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Nucleotide Metabolism by Microsomal UDP-Glucuronyltransferase and Nucleoside Diphosphatase as Determined by ^{31}P Nuclear-Magnetic-Resonance Spectroscopy

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^{31}P n.m.r. spectroscopy was used to study the nucleotide kinetics of UDP-glucuronyltransferase and associated reactions in the liver microsomal fraction. The effects of Mg^{2+} and EDTA on these reactions were investigated qualitatively. It was found that the rabbit microsomal fraction has no nucleoside pyrophosphatase activity, that UDP was immediately hydrolysed and that it was released from the microsomal surface. Reverse glucuronyltransferase could be demonstrated. The results are discussed with reference to functional coupling of UDP-glucuronyltransferase to other enzymes and the effects of Mg^{2+} and EDTA on the system.

Several enzymic reactions, such as hydrolysis of UDP-glucuronic acid and UDP, and hydrolysis of glucuronides, interfere with measurements of microsomal UDP-glucuronyltransferase. The physiological significance of these interferences (a possible functional linkage of membrane-bound enzymes) will remain unclear as long as basic facts about the locations of the enzymes relative to the membrane (sidedness), substrate access and product release are unknown. ^{31}P n.m.r., a direct non-destructive analytical technique, offers new possibilities to investigate these phenomena (Stier *et al.*, 1978*a*). The results demonstrate unequivocally that UDP is released on the cytoplasmic side of liver microsomes (microsomal fraction) during glucuronidation of *p*-nitrophenol.

Experimental

UDP-glucuronic acid, UDP, glucose 6-phosphate, phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) from rabbit muscle [crystalline suspension in $(NH_4)_2SO_4$ solution] were obtained from Boehringer Mannheim G.m.b.H., Mannheim, Germany. UMP, *p*-nitrophenyl β-D-glucuronide and D-saccharo-1,4-lactone were purchased from Sigma Chemie G.m.b.H., Munich, Germany; *p*-nitrophenol was from E. Merck, Darmstadt, Germany; pH 6–8 Ampholine was from LKB Produkter, Bromma, Sweden.

Proton-decoupled ^{31}P n.m.r. spectra were taken on a Bruker WH-270 spectrometer operating at 109.32 MHz. A 10 μs pulse followed by a 135 ms sampling time was used. Sample tubes (10 mm) contained internal ^2H_2O for the lock. In most cases the machine was set up to take eight consecutive spectra of 1024 transients each, total measuring time being 18.4 min.

The rabbit liver microsomal fraction was prepared by the method of Fouts (1971) from animals pretreated for 1 week with sodium phenobarbital (1 g/litre) in drinking water. Both food and phenobarbitol were withdrawn 24 h before slaughter. The microsomal preparation was stored for up to 1 week at −30°C. Before use the preparation was washed in 0.15 M-Tris/HCl (pH 7.6)/^2H_2O (4:1, v/v).

Reactions were started by addition of cofactor 1 min before the start of sampling, the time between being used to put the tube into the magnet and make final corrections to the field. The final volume of all samples was 2.5 ml. Final microsomal concentration was usually 1:9 (w/v) in 0.15 M-Tris/HCl (pH 7.6)/^2H_2O (4:1, v/v). The temperature was about 30°C in all experiments. Precise details are given in the Figure legends.

Abbreviations used: UDPase, uridine diphosphatase; UMPase, uridine monophosphatase.

Results

It is immediately apparent from the spectra of fresh microsomal suspensions taken during glucuronidation of *p*-nitrophenol that the final uridine product of the rabbit liver microsomal fraction is not UDP but UMP (and P_i) (Fig. 1*a*). There is no evidence for UDP either in the single spectra (of 1024 transients collected in 2.3 min) or in the sum of eight of these (the cumulative spectrum). The reason for this is the high UDPase activity of the preparation (attributable partially or wholly to nucleoside diphosphatase, EC 3.6.1.6): UDP breakdown outstrips the productive capacity of glucuronyl-

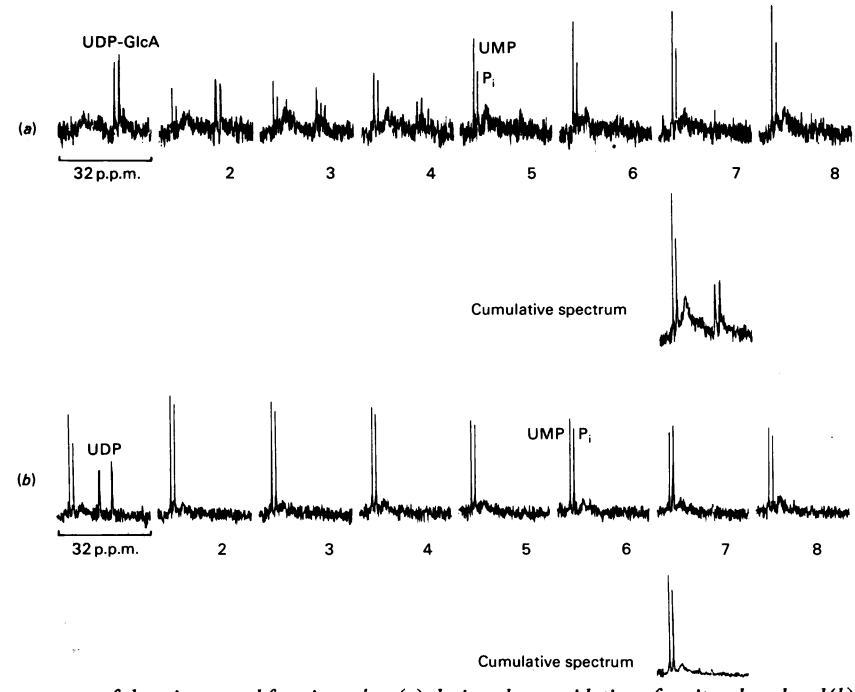

Fig. 1. *^{31}P n.m.r. spectra of the microsomal fraction taken (a) during glucuronidation of p-nitrophenol and (b) during hydrolysis of UDP*

Microsomal fraction (1:9, w/v) was incubated (*a*) with 4mM-UDP-glucuronic acid (UDP-GlcA) and 4mM-*p*-nitrophenol at 33°C and (*b*) with 4mM-UDP at 32°C. Each of the numbered spectra represents 1024 transients, total time for eight spectra being 18.4min. The cumulative spectra are the sum of the eight individual ones.

transferase in our preparations by a factor of 3–5 (Fig. 1*b*). In the presence of 4mM-Mg^{2+}, UDP metabolism is immeasurably fast. There is no measurable breakdown of either UDP-glucuronic acid by nucleotide pyrophosphatase (EC 3.6.1.9) in 20min, or UMP by the UMPase (probably 5′-nucleotidase, EC 3.1.3.5) over the same period without added Mg^{2+}. We have consistently observed that the signal-to-noise ratio is better for the UDP than for the UDP-glucuronic acid signals, probably as a result of binding of UDP-glucuronic acid to the microsomal fraction. This affects the accuracy with which signal integrals may be estimated and results quantified. For example, with a signal-to-noise ratio of 8 (from 3.3mM-UDP-glucuronic acid), we calculate a standard deviation of the integral over seven spectra of 7.1%.

Attempts to dissociate the two reactions and accumulate large quantities of UDP in suspensions of the fresh microsomal fraction met with limited success. With concentrations of EDTA at which the UDPase was partially or wholly inhibited (1–2mM), glucuronidation of *p*-nitrophenol was also adversely affected and only a small quantity of UDP was produced. Interestingly enough, this residual glucuronyltransferase reaction appeared to be EDTA-insensitive; increasing the concentration of EDTA to 4mM apparently had no effect on the amount of UDP produced (Fig. 2).

In contrast with the situation in a fresh microsomal fraction, if the preparation (diluted 1:1, w/v, with buffer) is allowed to age in air at 0°C for 8h or more, large quantities of UDP are observable in an EDTA-containing mixture during glucuronidation of *p*-nitrophenol (Fig. 3). This increase in the EDTA-insensitive reaction is not due to alteration of UDPase activity: this is apparently not significantly affected. Attempts to simulate the aging process with non-ionic detergents (Triton X-100 and N-101) and by sonication were partially successful, but we were unable to demonstrate as large an effect (Stier *et al.*, 1978*a*).

At first glance the system readily lends itself to estimation of nucleotide and P_i distribution across the microsomal membrane by use of shift reagents to separate the signals arising inside and outside the membrane. In practice, however, our attempts to employ the tervalent cations Pr^{3+}, Nd^{3+} and Eu^{3+} to this end were foiled by the impossibility of using a large enough quantity of metal to shift or broaden

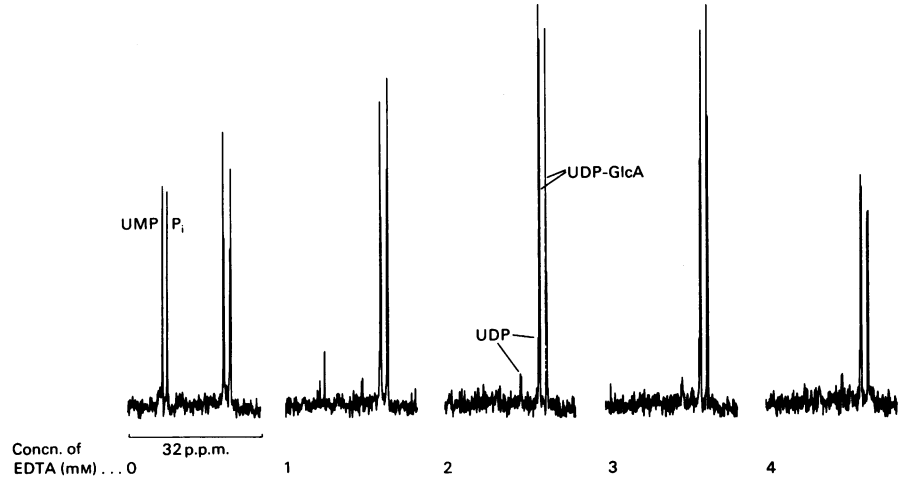

Fig. 2. *^{31}P n.m.r. spectra demonstrating the effect of EDTA on glucuronidation of p-nitrophenol*
The samples, microsomal fraction (1:9, w/v), 4mM-UDP-glucuronic acid (UDP-GlcA), 2mM-*p*-nitrophenol and EDTA as indicated were incubated for 20min at 30°C and then kept on ice until measured at 5°C; 4096 transients were collected over 9.2min for each spectrum.

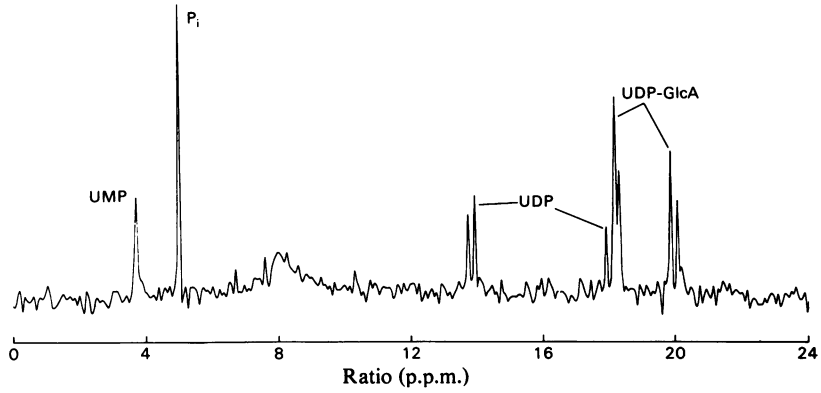

Fig. 3. *^{31}P n.m.r. spectrum showing accumulation of UDP in an aged microsomal preparation during glucuronidation of p-nitrophenol*
The microsomal fraction (1:1, w/v) was left in air at 0°C for 13h and then incubated in a 1:9 (w/v) dilution with 4mM-UDP-glucuronic acid (UDP-GlcA), 2mM-*p*-nitrophenol and 1mM-EDTA at 29°C. The spectrum shown represents 14336 transients collected over 32min.

the phosphorus signals without damaging the microsomal preparation (which aggregates and is precipitated). Similar difficulties attended our experiments with a number of derivatives of the spin label tetramethylpiperidine 1-oxide.

The pH-dependence of the ^{31}P chemical shift can also be exploited to distinguish the signals arising inside the microsomal membrane from those outside if the pH of one compartment can be altered relative to the other. To minimize the likelihood of pH equilibration we used pH6–8 Ampholine, a high-molecular-weight buffer substance that we consider unlikely to cross the microsomal membrane. Ampholine (400μl in a total volume of 2.5ml) shifted the pH of the medium from 7.6 to 7.3 and elicited a downfield shift of the ^{31}P resonances, especially those of UMP and P_i. Under these conditions we did not observe any splitting of the ^{31}P signals arising from *p*-nitrophenol glucuronidation (Fig. 4*a*). Similarly, both the glucose 6-phosphate and P_i signals of the control experiment were shifted without any evidence of splitting (Figs. 4*b* and 4*c*),

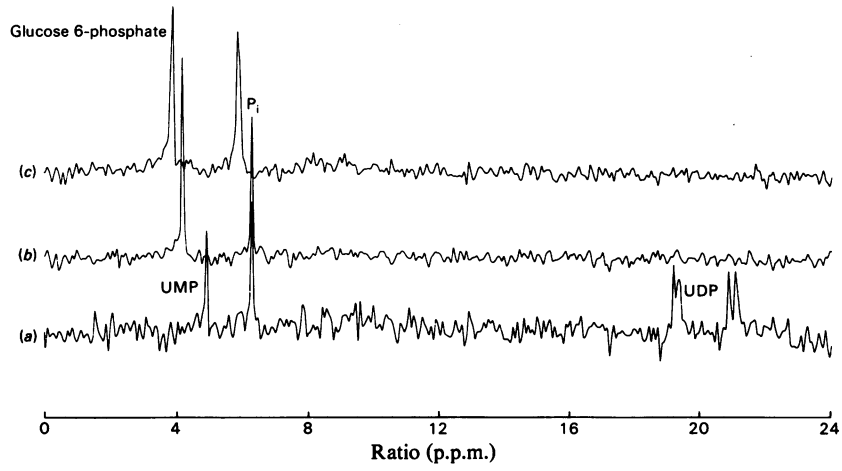

Fig. 4. *^{31}P n.m.r spectra demonstrating the effects of Ampholine on ^{31}P chemical shift*

(*a*) Cumulative spectrum over 18.4min of a sample containing microsomal fraction (1:9, w/v), 4mM-UDP-glucuronic acid, 4mM-*p*-nitrophenol, 1mM-$MgCl_2$ and 400μl of Ampholine at 28°C. (*b*) Cumulative spectrum of breakdown of 4mM-glucose 6-phosphate by a 1:9 (w/v) suspension of the microsomal fraction in the presence of 400μl of Ampholine over 27.6min at 30°C. (*c*) Cumulative spectrum over 18.4min of a similar sample without the Ampholine at 28°C. Clearly, in those samples containing Ampholine the P_i and glucose 6-phosphate signals are shifted upfield (the same is also true of the UMP signal).

though glucose 6-phosphatase has been shown to release P_i to the microsomal lumen (Leskes *et al.*, 1971; Lewis & Tata, 1973).

Further information about the sidedness of the reaction products can be obtained by using them in further enzymic reactions. The reaction of pyruvate kinase with UDP (Kornberg, 1951) has been used by Mulder & van Doorn (1975) in conjunction with glucuronyltransferase to convert the UDP produced into UTP at the expense of phosphoenolpyruvate. As shown in Fig. 5, when this reaction is coupled to the glucuronidation of *p*-nitrophenol the initial products are UTP and P_i (Fig. 5*b*). The UTP, however, is unstable, and is broken down to UDP: thus a cycle develops between UDP and UTP, of which only UTP is seen by virtue of its breakdown being the slower of the two reactions. This state of affairs persists until the phosphoenolpyruvate reservoir is exhausted, whereupon the UTP signals are replaced by those of UMP and P_i (Fig. 5*c*). As in the spectra of uncoupled reactions, no UDP is seen at any time. Although UMP is hydrolysed in microsomal suspensions containing 4mM-Mg^{2+} (over and above any endogenous Mg^{2+}) it was unexpectedly observed that the UMP arising on termination of the UDP-UTP cycle was stable, though the amount was rather less than that predicted by the simplest reaction stoicheiometry (Fig. 5*d*). We found that 10mM-P_i inhibits the hydrolysis. To confirm that no UMP is produced while phosphoenolpyruvate is present, the experiment was repeated with a microsomal fraction prepared in EDTA (i.e. as usual, but with 1mM-EDTA in all solutions of the preparative steps except the final washing buffer). In such preparations glucuronyltransferase and UDPase activities can be restored with 4mM-Mg^{2+} with little or no concomitant re-activation of UMPase (preparations vary in this respect). Under these conditions too no UMP is formed until the phosphoenolpyruvate is entirely consumed. Furthermore, the ratio of the P_i to the UMP signals is, as expected, 2:1. (From spectra of mixtures of P_i and UMP taken under identical conditions we know that the relation of signal height to molarity is 1:1 for these two species.)

Discussion

^{31}P n.m.r. is a direct-measurement non-destructive technique with considerable potential for general application in studies of nucleotide kinetics, as it provides information about the transformation of one or more phosphate-containing species in minutes as opposed to the hours or days required by conventional biochemical techniques. For a general discussion of the applications of ^{31}P n.m.r. in biological systems see Dwek *et al.* (1977).

Our observations (made with male Sprague–Dawley and male and female Gunn rats as well as with rabbits) that UDP is not normally seen as a product of glucuronidation and that the microsomal preparation contains high UDPase activity are indications for a coupling of glucuronyltransferase and a UDPase. In the rabbit liver microsomal fraction

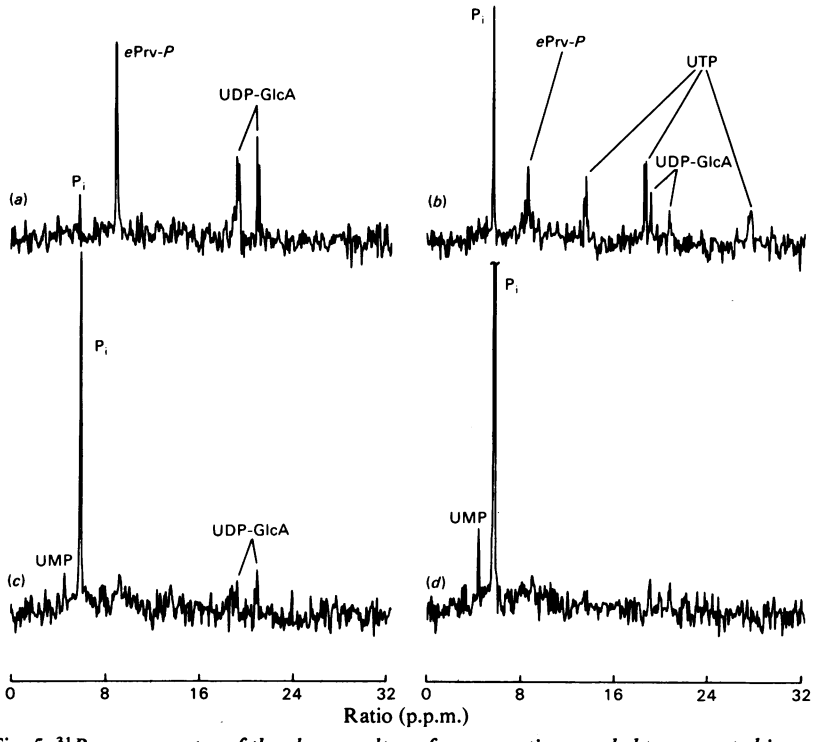

Fig. 5. *^{31}P n.m.r. spectra of the glucuronyltransferase reaction coupled to pyruvate kinase*

Microsomal fraction (1:9, w/v) was incubated with 4mM-UDP-glucuronic acid (UDP-GlcA), 4mM-*p*-nitrophenol, 4mM-phosphoenolpyruvate (*e*Prv-*P*), 4mM-$MgCl_2$ and 200 units (μmol/min) of pyruvate kinase at 35°C. The spectra (each of 1024 transients) represent (*a*) 1.0–3.3min, (*b*) 11.5–13.8min, (*c*) 25.3–27.6min and (*d*) 39.1–41.4min. Spectra (*a*) and (*b*) show that disappearance of phosphoenolpyruvate and UDP-glucuronic acid is accompanied by appearance of UTP and P_i signals. Eventually (*c*) when the phosphoenolpyruvate has been entirely consumed the UTP signals collapse and are replaced by those of UMP and P_i. The final ratio of UMP to P_i signal heights (*d*) is far removed from the expected value of 1:2.

in the presence of EDTA UDP is only seen in preparations disrupted by aging and detergent treatment, for example. The reverse reaction (glucuronidation of UDP with *p*-nitrophenyl glucuronide as donor) can only be performed in the presence of high concentrations of EDTA to inhibit the UDPase, and the amplitude is low (Fig. 6). So far these results support the suggestion of Berry & Hallinan (1976) that glucuronyltransferase is coupled to nucleoside diphosphatase (EC 3.6.1.6) and thus made effectively irreversible *in vivo*.

Although nucleoside diphosphatase is thought to be on the inner side of the microsomal membrane (Kuriyama, 1972; O'Toole, 1975), for which reason Berry & Hallinan (1976) place glucuronyltransferase in the same compartment, we have been unable to demonstrate any sidedness of the products in experiments with Ampholine. We can expect to detect a signal from 20% of the UMP or P_i in our sample, which, assuming 100μl of 'inside' volume in a sample of 2500μl, corresponds to 'inside' and 'outside' concentrations of 20 and 3.2 mM respectively. (This calculation disregards the effects of a possible difference in T_1 relaxation times inside and outside the microsomes: the longer the T_1 time the poorer the signal-to-noise ratio and the greater the detectable 'inside' concentration, and vice versa.) Thus it would appear that most of the UDP is not broken down inside the microsomes, or, if it is, that the products are rapidly transported outwards. The experiment is therefore inconclusive with regard to the location of UDP release. It is relevant to note here that accumulation of phosphates inside the microsomes might be expected to cause an acid pH shift and corresponding signal splitting: for this too we have no evidence. The pyruvate kinase experiment, on the other hand, indicates fairly unequivocally that UDP is released on the outside of the membrane, as it is not to be expected that pyruvate kinase will cross the microsomal membrane (Nilsson *et al.*, 1973). Since the K_m values of nucleoside diphosphatase and pyruvate kinase are of the same order, and the activities of the Mg^{2+}-activated UDPase [at a conservative estimate 2.5 units (μmol/min) concentrated in 125μl of microsomes] and pyruvate kinase [10 units (μmol/min)/125μl] are comparable, it is evident that pyru-

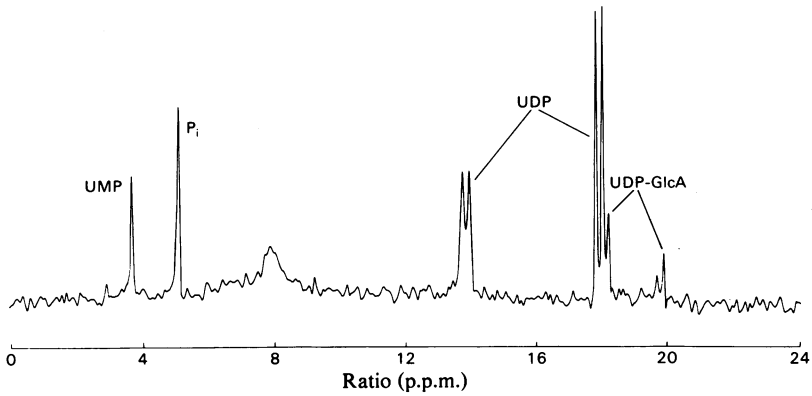

Fig. 6. *^{31}P n.m.r. spectrum showing glucuronidation of UDP with p-nitrophenyl glucuronide as donor*
Microsomal fraction (1:9, w/v) was incubated with 4mM-UDP, 4mM-*p*-nitrophenyl glucuronide, 10mM-saccharo lactone and 2mM-EDTA at 32°C. The spectrum represents 16384 transients collected over 36.9min. Abbreviation: UDP-GlcA, UDP-glucuronic acid.

vate kinase can only interfere with coupling of glucuronyltransferase and nucleoside diphosphatase to the point of complete capture of UDP if it has prior access to the substrate. It is difficult if not impossible to reconcile this result with a model of glucuronyltransferase in which the enzyme releases UDP to the microsomal lumen, which is also the site of the UDPase; one has to assume that UDP diffuses through membranes as readily as through water. One cannot, from these results, draw any conclusions about the sidedness of the UDPase, whether it is cytoplasmic, lumenal, or both. An answer to the question of functional linkage of glucuronyltransferase and UDPase depends on whether other UDP-consuming cellular enzymes effectively compete for the substrate.

These observations have obvious bearing on the NAD^+-linked assay for glucuronyltransferase proposed by Mulder & van Doorn (1975). Fortuitously, one source of error (hydrolysis of UDP) seems not to be important. The breakdown of UTP to UDP, however, will render the assay useless in rabbits (we have not checked it in rats), causing gross overestimations of activity unless UTP recycling can be prevented.

The divers actions of EDTA in the system are difficult to analyse, but seem in the main to be attributable to its Mg^{2+}-sequestering activity. EDTA inhibits glucuronidation, di- and mono-nucleotide hydrolysis in normal preparations, whereas Mg^{2+} activates all three. In microsomal fractions prepared with 1mM-EDTA in the buffer (except for the final wash), glucuronyltransferase and UDPase activities seem to be restored to normal values by 4mM-Mg^{2+} (because of considerable variation in enzyme activity from different rabbits, the effect cannot easily be precisely quantified), though UMPase activity appears to be irreversibly destroyed. The reasons for these effects are unclear: Mg^{2+} may be active in the catalytic step, but it should also be noted that Mg^{2+} effects alterations to the gross lipid structure of microsomal membranes, increasing the amount of a non-lamellar lipid phase which may function as a non-specific 'permease' for cofactors (Stier *et al.*, 1978*b*, 1979).

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