activated microsomes or Emulgen 911-solubilized enzymic preparations were used no stimulation was observed with either substrate. The inhibition was half-maximal at $8 \cdot 10^{-4}$ and $4 \cdot 10^{-4}$ μmoles RMI 12330 A/μg protein respectively for the digitonin-activated and Emulgen 911–solubilized *p*-nitrophenol UDP-GTA assays, and $3 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$ μmoles/μg for the digitonin-activated and Emulgen 911-solubilized bilirubin UDP-GTA assays respectively. All these inhibitions were total for a ratio of $2 \cdot 10^{-3}$ μmoles RMI 12330 A/μg protein.

The presence of albumin in the bilirubin UDP-GTA assays did not account for the difference observed between the two substrates, since increasing the amount of albumin up to 3.5 mM inhibited slightly the reaction, without changing the relative inhibition exerted by the drug (results not shown). It is therefore most likely that RMI 12330 A interacts with membrane structure and not with the enzyme protein structure *per se*.

Effect of RMI 12330 A upon the rat liver microsomal glucose-6-phosphatase. The biphasic effect of RMI 12330 A upon the enzyme UDP-GT existed also upon the microsomal glucose-6-phosphatase activity (Fig. 5). This supports further a non-specific interaction of RMI 12330 A with membrane structures. The maximal activation (144 per cent) was observed with an inhibitor–protein ratio of 10^{-3} μmoles/μg, while complete inhibition was obtained at 10^{-2} μmoles/μg, slightly more than that required for complete inhibition of the UDP-glucuronosyltransferase activity.

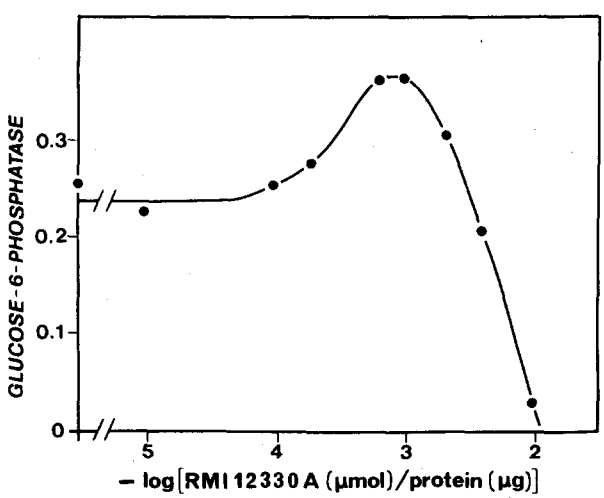

Fig. 5. Effect of increasing amounts of RMI 12330 A upon the glucose-6-phosphatase activity in a non-activated rat liver microsomal preparation. The specific activity (expressed as μmoles of inorganic phosphate liberated per min per mg protein) is plotted against the ratio of inhibitor content over microsomal protein. RMI 12330 A was dissolved as described in the text, and all the assays contained the same amount of ethanol. The assays were performed as described in the text.

DISCUSSION

We have shown here the inhibition by the organic cycloalkyl compound RMI 12330 A on the adenylate cyclase, ATPase, and leucyl-β-naphthylamidase liver plasma membrane enzyme activities (Fig. 2). The similar effects of RMI 12330 A on the activity of the adenylate cyclase in particulate fractions from spleen, brain, heart and kidney (Fig. 3) suggested that the action of this compound was not tissue specific. The drug, however, did not affect the 5′-nucleotidase activity from rat liver plasma membrane.

The action of RMI 12330 A resembled that of a series of drugs which interact with the hydrophobic regions of plasma membranes altering various enzyme activities [20–25]. These drugs are embedded to varying extents in the lipid phase and probably interact with discrete membrane components as exemplified by filipin. This polyene antibiotic specifically interacts with cholesterol resulting in uncoupling of the epinephrine-sensitive cyclase [22] but with less effect on the fluoride stimulated activity [26]. However, unlike filipin, RMI 12330 A affected all forms of cyclase activity probably due to the large number of membrane binding sites (370 nmoles of RMI 12330 A/mg liver plasma membrane) [3]. We have previously shown [3] that RMI 12330 A interacted irreversibly with plasma membranes, with hydrophobic bonds presumably playing a major role in the interaction. RMI 12330 A, a highly hydrophobic compound, was very soluble in organic solvents. The drug interacted readily with the fluorescent probe 8-aniline-1-naphthalene sulfonic acid, increasing the fluorescence of the dye 20-fold and shifting the emission maximum from 520 to 470 nm (data not shown). Similar effects were observed when the fluorescent probe was incubated with plasma membranes. The mild effects of RMI 12330 A upon the activities of the plasma membrane ecto-enzymes, leucyl-β-naphthylamidase and 5′nucleotidase (Fig. 2) further suggest that the drug primarily interacts with the membrane conformation.

The general perturbant effect on membrane-associated enzyme activities is further illustrated by the action of the drug upon the microsomal enzyme UDP-GTA. This enzyme is tightly bound to the microsomal membrane, and its active site may be located behind a hydrophobic barrier [27, 28]. This is the likely explanation for the enzyme activation which is observed in presence of detergents [6], such as digitonin or Emulgen 911. As shown in Fig. 4, low concentrations of RMI 12330 A activated the membrane bound UDP-GTA while high concentrations were inhibitory. However, the drug exhibited only inhibitory effects on the digitonin-activated or the Emulgen 911-solubilized forms of the enzyme. Glucose-6-phosphatase, another tightly bound

enzyme [29] also exhibited a biphasic response in its activity to RMI 12330 A. It has been suggested by Mulder [30] that lipid soluble, and/or detergent like drugs which bind to microsomal membrane, affect their conformation and lead to biphasic responses. Thus, stimulatory effects of such membrane perturbants might result from freer access for hydrophobic substrates to the catalytic site of UDP-GT behind a permeability barrier [28, 31–33]. Alternatively, the perturbant might release the enzyme from a conformation with sub-optimal catalytic activity imposed by membrane components [34]. Finally the biphasic effect observed with the microsomal enzymes UDP-GT and glucose-6-phosphatase but not with the enzymes from the plasma membrane may reflect differences in the enzyme environments in the two membranes, rather than differences in the properties of the various enzymes which were assayed.

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