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Structure–Activity Studies of Glucose Transfer: Determination of the Spontaneous Rates of Hydrolysis of Uridine 5′-Diphospho-α-D-glucose (UDPG) and Uridine 5′-Diphospho-α-D-glucuronic Acid (UDPGA)

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Abstract—The pH-rate profiles for the hydrolysis of uridine 5′-diphospho-α-D-glucose (UDPG) and uridine 5′-diphospho-α-D-glucuronic acid (UDPGA) in aqueous solution have been measured. The results obtained and a comparison with other data suggests that the mechanism of hydrolysis of each activated glycosyl-donor at pH 1–4 probably involves the slow ionisation, via an S_N1 process, of the neutral molecule to a glycosyl ion and UDP. From these data, the catalytic power (k_{cat}/k_{uncat}) of the glycosyltransferases has been estimated for the first time to be in the order of 10^{11-13}.

Introduction

'Active' glucose has a pyrophosphate leaving group

Biological glycosylation is largely accomplished by enzymes that utilise as activated glycosyl-donors the glycosyl derivatives of either nucleoside pyrophosphates or polyprenol pyrophosphates.[1] The former class of activated glycosyl-donors form the largest group, for one of several nucleosides (e.g., uridine, cytidine, adenine) may be linked to one of a whole range of glycosyl groups (e.g., glucosyl, galactosyl, mannosyl, glucuronyl, ribosyl). Our interest in this area stemmed from our work with uridine 5′-diphospho-α-D-glucuronic acid (UDPGA), which can be used routinely to biosynthesise glucuronide conjugates of drug metabolites (and other foreign compounds). It is well known that incubation of an aglycone with boiled enzyme preparations plus UDPGA produces no glucuronides,[2] and we became interested in investigating the catalytic power of the enzymes involved, the glucuronyltransferases. To this end, we began a structure–activity study of glucuronic acid transfer by determining the spontaneous (non-enzymic) glucuronylating reactivity of UDPGA. This was achieved by determining its pH-rate profile, and hence its glucuronylating reactivity towards water. Because it lacked the ionizable carboxyl group of UDPGA, the prototype of the series, uridine 5′-diphospho-α-D-glucose (UDPG), was also studied. This was of interest in its own right, of course, since UDPG is the activated glucosyl-donor employed in the biosynthesis of glycogen and in many other polysaccharides, glycolipids, and glycoproteins. More mundanely, UDPG is also utilised in the biotransformation in insects and plants of natural, and xenobiotic, alcohols and phenols into 'glucose conjugates' (analogous to the role of UDPGA in the formation of 'glucuronide conjugates' in mammals).

In the event, because it lacked the complicating ionisable carboxyl group, we conducted a comprehensive study of UDPG, and collected only a few data for UDPGA.

Mechanism of biological glycosylation

In general, these UDPGly-dependent processes effect, in the main, either *O*-glycosylation or *N*-glycosylation of a substrate. In all cases, whether the substrate be a

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large or small (bio)molecule, the sites (e.g., hydroxyl or amino groups) of glycosylation are, by definition, nucleophilic groupings. Since *O*- and *N*-glycosylation are mechanistically congeneric, the prototype of biological glycosylation reactions can be taken as the glucosyltransferase-mediated glucosylation of an alcohol, ROH, by UDPG. Since, moreover, all of the nucleotide glycosyl-donors are α-XDP-glycosides (none is β-), the prototypical formation from an alcohol (ROH) of its β-glucoside can, as Axelrod and co-workers[3] (in their work on mammalian steroidal β-glucuronides) recognised more than 40 years ago, be viewed as a glucosyltransferase-mediated inverting nucleophilic displacement at C-1 of UDPG with expulsion of uridine 5′-diphosphate (UDP) (Scheme 1). (Clearly, when α-glucosides are formed, a double displacement reaction of UDPG is required, presumably involving the intermediacy of a glucosylated enzyme. That type of process will not concern us here.)

Related biological transfer processes—methylation, prenylation, acetylation, phosphorylation and sulfation

Biological glycosylation can be grouped with the two other major biological alkylation processes, methylation and prenylation. Moreover, mechanistically we can group these three biological alkylation reactions with three important biological 'acylation' processes, acetylation, phosphorylation and sulfation. For each effects the alkylation or acylation of a nucleophilic group in a biomolecule by means of a coenzyme—or an activated donor-molecule (ADM)—in which the group to be transferred is attached to a good leaving group. The combinations of coenzyme/enzyme (or ADM/enzyme) involved are *S*-adenosylmethionine (SAM)/methyltransferases, geranyl or farnesyl pyrophosphate (GPP or FPP)/prenyltransferases, acetyl coenzyme A (acetyl CoA)/acetyltransferases, adenosine 5′-triphosphate (ATP)/phosphotransferases and 3′-phosphoadenosine-5′-phosphosulfate (PAPS)/sulfotransferases.

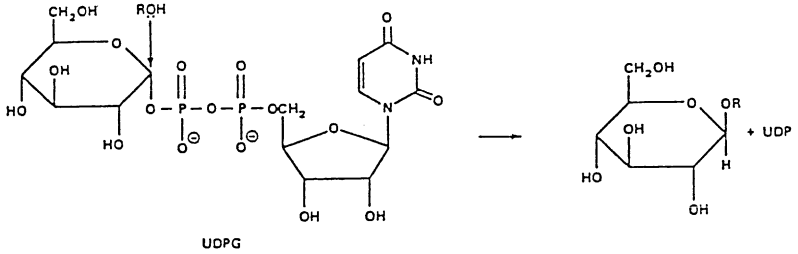
The catalytic power of these transferases has long been of interest to mechanistic bioorganic chemists, and more than 30 years ago that of the phosphotransferases (kinases) came under close scrutiny. By elucidating the weak spontaneous (non-enzymic) phosphorylating activity towards water of adenosine 5′-triphosphate (ATP) at pH 7, it was possible to calculate from known enzymic rates that the catalytic power (k_{cat}/k_{uncat}) of the phosphotransferases was in the order of 10^{10}–10^{12} (e.g., see ref 4). Subsequently, Schowen and Coward and co-workers[5] determined the spontaneous methylating reactivity of *S*-adenosylmethionine (SAM) and estimated the catalytic power (k_{cat}/k_{uncat}) of the methyltransferases to be in the order of 10^{16}. No attempt has been made to evaluate the catalytic power of the prenyltransferases, although Tidd's determination of the spontaneous alkylating reactivity of dimethylallyl pyrophosphate (DMAPP)[6]—which is a good model for GPP and FPP—provides the necessary information. Recently we determined the spontaneous sulfating reactivity of PAPS and estimated[7] the catalytic power (k_{cat}/k_{uncat}) of the sulfotransferases to be in the order of 10^{10-2}—in the event, identical to the range estimated for the phosphotransferases.[4] However, there have been, to our knowledge, no studies of the measurement of the spontaneous glycosylating reactivity of any glycosyl derivative of a nucleoside pyrophosphate which would yield similar data pertaining to the magnitude of the catalytic role of the glycosyltransferases. We have now accomplished this task, and report rates of hydrolysis of UDPG and UDPGA in the pH range 1–10 and the magnitude of the spontaneous (non-enzymic) glycosylating reactivity (towards water) of UDPG and UDPGA and the pH range at which these reactivities prevail. A preliminary report of the results of the UDPG study has been made.[8]

Results

Hydrolysis experiments

In order to follow the hydrolysis of UDPG and of UDPGA by quantitative HPLC, using spectrophotometric detection of the uridine chromophore, a mixed ion-pair system capable of resolving UDPG and UDPGA from their likely uridine-containing hydrolysis products, UDP, uridine 5′-phosphate (UMP), and uridine 3′,5′-cyclic-phosphate (cUMP) was developed (Fig. 1).[9]

From the results of pilot experiments, a temperature of 60 °C was chosen for the determination of the pH rate profile of UDPG and UDPGA. The rate data that were obtained are shown in Tables 1 and 2 and plotted in Figure 2. Some rate data of the hydrolysis of the common hydrolysis product, UDP, were also obtained at 60 °C at pH 1.5–6 and these are shown in Table 3 and plotted in Figure 2.



UDPG

Scheme 1.

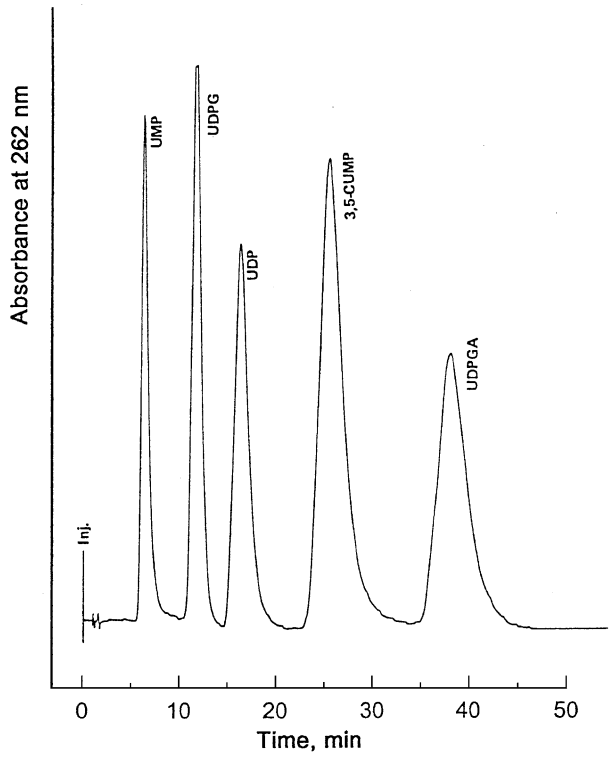


Figure 1. Mixed ion-pair reversed-phase HPLC trace of a mixture of UMP, UDPG, UDP, cUMP, and UDPGA. (Conditions: 10 μL from an aqueous solution of about 1 mg/mL injected by valve onto a 200×4.5 mm column packed with Partisil 10 ODS 2. The mobile phase was 0.002375 M tetrabutylammonium hydroxide, 0.002375 M tetraethylammonium hydroxide, 0.0475 M ammonium dihydrogen orthophosphate, adjusted to pH 6 with KOH in aqueous 10% methanol.)

Table 1. Observed rate constants for the hydrolysis of UDPG in aqueous solution at 60 and 95 °C (μ = 1.0)

Buffer	Molarity (M)	pH	Temp (°C)	$10^5 k_{obs}$ (s^{-1})
HCl	0.10	1.01	60	168.8
HCl	0.05	1.50	60	49.24
HCl	0.027	1.84	60	19.52
HCl	0.0063	2.28	60	7.652
HCl	0.0063	2.39	60	5.994
HCl	0.006	2.61	60	3.912
Formate	1.0	2.97	60	1.581
Formate	0.10	3.61	60	0.4002
Acetate	1.0	3.76	60	0.3242
Acetate	0.10	3.80	60	0.2415
Succinate	0.33	5.64	60	0.0400
Tris	0.05	6.97	60	0.2788
Tris	0.10	8.07	60	1.365
Carbonate	0.33	9.42	60	19.21
NaOH	0.10	11.78	60	1982
HCl	0.016	2.08	95	934.5
Acetate	1.0	3.90	95	14.75

Isotope experiments

The positions of bond fission of UDPGA were determined from the results of FAB mass spectral analysis of UDP isolated from reactions run in water enriched with ^{18}O. The negative ion FAB mass spectrum of authentic UDP has been reported[10] to show an intense ion at m/z 403 due to the UDP monoanion with an equally intense fragment at m/z 323 due to the corresponding UMP monoanion. Peak ratio measurements for each sample were therefore carried out on m/z 403, 404 and 405, which should indicate any enrichment, and on m/z 323, 324, and 325, which provided an internal check since it would not be expected to contain any ^{18}O. (No enhancement of peak area was expected of m/z 404 or of m/z 324, and this provided an additional internal check.)

Comparison of the mass spectral data of duplicate samples of UDP obtained by hydrolysis of UDPGA at pH 1.5 at 60 °C for 0.5 h in ^{18}O water (50% ^{18}O) with those of authentic UDP and of a blank obtained by heating UDP under similar conditions revealed that no incorporation of label had occurred.

Table 2. Observed rate constants for the hydrolysis of UDPGA in aqueous solution at 60 °C (μ = 1.0)

Buffer	Molarity (M)	pH	$10^5 k_{obs}$ (s^{-1})
HCl	1.0	0.03	403.3
HCl	0.10	1.10	12.71
HCl	0.04	1.51	4.245
HCl	0.0063	2.53	1.060
Succinate	0.33	5.63	0.0472
Tris	0.5	6.96	0.3814

Discussion

The pH-rate profiles of UDPG and UDPGA

Clearly, amongst the several possible C–O and P–O hydrolytic cleavage reactions of UDPG, there was a need to identify and quantify the hydrolytic process yielding uridine 5′-diphosphate (UDP) by C–O fission (Scheme 1, HOH for ROH). The quantitative HPLC method[9] that was used for the assay of UDPG yielded, simultaneously, an assay of both UMP and UDP (see Fig. 1), and this permitted an estimate to be made of the amounts of each of these reaction products formed during each run at a given pH value.

At pH 1–3, the major hydrolysis product of UDPG was found to be UDP (ca. 95%), with minor amounts of UMP. Moreover the rate of formation of UDP in this pH range was virtually identical to the rate of disappearance of UDPG, once allowance had been made for the rate of hydrolysis of UDP (to UMP), which we also measured. The rate profile at 60 °C for UDP in the pH range 1–6 is, for ease of comparison, superimposed on the UDPG rate profile in Figure 2, and was very similar to that of adenosine 5′-diphosphate (ADP) determined by Miller and Westheimer[11] at 95 °C. [We made two determinations of the hydrolysis rate of UDP at 95 °C as a check on our methodology (see Table 3) and we obtained essentially identical results to those obtained by Miller and Westheimer.[11]]

At pH 4, the major hydrolysis product was found to be UMP, with minor amounts of UDP. As is evident from Figure 2, the rate of hydrolysis of UDPG to UDP is

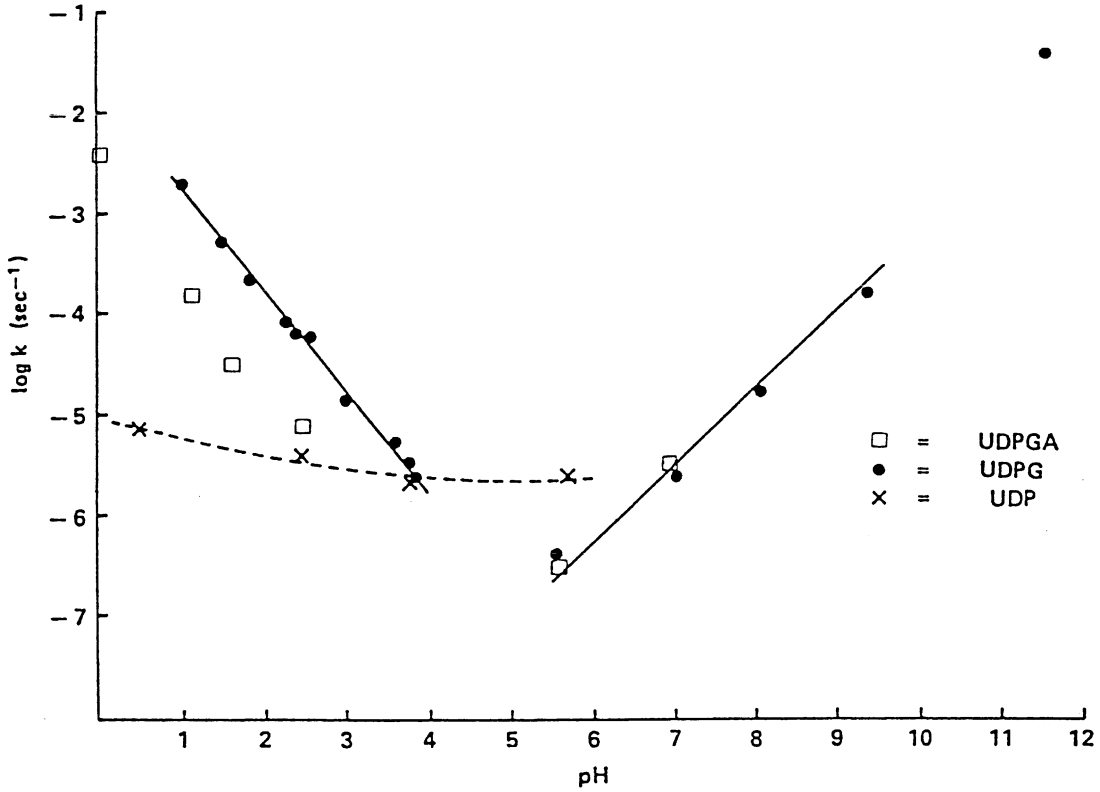


Figure 2. The pH-rate profiles at 60 °C of the hydrolysis of UDPG, UDPGA and UDP.

Table 3. Observed rate constants for the hydrolysis of UDP in aqueous solution at 60 and 95 °C ($\mu = 1.0$)

Buffer	Molarity (M)	pH	Temp (°C)	$10^5 k_{obs}$ (s^{-1})
HCl	0.05	1.50	60	0.692
HCl	0.0063	2.39	60	0.380
Acetate	0.10	3.80	60	0.263
Succinate	0.33	5.64	60	0.245
HCl	0.016	2.08	95	17.0

about equal to that of the hydrolysis of UDP to UMP at pH 4, and this means that UDP at this pH would be hydrolysed as fast as it was formed. Clearly, although all, or a part of, the UMP could have been formed directly from UDPG, it seems more likely that all of it is formed via the hydrolysis of initially-formed UDP.

Less data were obtained for UDPGA, but as can be seen from Figure 2, the rates of hydrolysis of UDPGA and UDPG differ by a factor of about 10 in the range pH 1–2.5, UDPGA being the less reactive. At low pH (<3), the carboxylic acid group of UDPGA will be fully protonated, and this electron-withdrawing group is exhibiting its expected rate-decreasing destabilising effect upon the incipient glycosyl cation (vis à vis that formed from UDPG).

We can safely conclude, therefore, that the nucleotide hydrolysis product at pH 1–4 of UDPG and UDPGA is predominantly, if not solely, UDP, and that the rate of these reactions, as Figure 2 shows, increases in approximate proportion to the hydrogen-ion concentration. UDP could have been formed from either substrate via P–O or C–O fission, but we have confirmed that C–O fission was occurring by means of a labelling experiment with UDPGA using $H_2{}^{18}O$ (in view of their closely related structures, we have assumed—though not conducted the experiment— that UDPG would have yielded a similar result).

In the pH range 6–10, UDP, which is stable under mild alkaline conditions (like ADP[11]) and would have been expected to have persisted if it had been formed, was not a hydrolysis product of UDPG or of UDPGA. Consequently, only a few rate measurements in this pH range were made and no product studies were undertaken. The rate profile for UDPG in the pH range 7–10 (Fig. 2) is probably best explained by hydroxide ion-catalysed formation of glucose-1,2-cyclic monophosphate and uridine 5′-monophosphate (UMP), as observed by Leloir and co-workers[12] during their structural studies of UDPG. This reaction, as would be expected, increases in rate in approximate proportion to the hydroxide-ion concentration. The remoteness from the reaction centre of the carboxylate group of UDPGA has no effect, predictably, on the rate of the analogous reaction of UDPGA, the rates of both substrates being identical at pH 5.5 and 7 (see Fig. 2).

Comparison of UDPG rate data with that of other phosphates

For comparison, our rate data for UDPG in the pH range 1–4 and those for α-D-glucose-1-phosphate,[13a]

methyl phosphate,[13b] dimethylallyl phosphate,[6] and dimethylallyl pyrophosphate[6] in the pH range 1–8 (all extrapolated to 60 °C) appear in Figure 3.

Gratifyingly, the rectilinear profile that we obtained for UDPG at pH 1–4 parallels that for α-D-glucose-1-phosphate (α-GP) at pH 1–4 determined by Bunton and co-workers,[13a] and our demonstration of C-O fission in that pH region reproduces the finding, in experiments using $H_2{}^{18}O$, that the hydrolysis of α-GP at pH 1–4 also occurred wholly via C–O fission.[13a]

The near-identical slopes of the profiles for UDPG and α-GP are closely similar to the slopes of the profiles for dimethylallyl phosphate and dimethylallyl pyrophosphate (DMAPP) in the pH range 1–7, which Tidd has shown[6] to be due to C–O fission of each. However, DMAPP is, at pH 4, about a 100-fold more labile hydrolytically than UDPG. Put another way, this shows that the prototypes of the activated donor-molecules deployed for biological glycosylation and prenylation, UDPG and DMAPP respectively, possess alkylating reactivities (here measured towards water) that differ by about two orders of magnitude, DMAPP being the more reactive.

It is noteworthy that for dimethylallyl esters, monophosphate appears to be about as good a leaving group as pyrophosphate, but for glucosyl esters monophosphate is about 5-fold less good as a leaving group than is (nucleotidyl) pyrophosphate. The pH-rate profile of methyl phosphate (see Fig. 3) does not show any features at pH 1–4 indicating C–O fission, and labelling studies have shown that P–O fission is the exclusive mode in this pH range.[13b] Put another way, methyl phosphate is not a methylating agent in this pH range. Indeed, in contrast to biological glycosylation and prenylation where pyrophosphates are deployed as activated donor-molecules in S_N1-type processes, biological methylation, most commonly, deploys as coenzyme

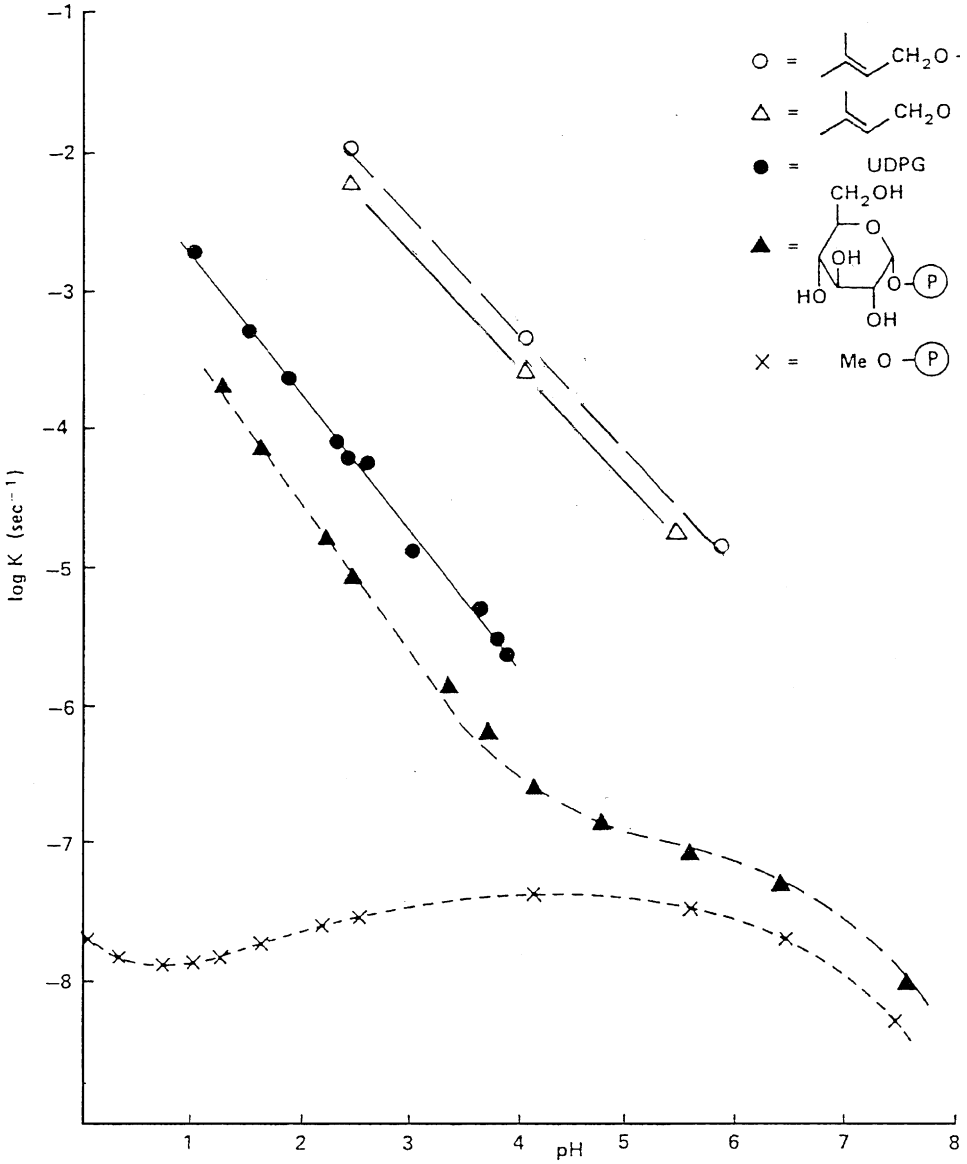


Figure 3. The pH-rate profiles at 60 °C of the hydrolysis of methyl phosphate, dimethylallyl phosphate, dimethylallyl pyrophosphate, α-D-glucose-1-phosphate and UDPG.

S-adenosylmethionine (SAM), a methylsulphonium compound, MeR_2S^+, with neutral R_2S as a leaving group in an S_N2 process.

Mechanism of hydrolysis of UDPG and UDPGA

The mechanism of the hydrolysis of UDPG and UDPGA at pH 1–4 would be expected to be analogous to that proposed[13a] for α-GP and therefore probably involves the slow ionisation via an $S_N1(C)$ process of the neutral molecule (Scheme 2), or less likely of the mono-anion, to a glycosyl ion and UDP.

The catalytic power of the glycosyltransferases

In summary, these results reveal that UDPG is a glucosylating agent only at pH 1–4 with a reactivity only about five-fold greater than that of α-D-glucose-l-phosphate. Extrapolation to pH 7 and to 37 °C of the rate data and conversion to a second-order rate constant gives a value of about 5×10^{-12} M^{-1} s^{-1} for the glucosylating reactivity (towards water) of UDPG. Expressing this in a way suitable for comparison with an enzymic reaction and converting to units familiar to enzymologists, we can say that the spontaneous (non-enzymic) glucosylation of water by UDPG occurs at a rate of about 2×10^{-11} mM^{-1} min^{-1}. Enzymic rate data of glycosyltransferases are sparse,[14] but are in the range 5–500 mM^{-1} min^{-1}. By assuming that the magnitude of the glucosylating reactivity of UDPG towards a typical hydroxyl-containing substrate, ROH, is similar to that of water, we can estimate that the catalytic power (k_{cat}/k_{uncat}) of the glucosyltransferases—and, by analogy, of all of the glycosyltransferases—is in the order of 10^{11-13}.

Experimental

Materials

Uridine 5′-diphospho-α-D-glucose (UDPG), uridine 5′-diphospho-α-D-glucuronic acid (UDPGA), uridine 5′-diphosphate (UDP), uridine 3′,5′-cyclic-phosphate (c-UMP) and uridine 5′-phosphate (UMP) were obtained as sodium salts from Sigma Ltd. Tetrabutylammonium hydroxide and tetraethyl-ammonium hydroxide were obtained as aqueous solutions from BDH Ltd. All other reagents were of AR grade.

High-performance liquid chromatograph

The analytical HPLC system was composed of a Laboratory Data Control (LDC) gradient solvent-delivery system, a Rheodyne injection valve fitted with a 20 μL loop, a Pye LC3 spectrophotometric detector operating at 262 nm and a Hewlett Packard 338S Integrator. The columns (200×4.5 mm) were either Whatman Partisil 10 SAX for ion-exchange or Partisil 10 ODS2 for paired-ion chromatography. Ion-exchange analyses were run at room temperature, but paired-ion analyses were run with the column maintained at 30 °C by means of a Magnus Scientific water jacket and a Churchill water pump and heater. The flow rate was 2 mL/min.

Ion-exchange chromatography

For isocratic elution, optimal resolution was obtained using as a mobile phase: 0.05 M KCl and 0.05 M KH_2PO_4, adjusted to pH 6.0 with 1 M KOH.

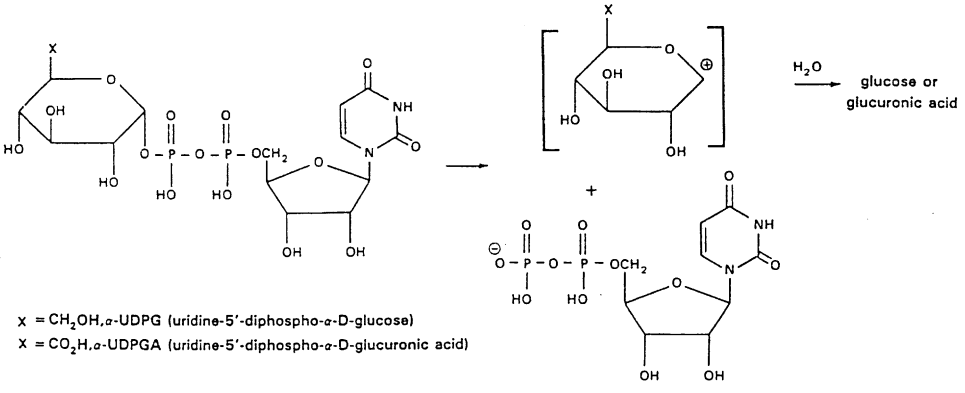
Paired-ion chromatography

Using a single ion-pairing reagent. Optimum isocratic conditions for separating UDPG, UDP and UMP from each other and also c-UMP were found to be a mobile phase of: 0.0475 M NH_4PO_4, 0.00475 M tetrabutyl ammonium hydroxide, adjusted to pH 6.0 with 1 M HCl in aqueous 5% methanol.

Using two ion-pairing reagents. The optimal isocratic conditions for separating all of the phosphates was found to be a mobile phase of: 0.002375 M tetraethylammonium hydroxide, 0.002375 M tetrabutylammonium hydroxide, 0.0475 M NH_4PO_4, adjusted to pH 6.0 with 1 M HCl in aqueous 5% methanol.

Kinetic studies

The kinetics of the hydrolysis of UDPG or UDPGA were followed by quantitative HPLC either by periodically measuring the decrease in peak area of UDPG or UDPGA or the increase in peak area of UDP and/or



Scheme 2.

UMP (the only detectable products). All reactions were studied at either 60 or 95 °C and were initiated by adding a known volume of pre-heated buffer to a known amount of substrate. In all cases the substrate dissolved immediately. Buffers employed for the hydrolyses were HCl (pH < 3.5), formate (0.1–1.0 M), acetate (0.1–1.0 M), succinate (0.33 M), Tris (0.5–1.0 M), carbonate (0.33 M), and NaOH (pH > 11), with ionic strengths of 1.0 made up with potassium chloride. No corrections of the rate data for buffer catalysis were necessary, since representative checks for buffer catalysis at pH 3.6 (formate), pH 3.8 (acetate) and pH 8.0 (Tris) showed there to be none. All pHs were measured using a Russel glass electrode and a Corning pH meter. Pseudo-first-order rate constants were calculated from data acquired over at least four half-lives.

Mass spectrometry

Analyses by fast-atom bombardment (FAB) were carried out in a VG 7070 spectrometer in negative ion mode using glycerol as the matrix material. A duplicate measurement of each sample of UDP was recorded and the mean result determined.

Acknowledgements

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References and Notes

1. Davies, G.; Sinnott, M. L.; Withers, S. G. In *Comprehensive Biological Catalysis;* Sinnott, M. L., Ed., Academic: New York, 1998; Chapter 3, p 119.
2. Mulder, G. J.; Coughtrie, M. W. H.; Burchell, B. In *Conjugation Reactions in Drug Metabolism*; Mulder, G. J., Ed.; Taylor and Francis: London, 1990; Chapter 4, p 51.
3. Axelrod, J.; Inscoe, J. K.; Tompkins, G. M. *J. Biol. Chem.* **1958**, *232*, 835.
4. Benkovic, S. J.; Schray, K. J. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic: New York, 1970; Vol. VIII, Chapter 6, p 201.
5. Mihel, I.; Knipe, J. O.; Coward, J. K.; Schowen, R. L. *J. Amer. Chem. Soc.* **1979**, *101*, 4349.
6. Tidd, B. K. *J. Chem. Soc. (B)* **1971**, 1168.
7. Bedford, C. T.; Kirby, A. J.; Logan, C. J.; Drummond, J. N. *Bioorg. Med. Chem.* **1995**, *3*, 167.
8. Bedford, C. T.; Hickman, A.; Logan, C. *J. Bioorg. Med. Chem. Lett.* **1992**, *2*, 1513.
9. Bedford, C. T.; Hickman, A.; Logan, C. J. *J. Chromatogr. A* **1995**, *695*, 123.
10. Fenselau, C.; Feng, P. C. C.; Chen, T.; Johnson, L. P. *Drug Metab. Dispos.* **1982**, *10*, 316.
11. Miller, D. L.; Westheimer, F. H. *J. Am. Chem. Soc.* **1966**, *88*, 1507.
12. Caputto, R.; Leloir, L. F.; Cardini, C. E.; Paladini, A. C. *J. Biol. Chem.* **1950**, *184*, 333.
13. (a) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc.* **1958**, 3588. (b) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc.* **1958**, 3574.
14. Tvaroska, I.; Andre, I.; Carver, J. P. *J. Am. Chem. Soc.* **2000**, *122*, 8762.