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NOVOBIOCIN-INHIBITION AND MAGNESIUM-INTERACTION OF RAT LIVER MICROSOMAL BILIRUBIN UDP–GLUCURONOSYLTRANSFERASE

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Abstract—Novobiocin inhibited bilirubin UDP-glucuronosyltransferase (EC 2.4.1.17) from rat liver both *in vitro* and *in vivo*, in a dose-dependent fashion. This inhibition was immediate, and was fully reversed when novobiocin was removed by dialysis or by ultracentrifugation through 0.6 M sucrose. The inhibition could not be explained by an alteration in the membrane conformation of this enzyme, since the same kinetic changes were observed in digitonin-activated and in non-activated microsomes. Novobiocin exerted a non-competitive inhibition of bilirubin UDP-glucuronosyltransferase with either bilirubin or UDP-glucuronic acid as the substrate. Kinetic studies demonstrated uncompetitive inhibition of novobiocin on bilirubin UDP-glucuronosyltransferase as a function of Mg^{2+} concentration, whether the assays were EDTA-free or not. Thus, similarities seem to exist between the known effect of novobiocin on membrane-bound enzymes of the bacterial wall and its inhibitory effect on bilirubin UDP-glucuronosyltransferase: both these enzymic systems require metal divalent cations for maximal activity. The uncompetitive inhibition pattern observed with novobiocin with regard to Mg^{2+} suggests that this antibiotic acts on bilirubin conjugation by affecting Mg^{2+}–enzyme complexes.

Novobiocin causes unconjugated hyperbilirubinemia in several species [1, 2], including man [3]. Inhibition of liver bilirubin UDP-glucuronosyltransferase activity (EC 2.4.1.17) (UDP-GTA) could play a role in the genesis of such hyperbilirubinemia [2, 4–7]. However, experimental data on novobiocin inhibition of UDP-GTA remain controversial, since in only a few studies was bilirubin used as the substrate. Such results cannot be extrapolated to bilirubin since it is probable that this enzyme is heterogenous [8]. In addition, further difficulties exist, since an enhancement of UDP-GTA was found when the drug was given *in vivo* to newborn rats and guinea-pigs [2].

The precise mechanism by which novobiocin may inhibit bilirubin UDP-GTA remains unknown. In bacteria, novobiocin affects a wide variety of biochemical events, whereas most other antibiotics seem to affect only one specific process [9]. An attractive possibility would be that all the events known to be affected by novobiocin could be explained by a common mechanism. In this respect, it is relevant that all the inhibitory effects exerted by novobiocin on the bacterial wall are Mg^{2+}-dependent [10]: this action might therefore be related to chelation of this cation by novobiocin and was indeed reversible by further addition of Mg^{2+}.

Maximal activity of bilirubin UDP-GTA necessarily requires Mg^{2+} or other metal divalent cations, such as Ca^{2+} or Mn^{2+} [11]. Therefore it was of interest to determine whether the inhibitory effect of novobiocin on bilirubin UDP-GTA was mediated by a metal divalent cation and further to investigate how such a model of inhibition might assist in the delineation of the molecular events leading to the glucuronidation of bilirubin.

MATERIALS AND METHODS

Chemicals. Novobiocin sodium salt, (Cathomycine®) was obtained from Specia, Paris, France. Sodium pentobarbital, (Nembutal®) was purchased from Abbott, Saint-Remy sur Avre, France. Bilirubin was obtained from the British Drug Houses Ltd, Poole, England; when controlled, its molar extinction was found to be greater than 58,000 and could not be further increased by recrystallisation. UDP-glucuronic acid, triammonium salt, was purchased from Sigma, St. Louis, Mo, U.S.A.; human serum albumin from CDTS, Paris, France; ethyl anthranilate from Eastman Kodak, Rochester, N.Y., U.S.A. All other reagents were from Merck, Darmstadt, Germany and of analytical grade.

Animals. Male Sprague–Dawley rats, Charles River CD, weighing 180–250 g were fed *ad lib.* for 2 weeks and starved for 12 hr prior to the experiments.

In vivo *experiments.* Animals acted as their own controls: a first liver biopsy was taken under light ether anesthesia, and novobiocin, diluted in 0.15 M NaCl, was injected intravenously (penian vein) at doses of 30, 60, 150 or 300 μmoles/kg body wt, in a volume of approximately 0.5 ml. Ten min after the injection, a second liver biopsy was taken. Both the liver specimens were quickly homogenized in 4 vol of ice-cold 0.25 M sucrose containing 1 mM EDTA, pH 7.4 using a glass Potter homogenizer. In these experiments, an equal vol. of a 2% (w/v) suspension of digitonin was added to the homogenates prior to the enzymic determinations. The small size of the biopsies, around 20 mg, led us to assay bilirubin UDP-GTA in the homogenates.

In vitro *experiments.* These were all performed on liver microsomal suspensions. The rats were anesthe-

tized with sodium pentobarbital, 4 mg/100 g body wt., i.p. Microsomes were prepared as previously described [13], the pellet being resuspended in 0.25 M sucrose–1 mM EDTA, pH 7.4, so as to contain 4–8 mg protein/ml. For the various *in vitro* studies, the microsomes which were used as the enzymic source were diluted before the assays with 0.25 M sucrose–EDTA in the presence or absence of a 2% (w/v) suspension of digitonin; this corresponded to a digitonin concentration of 0.35% in the incubation mixtures.

Enzymic assays. Bilirubin UDP-GTA was measured according to a micromodification [12] of the method of Van Roy and Heirwegh [13] and Black, Billing and Heirwegh [14] i.e. a system using diazotized ethyl anthranilate and in which the excess of unconjugated bilirubin does not react [15]. The final concentrations in the assaying mixtures were 80 mM triethanolamine buffer; 9 mM $MgCl_2$; 0.3 mM bilirubin; 0.16 mM human serum albumin; 3 mM UDP-glucuronic acid; 0.7 mM EDTA. The reaction was initiated by the addition of an equivalent of 35 mg of liver, or of 1.5–3 mg of microsomal protein, per ml of assaying mixture.

Varying amounts of novobiocin, dissolved in 0.1 M Na_2CO_3, were added in a minute volume to the incubation mixtures. The final concentration of the antibiotic varied from 0 to 10^{-3} M. It was determined that identical volumes of 0.1 M Na_2CO_3 affected neither the final pH nor the enzymic activity of the system.

Reversibility of novobiocin effects. In order to study a possible binding of the drug to microsomes, novobiocin (20 nmoles per mg of microsomal protein) was added to 250 μl of a digitonin-activated microsomal suspension in 0.15 M KCl, and dialysed in cellophan bags against 500 ml of 0.15 M KCl at 4° for 20 hr. In these experiments, the final concentration of novobiocin was 50 μM. The UDP-GTA was then determined and compared with that in (i) microsomes dialyzed without addition of novobiocin, and in (ii) microsomes stored under similar conditions, but without dialysis. Novobiocin binding to microsomes was also studied by ultracentrifugation experiments, novobiocin being added to microsomal suspensions in 0.25 M sucrose. One ml of this microsomal suspension was then ultracentrifuged over 9 ml of 0.6 M sucrose at 100,000 *g* for 60 min. The resulting microsomal pellet was resuspended in 0.25 M sucrose-EDTA, bilirubin UDP-GTA assayed and its activity compared to that of microsomes which had been centrifuged in an identical manner, but in the absence of novobiocin.

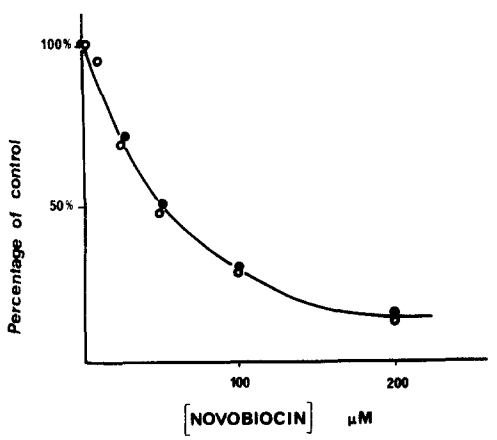

Fig. 1. Inhibition of the formation of bilirubin glucuronide by novobiocin in digitonin-activated (●) and non-activated (○) microsomal preparations. The concentration of bilirubin was 0.3 mM.

Kinetic studies. When the final concentrations of bilirubin, UDP-glucuronic acid, magnesium and other divalent cations differed from the conditions of the standard enzymic assay described above, then the exact conditions are given in the legends to the figures. When variable concentrations of bilirubin were used, then the bilirubin: albumin molar ratio was kept constant in order to avoid deviation from Michaelis–Menten kinetics that an excess of albumin would create [16, 17]. The initial rates of velocity were studied as a function of metal divalent cations and assayed in parallel in the presence and absence of EDTA. All the incubation periods were of 15 min duration, since activity was still linear with time at this point.

Endogenous Mg^{2+} content of microsomes. The concentration of Mg^{2+} was measured by atomic absorption in two microsomal suspensions which had been prepared in a different manner. One preparation was isolated from a liver specimen homogenized in 0.25 M sucrose containing 1 mM EDTA and washed twice in 0.15 M KCl before final resuspension of the microsomes in EDTA-free sucrose, while the other was isolated in the absence of EDTA. Both preparations were made to a final concentration of 5‰ (v/v) of sodium dodecyl sulfate before the Mg^{2+} measurements were made against corresponding blanks.

RESULTS

In vivo *studies.* There was a definite, although moderate, inhibitory effect of novobiocin on bilirubin UDP-GTA. This inhibition appeared to be dose-related. Thus at 30, 75, 150 and 300 μmoles of novobiocin per kg body wt, the inhibitions were 0, 18, 24 and 28 per cent of the initial activities, respectively (mean of four rats for each dose). The amounts of novobiocin which were chosen correspond to the therapeutic range used in man (around 100 μmoles/kg).

In vitro *studies.* When novobiocin was added to the incubate, at final concentrations varying from 10 to 200 μM, it inhibited the enzyme activity in a dose-dependent fashion. The antibiotic exerted an inhibition of bilirubin UDP-GTA when assayed on digitonin-activated microsomes at a fixed concentration of 0.3 mM bilirubin, effecting a 50 per cent inhibition at a concentration of 45 μM (Fig. 1). Novobiocin exerted a similar inhibition when non-activated microsomes were used, yielding 50 per cent inhibition at 50 μM (Fig. 1). When added during the course (at the 5th min) of the enzymic assays, the inhibitory effect of novobiocin was immediate and sustained for the whole period of incubation (20 min) (Fig. 2).

The novobiocin-induced alteration of bilirubin UDP-GTA was reversible, since in microsomal suspensions to which novobiocin has been added, inhibition was no longer seen after dialysis (99 and 102 per cent of the control values). In contrast, when di-

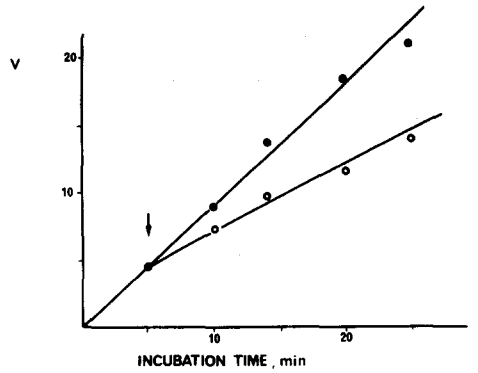

Fig. 2. Effect of 50 μM novobiocin (○) or of an equal volume of solvent (0.1 M Na_2CO_3) (●) added after 5 min to the incubation assay. Velocity (V) is expressed as nmoles of bilirubin conjugated per mg protein. Assays were carried out on activated microsomes; other conditions are described under Methods.

alysis was omitted, 50 per cent inhibition of enzyme activity persisted at the 50 μM novobiocin concentration. Similarly, the basal level of enzyme activity in three different microsomal suspensions, which had been mixed with novobiocin, was fully restored by ultracentrifugation through 0.6 M sucrose.

Rate of synthesis of bilirubin glucuronide as a function of bilirubin concentration. When assayed in activated microsomes, novobiocin appeared as a non-competitive inhibitor of bilirubin UDP-GTA, since inhibition consisted chiefly of a decrease in the maximal velocity (Fig. 3). Higher concentrations of bilirubin could not be assayed in these experiments because of the low solubility of this substrate. Provided that the molar ratio of bilirubin to albumin was maintained constant, then linearity was observed in double reciprocal plots whether novobiocin was present or not. Concentrations of 20, 40 and 60 μM novobiocin produced a lowering of the calculated V_{max}, and only a slight increase in the K_m at the highest concentration of novobiocin (60 μM) (Fig. 3). An apparent non-competitive inhibition, which appeared to be a function of bilirubin concentration was also observed with non-activated microsomes (Fig. 4).

Rate of synthesis of bilirubin glucuronide as a function of UDP-glucuronic acid. These studies produced hyperbolic curves, and double reciprocal plots gave further evidence of non-competitive inhibition of novobiocin as a function of the nucleotide (Fig. 5). The changes in calculated V_{max} resembled those observed in kinetic studies as a function of bilirubin. The apparent K_m's for UDP-glucuronic acid were 0.77 and 0.80 mM in the absence and in the presence of 50 μM novobiocin, respectively.

Rate of synthesis of bilirubin glucuronide as a function of Mg^{2+} or other metal divalent cations. The comparative stimulatory effect of Mg^{2+}, Ca^{2+} or Mn^{2+} on UDP-GTA is shown in Fig. 6. Mg^{2+} and Ca^{2+} induced the same type of activation which was maximal around 10 mM. For Mn^{2+} however, the highest enzyme activity was obtained at 1 mM, whereas higher concentrations rapidly inhibited bilirubin UDP-GTA. When bilirubin UDP-GTA was assayed either with 9 mM Mg^{2+}, 10 mM Ca^{2+} or 1 mM Mn^{2+}, it appeared that the inhibition produced by novobiocin was different for each cation: the 50 per cent inhibitory effect was produced at a concentration of novobiocin about twice lower with Ca^{2+} or Mn^{2+} than that with Mg^{2+} (Fig. 7).

When bilirubin UDP-GTA was assayed in the absence of novobiocin as a function of the concentration of added Mg^{2+}, and in EDTA-free assay mixtures, double reciprocal plots exhibited to different slopes (Fig. 8). Such slopes exhibited a descending concavity, and are usually considered as suggesting either negative cooperativity or two distinct binding sites. In the absence of any inhibitor, the apparent K_m corresponding to the two slopes was 1.72 and 0.59 mM, respectively. However, it is known that in the absence of any added Mg^{2+}, some transferase activity is still found. Such activity could well be related to the highly-bound endogenous divalent cations that we found in the microsomal suspensions, even when they were prepared in the presence of 1 mM EDTA (Table 1). It is therefore conceivable that the non-linearity of the curves shown in Fig. 8 could be a consequence of the endogenous Mg^{2+} pool, the availability of which could differ from that of the added cation. It should also be noted that endogenous Mg^{2+} will represent a high proportion of the total Mg^{2+} when low concentrations of the cation are added, thus altering the shape of the curves which refer only to the actual amount added. An argument favoring this theory is as follows: when the same experiments were conducted in the presence of 0.7 mM EDTA, a linear reciprocal plot was obtained, the K_m of which (1.95 mM) was quite similar to the lowest affinity constant observed when EDTA was omitted (1.72 mM).

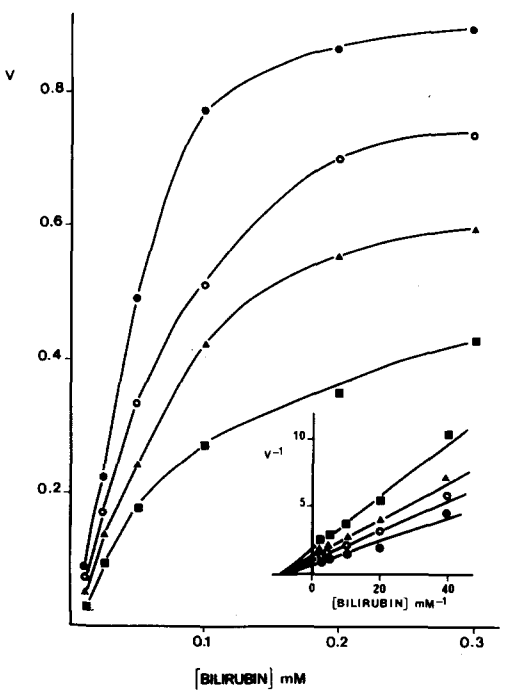

Fig. 3. Inhibition of the formation of bilirubin glucuronide by zero (●), 20 (○), 40 (▲) and 60 (■) μM novobiocin. Velocity is expressed as nmoles of bilirubin conjugated per min per mg protein. Activated microsomes were used in these experiments.

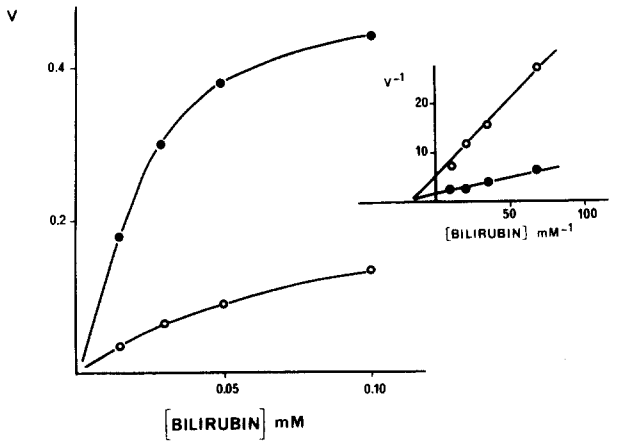

Fig. 4. Inhibition of the formation of bilirubin glucuronide by zero (●) and 50 (○) μM novobiocin in non-activated microsomes. Velocities are expressed as nmoles of bilirubin conjugated per min per mg protein.

Whatever the actual number of Mg^{2+} binding sites, fixed concentrations of novobiocin (40 or 80 μM) exerted a similar effect, causing a parallel shift of the curves whether EDTA was present (data not shown) or not (Fig. 8). Thus, novobiocin, with regards to Mg^{2+}, exerted an inhibition of the so-called uncompetitive type [18]. When bilirubin UDP-GTA was tested as a function of either Ca^{2+} or Mn^{2+}, two slopes were again found in the absence of EDTA, the calculated K_m being 1.04 and 0.27 mM for Ca^{2+}, and 0.18 and 0.01 mM for Mn^{2+}. Novobiocin still exerted an uncompetitive inhibition with regard to either of these cations.

DISCUSSION

The present experiments demonstrate that novobiocin exerts an inhibition on bilirubin UDP-GTA both *in vivo* and *in vitro*. However, no more than 28 per cent inhibition could be achieved at the highest dose *in vivo*. This inhibitory effect of novobiocin was dose-related and was of the same order of magnitude as that observed at low concentration (25 μM) *in vitro*. The moderate *in vivo* effect of novobiocin could be due to a low intracellular concentration of novobiocin at the active site of the enzyme. No precise data are available with regard to novobiocin disposition, and the 10-min interval which was arbitrarily chosen between novobiocin administration and liver biopsy may not correspond to the time at which the highest intrahepatocytic concentration of the antibiotic was reached.

Novobiocin does not cause any permanent alteration in microsomal membrane structure as its inhibitory effect *in vitro* was reversible by either dialysis of the microsomal preparation, or by ultracentrifugation of the microsomes through hypertonic sucrose. This seems of importance, since novobiocin contains phenolic groups which theoretically possess a membrane-perturbing effect, at least at high concentration [10].

UDP-glucuronosyltransferase is a tightly membrane-bound enzyme the active site of which may be located behind a hydrophobic barrier [19]. The acti-

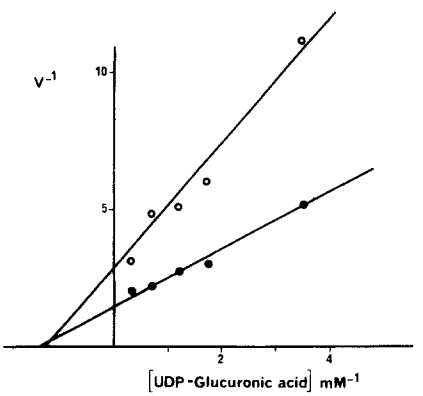

Fig. 5. Inhibition of the formation of bilirubin glucuronide by novobiocin. Double reciprocal plots of initial velocities as a function of the concentration of UDP-glucuronic acid in the absence (●) and presence (○) of 50 μM novobiocin. Velocities (V) are expressed as nmoles of bilirubin conjugated per min per mg protein. The microsomes used were digitonin-activated.

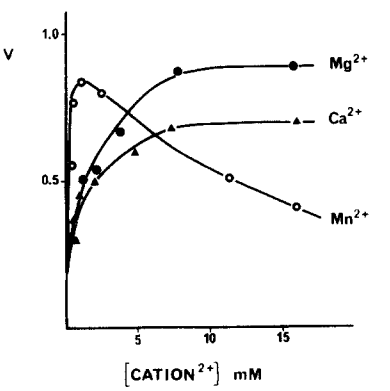

Fig. 6. Stimulating effect on bilirubin-UDP-glucuronosyl transferase of various concentrations of the chloride salts of the three following metal divalent cations: Mg^{2+} (●), Ca^{2+} (▲) or Mn^{2+} (○). Velocity is expressed as nmoles of bilirubin conjugated per min per mg protein. No EDTA was added in any of these assays; activated microsomes were used.

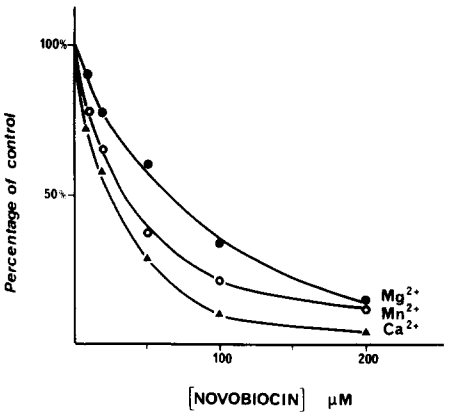

Fig. 7. Inhibition of the formation of bilirubin glucuronide by novobiocin in the presence of optimal concentration of the three following metal divalent cations: 9 mM Mg^{2+} (●), 10 mM Ca^{2+} (▲) or 1.5 mM Mn^{2+} (○). Velocity is expressed as the per cent of the control corresponding to each of the cations which were used. Assays were carried out on activated microsomes; EDTA was omitted in these experiments.

vity of this enzyme greatly depends on (i) the aglycone which is used [8] and especially its lipid solubility; (ii) the conformation of the microsomal membrane, which may be altered by many procedures [20], including detergents and compounds that readily bind to membranes. Therefore, it still remains difficult to define whether an inhibitor either acts on the enzyme protein, or alter its membrane environment, thus secondarily affecting the enzyme. As a consequence, it is questionable whether an inhibition observed *in vitro* is of any significance in the *in vivo* situation [20]. In the present experiments, however, such an action on membrane environment seems unlikely for the following two reasons: (i) the apparent non-competitive inhibitory effect of novobiocin towards bilirubin and UDP-glucuronic acid was also observed after digitonin pretreatment of microsomes; (ii) compounds inhibiting the enzyme by an alteration of the membrane conformation frequently exhibit opposite effects on native microsomes, i.e. an activation at low concentration followed by an inhibition at high concentration [20, 21]; novobiocin failed to show such a diphasic action. A direct interaction of novobiocin with the enzyme protein appears therefore to be the most likely possibility. It is, however, notable that kinetics of membrane-bound enzymes are not fully reliable [22], and may, for instance, be greatly hampered by the diffusion of the substrates within the membrane. Thus, novobiocin may act in part as a competitive substrate, thereby contributing to the inhibitory effect. In fact, this remains an open possibility, for novobiocin is glucuronidated by the liver, although to a limited extent (unpublished personal data).

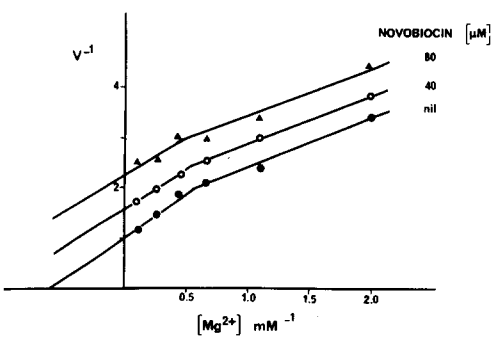

Fig. 8. Double reciprocal plot of initial velocities of bilirubin glucuronide formation in activated microsomal preparations as a function of varying concentrations of added Mg^{2+} in the absence (●) and presence of 40 (○) or 80 (▲) μM novobiocin. The enzymic sources used were prepared in the absence of any EDTA.

Table 1. Endogenous Mg^{2+} content of rat liver microsomes

	Mg^{2+} content (nmoles/mg microsomal protein)
Microsomes prepared in EDTA-free sucrose	53.0 51.6
Microsomes prepared in 1 mM EDTA–sucrose	23.0 22.6

The results are individual values from two preparations.

Kinetic analysis as a function of Mg^{2+} exhibited a peculiar mechanism of inhibition since it appeared to be an uncompetitive one. This suggests that novobiocin inhibition is dependent on Mg^{2+}, or on other metal divalent cation such as Ca^{2+} or Mn^{2+} for which a similar uncompetitive inhibition could be demonstrated. This type of inhibition may reflect a reaction of novobiocin with an enzyme–Mg^{2+} complex. Such an interaction between Mg^{2+} and novobiocin actually resembles that found in bacteria, which was attributed to the ability of the antibiotic to complex Mg^{2+} [9], thus acting on bound magnesium in a particular class of enzymes of the bacterial membrane [10].

The mechanism of the inhibition that novobiocin exerts on bilirubin UDP-GTA could well be explained by chelation of Mg^{2+}. However, 50 μM novobiocin would have very little effect on the overall Mg^{2+} concentration (9 mM added); thus, a specific interaction with only a bound fraction of the Mg^{2+} is more likely, and such a hypothesis is quite conceivable for the following reasons. Firstly, Zakim *et al.* [23] have suggested two distinct sites at which divalent metal ions may act to alter the kinetics of *p*-nitrophenol glucuronidation; following EDTA treatment, the microsomes had endogenous metal ions still attached at only one of these sites. This finding is further supported by the presence of still about half the amount of endogenous Mg^{2+} that we obtained in microsomes prepared in sucrose containing 1 mM EDTA as compared to that in EDTA-free preparation. Secondly, we observed two slopes when bilirubin UDP-GTA was assayed in the presence of increasing amounts of added Mg^{2+}. When EDTA was added, only one slope was achieved. This indeed suggests two different binding sites, in accordance with the non-linearity already observed in non-activated microsomes [17]. As a consequence, the kinetics as a function of Mg^{2+} must be interpreted with cau-

tion, since the contribution of endogenous Mg^{2+} to the total Mg^{2+} concentration is non-negligible at low concentrations of added cation.

Whether or not the interaction between novobiocin and Mg^{2+} can be mediated through Mg^{2+}–UDP–glucuronic acid complexes remains an open possibility which is now under investigation in our laboratory. Novobiocin does however seem to interact with an enzyme–Mg^{2+} complex, and this effect may be used to clarify the role of Mg^{2+} in glucuronidation.

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