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ASSAY OF UDPGlcUA PYROPHOSPHATASE AND ITS RELATION TO TRANSGLUCURONIDATION

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SUMMARY

The activity of UDPGlcUA pyrophosphatase in liver and kidney was measured by a fluorimetric method which is based on the conjugation of the aglycone, harmol, with the unreacted UDPGlcUA. When labelled nucleotide was used in the reaction, good correlation was established between the fluorimetric and isotopic analyses of the glucuronide produced. Under the same experimental assay conditions, there is an inverse relationship between the hepatic activities of the pyrophosphatase and glucuronyltransferase in four species of animals. The reduction in the rate of transglucuronidation by the interfering pyrophosphatase was most significant in rat liver homogenate. In the presence of 10 mM of EDTA, a concentration which completely inhibits the pyrophosphatase, the "full" transglucuronidating activity of homogenate could be ascertained. The effects of ATP on both of these enzymatic reactions were also studied.

INTRODUCTION

A nucleotide pyrophosphatase capable of degrading NAD was first demonstrated by KORNBERG AND LINDBERG[1]. Similar hydrolases have been shown to act on the pyrophosphate bond of the uridine nucleotides like UDPGlcUA[2,3], UDPG[4,5] and UDPGlcNHAc[6]. It is not known whether the same enzyme is involved in these reactions, although their pH optima are similar and they are inhibited to variable extent by EDTA[5–7]. In each instance, the products are UMP and the phosphorylated sugar.

UDPGlcUA pyrophosphatase has been implicated in interfering with the transglucuronidating reaction[8–11]. The quantitative significance of this has never been evaluated. This could be due to the lack of a simple sensitive assay procedure. Hitherto, two attempts have been made to measure this enzyme, namely by HOLLMAN AND TOUSTER[12] and by OGAWA *et al.*[7]. The former depends on the reaction of the residual UDPGlcUA with *p*-nitrophenol, while the latter involves the analysis by the naphthoresorcinol reaction of the glucuronic acid-1-*P* produced. In this study, a fluorimetric method was developed. The procedure is essentially that of a back-titration: the

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unreacted UDPGlcUA being determined by its conjugation with harmol[13]. (Harmol, $C_{12}H_{10}N_2O$, is the oxidized form of the alkaloid, harmalol, $C_{12}H_{12}N_2O$.)

It was thought that the counteracting action of pyrophosphatase present in an enzyme preparation could be principally responsible for the differences in the enzyme activity of glucuronyl transferase measured *in vitro*. It is therefore imperative that in such an investigation the pyrophosphatase activity be determined under the same experimental conditions as those normally employed in the transglucuronidating reaction, *i.e.* with respect to pH, extraction medium and nucleotide concentration. Accordingly, the pyrophosphatase activities of the liver and kidney of a few species of animals were compared and the results discussed in relation to their capacities for transglucuronidation.

MATERIALS

UDPGlcUA, ammonium salt, ATP, glucuronic acid and glucuronic acid-1-*P* were obtained from Sigma Chem. Co.; harmol·HCl from Fluka, A.G., Buchs, Switzerland; cellulose MN-300 for thin-layer chromatography from Macherey, Nagel and Co., Germany and UDP-[^{14}C]glucuronic acid, specific activity of 33.4 mC/mM from the Radiochemical Centre, Amersham.

METHODS

A known quantity of UDPGlcUA was initially incubated in a suitable medium with the enzyme preparation of liver or kidney. The reaction was terminated by boiling for 2 min, and a fraction of the reacted mixture was subsequently subjected to a conjugating reaction with harmol and guinea pig liver microsomes which provided the glucuronyl transferase. Controls were carried out simultaneously and differed only in that the UDPGlcUA was added after boiling. The amount of glucuronide formed is directly proportional to the residual nucleotide present. Consequently, the difference between the experiment and the control represents the amount of nucleotide hydrolysed by the pyrophosphatase, and this was determined from a standard curve where 5–25 μg of UDPGlcUA was used in the transglucuronidating reaction. The time of incubation varied from 10 to 30 min depending on the enzymic activity of the preparation. For the assay of the pyrophosphatase, the reaction mixture contained the following: 100 μg UDPGlcUA, 0.2 ml Tris buffer, (0.5 M) (pH 7.8), 0.2 ml of enzyme preparation containing 1–10 mg fresh weight tissue extracted with 0.15 M KCl. The total volume was made up to 1 ml with water. After centrifugation of the boiled reacted mixture, 0.25 ml of the supernatant was carried over to the next step. This second incubation mixture contained 0.2 ml Tris buffer and 0.1 ml harmol, the final concentration of which was $1.42 \cdot 10^{-4}$ M. The reaction was started with 0.1 ml of a microsomal preparation of guinea pig liver, obtained by the procedure of WONG AND SOURKES[13]. The final volume of this second reaction mixture was 0.9 ml. After incubation at 37° in a Dubnoff shaker bath for 30 min, the reaction was stopped by adding 0.1 ml of a 5% solution of $ZnSO_4$.

Isotopic procedure

The reaction procedure is essentially the same except that the final volumes of

the two reaction mixtures were 0.4 and 0.45 ml, respectively, and half of the first-reacted mixture was carried over to the second step. The stock solution of UDPGlcUA of 1 mg/ml was mixed with 1/24th of its volume of labelled UDPGlcUA, whose radioactive concentration was 50 μC/ml (specific activity = 33.4 mC/mM).

Measurement of harmol glucuronide

(a) Fluorimetric analysis: As reported by WONG AND SOURKES[13]. (b) Isotopic analysis: A 15-μl aliquot of the reacted mixture was subjected to paper chromatography on Whatman No. 1 paper. Development in 0.1 M HCl took 6 h. Five strips of 1 cm each (the central segment of which corresponds to the region of highest intensity of the glucuronide spot) were counted in the Unilux liquid scintillator, Nuclear Chicago. 40-min counts were taken. The scintillator contained 0.02% 1,4-bis-(5-phenyloxazolyl-2)benzene and 0.3% 2,5-diphenyloxazolyl in toluene.

Location of UDPGlcUA and its metabolites on paper and thin-layer chromatograms

To ensure that UDPGlcUA and some of its known breakdown products did not interfere with the counting of harmol glucuronide, their positions on the paper chromatogram were determined; glucuronic acid by the naphthoresorcinol reagent[14] and glucuronic acid-1-*P* by the ammonium molybdate reagent[15]. To locate the position of UDPGlcUA, fractions of 1 inch of the paper chromatogram from the origin to the solvent front were eluted and these eluates were tested for their ability to conjugate with harmol.

RESULTS

The activity curves of UDPGlcUA pyrophosphatase of rat liver determined by

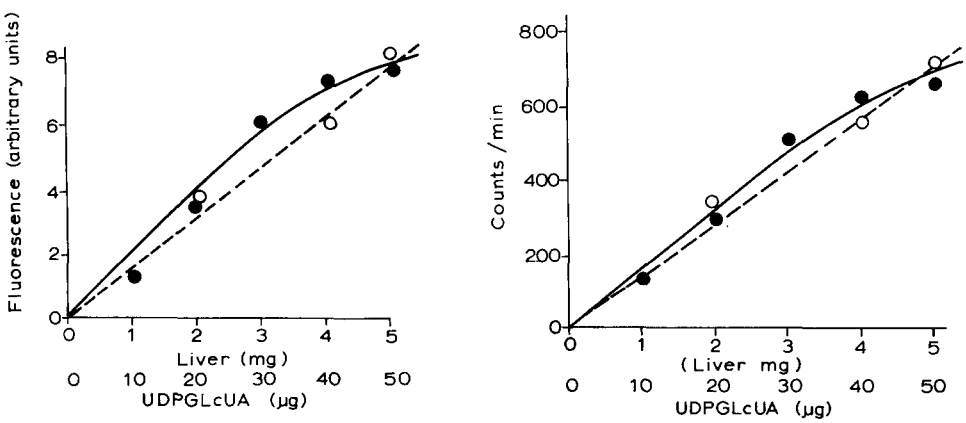


Fig. 1. Measurement of UDPGlcUA pyrophosphatase of rat liver by the fluorimetric procedure. The fluorescence units given represent the difference between the control (in which the enzyme was omitted) and experimental tubes. The reaction mixture contained the following: 100 μg UDPGlcUA, 0.1 ml Tris buffer (pH 7.8) and 0.2 ml enzyme preparation containing 1–10 mg wet weight liver, in a total volume of 0.4 ml. Incubation was carried out for 10 min at 37°. Standards containing 20, 40 and 50 μg UDPGlcUA (○— - —○) were carried through the transglucuronidating reaction (see text).

Fig. 2. Measurement of UDPGlcUA pyrophosphatase by the isotopic procedure. Reaction conditions and symbols are the same as in Fig. 1.

TABLE I

R_F VALUES OF HARMOL, UDPGlcUA AND THEIR METABOLITES

Chromatograms were developed in 0.1 M HCl.

Compound	*R_F Value*	
	On paper chromatogram	*On thin-layer chromatogram*
UDPGlcUA	0.95	1.0
Glucuronic acid-1-*P*	1.0	1.0
Glucuronic acid	0.82	0.73
Harmol	0.23	0.18
Harmol glucuronide	0.53	0.45

the fluorimetric and isotopic analyses are shown in Figs. 1 and 2, respectively. There was no discrepancy in the two analytical procedures. The fluorimetric assay was favoured in subsequent experiments because it is simpler and more economical.

The positions of the following compounds were located on paper and thin-layer chromatograms: UDPGlcUA, glucuronic acid, glucuronic acid-1-*P*, harmol and harmol glucuronide. From the R_F values of Table I, it can be inferred that UDPGlcUA and its metabolites are unlikely to interfere with the measurement of the glucuronide. This is further confirmed in the isotopic analysis by the fact that the end strips of each glucuronide band approach background counts as shown in Fig. 3.

The hydrolysis of UDPGlcUA using rat liver homogenate is shown as a function of time in Fig. 4. After 5 min of incubation, about 33% of the nucleotide added to the system had been degraded. This progressive breakdown of the conjugating agent undoubtedly diminished the rate of transglucuronidation. A linear increase in activity was demonstrated for enzyme concentration up to 1 mg fresh weight of liver (Fig. 5). Enzyme extracted with 0.15 M KCl and 0.25 M sucrose exhibited almost equal activity

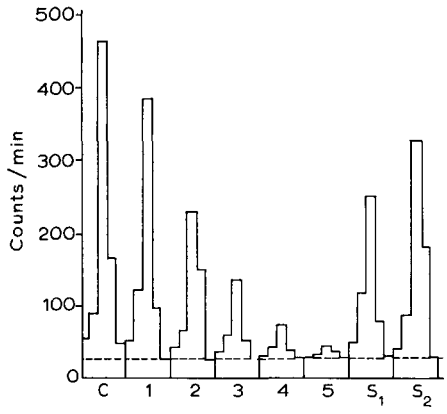


Fig. 3. Diagrammatic representation of the glucuronide peaks as determined by the isotopic procedure. C is control in which the enzyme is omitted and 1–5 are experimental tubes containing 1–5 mg of wet weight rat liver, respectively. Standards, S_1 and S_2 correspond to 20 and 40 μg UDPGlcUA (added to the transglucuronidating reaction). The background of 25 counts/min is shown by the dotted line.

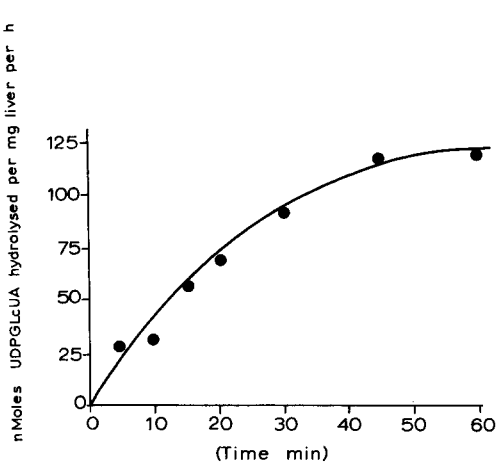


Fig. 4. UDPGlcUA pyrophosphatase activity as a function of time.

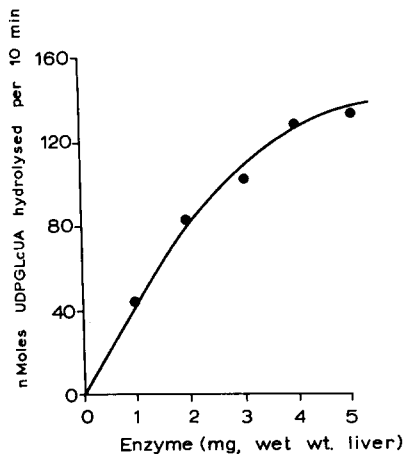


Fig. 5. Effect of enzyme concentration on the pyrophosphatase activity.

(contrast HOLLMAN AND TOUSTER[12]). The specific activities of the various enzyme preparations of liver and kidney are summarized in Table II. Unless otherwise stated, extraction of the enzyme was carried out with 0.15 M KCl.

EDTA at a concentration between 1 and 10 mM inhibited the pyrophosphatase completely but had negligible effect on the glucuronyl transferase, as shown in Fig. 6. This effect appeared to be immediate as the incubation period was only 10 min.

TABLE II

ACTIVITY OF UDPGlcUA PYROPHOSPHATASE IN LIVER AND KIDNEY OF VARIOUS ANIMALS

Species	*Tissues*	*Time of incubation (min)*	*Fresh wt. of tissue (mg)*	*Activity* (nmoles UDPGlcUA hydrolysed/mg per h)*
Rat	Liver	10	1.0	292
	Liver	10	2.0	257.6; 268.5, 270 241.1**
	Liver	10	1.0	225 ***
	Kidney	10	2.0	276.8
Mouse	Liver	30	10	25
	Kidney	10	1.0	428.6
	Kidney	10	2.0	285.7
Monkey	Liver	30	5.0	18.5
	Liver	30	10	13.9; 15.2
	Kidney	30	10	14
Rabbit	Liver	30	10	1.2
	Kidney	30	10	13.9
Guinea pig	Liver	30	10	Not measurable
	Kidney	30	10	Not measurable

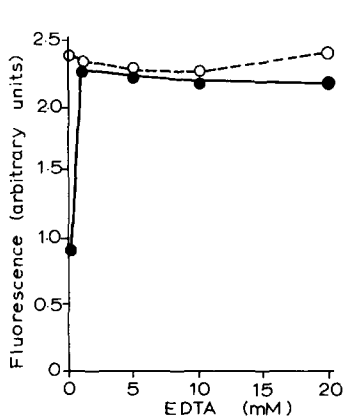
* Where two or more sets of data are given, they were obtained from independent experiments.

** Enzyme extracted with 0.25 M sucrose.

*** Value obtained from radioactive experiment.

ATP has been found by POGELL AND LELOIR[11] to activate the transglucuronidating reaction of rabbit and rat microsomes. An inhibition of the pyrophosphatase would elicit the same kind of response in an *in vitro* system. The effect of ATP was tested on both reactions as shown in Fig. 7. Results are expressed in fluorescence units obtained directly. This allows for a better visualization of the correlation between the two sets of results. The pyrophosphatase activity was progressively inhibited by increasing concentrations of ATP from 0.09 to 0.36 mM; complete inhibition being observed at the higher concentration. If the effect of ATP on the conjugating system were taken into consideration, its inhibitory action on the pyrophosphatase would be more pronounced in the range below 0.36 mM and saturation effect would be shown at and above this concentration.

From the above observations, an indirect or apparent increase in transferase activity would be produced by preincubating rat liver homogenate with EDTA or ATP. In one experiment, an activation of 2.7- and 1.4-fold were shown in a 10-min



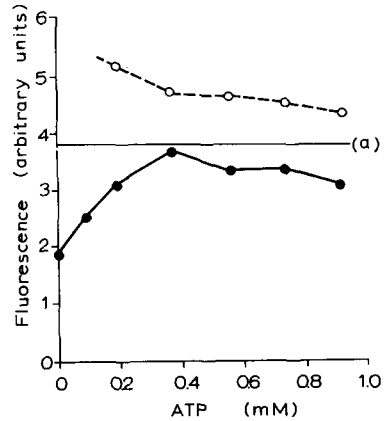


Fig. 6. Effects of EDTA on the activities of glucuronyl transferase (○— — —○) and UDPGlcUA pyrophosphatase (●—●). The transglucuronidating reaction mixture contained 25 μg UDPGlcUA; 0.2 ml Tris buffer (pH 7.8); harmol at a final concentration of $1.42 \cdot 10^{-4}$ M; microsomes equivalent to 50 mg fresh weight of guinea pig liver and varying concentrations of EDTA in a final volume of 0.9 ml. Time of incubation was 30 min. The incubation mixture for the pyrophosphatase assay contained 100 μg UDPGlcUA; 0.2 ml Tris buffer (pH 7.8) and 0.2 ml homogenate, equivalent to 2 mg wet weight of rat liver, in a total volume of 1 ml. After an incubation of 20 min, 0.25 ml of the reacted mixture was subjected to the above transglucuronidating reaction.

Fig. 7. Effects of ATP on glucuronyl transferase (○— — —○) and pyrophosphatase (●—●). The fluorimetric reading for the control tube where the enzyme was omitted in the pyrophosphatase reaction is indicated by (a). The reaction conditions were similar to those given in Fig. 6 except that ATP of 0.1–0.9 mM substituted for EDTA and 100 μg of UDPGlcUA was used in the transglucuronidating reaction.

preincubation with 10 mM EDTA and 0.36 mM ATP, respectively. In contrast, no effect was demonstrated on mouse liver homogenate similarly treated. This could be the result of the differential inhibition of the pyrophosphatases in the two systems. Thus the activation of EDTA on transglucuronidation appears to be mediated through the inhibition of the pyrophosphatase. Further suggestive evidence for this will be discussed.

DISCUSSION

From the fluorimetric and isotopic analyses, essentially similar results were obtained for the UDPGlcUA pyrophosphatase activity of rat liver. Both techniques are comparable in sensitivity and are free from the interference of the known metabolites of UDPGlcUA. Other advantages of the fluorimetric method have been elaborated[16,17], and all these considerations have contributed to its adoption in this study.

There is a disparity between the two sets of data obtained for the hepatic activity of pyrophosphatase of Wistar rats. The specific activities as measured by OGAWA *et al.*[7] and HOLLMAN AND TOUSTER[12] are, respectively, 79.5 nmoles glucuronic acid-1-*P* formed/mg protein per min for the microsomal preparation and 4.0 μmoles UDPGlcUA split/20 min per g wet weight liver. When expressed on the same basis as those values given in Table II, these would correspond to 286 nmoles glucuronic acid-1-*P* formed/mg wet weight per h and 12 nmoles UDPGlcUA split/mg wet weight per h. The first value is in close agreement with our results determined for the Sprague–Dawley rats (see Table II).

Great species differences of hepatic glucuronyltransferase have been reported[18–20] and it is difficult to evaluate whether these differences were "true" or "apparent", for the presence of active β-glucuronidase or pyrophosphatase in an enzyme preparation would inadvertently reduce the overall rate of transglucuronidation. In this respect the counteracting effect of β-glucuronidase is negligible as it showed only 5% of its activity at the pH optimum of transglucuronidation[16]; this was assessed by the hydrolysis of harmol glucuronide[21]. In contrast, rat liver homogenate exhibited more than 70% of its pyrophosphorylytic activity under similar conditions.

For the various species of animals studied, their pyrophosphatase activities measured in liver homogenates, in decreasing order, are as follows: rat, mouse, monkey, rabbit, and guinea pig (Table II). There exists in the guinea pig liver a situation where there is a high transferase activity[16] accompanied by very low pyrophosphatase activity. The reverse pattern occurs in the rat while intermediate values of both enzymes prevail in the mouse and rabbit. There is thus a reciprocal relationship of these two enzymes in these species of animals. On this basis, the high pyrophosphorylytic activity of kidney homogenate could explain its relatively low transglucuronidating capacity as compared to that of the liver[22–24].

The inhibition of pyrophosphatase induced by EDTA is shown in Fig. 6. It is conceivable that the activation of the transglucuronidating system by EDTA[25,26] is the result of such an inhibitory action. The preincubation experiments with EDTA clearly illustrated this point. Without a resolution of these two enzymes, it would not be possible to distinguish between activation of transferase *per se* from activation resulting from an inhibition of pyrophosphatase. Consequently, the removal or inhibition of pyrophosphatase becomes a prerequisite in the assay of glucuronyl transferase. For this purpose, the addition of EDTA in a final concentration of 10 mM to the conjugating system seems a reasonable proposition.

The activating effect of ATP on transglucuronidation is confirmed in this study. It is unlikely that the channelling of UDPGlcUA to the formation of glucuronic acid by ATP, as suggested by POGELL AND LELOIR[11], is significant, for the residual UDPGlcUA remains high in the presence of ATP (see Fig. 7).

In view of the participation of free glucuronic acid-1-*P* in the proposed biosynthetic pathway of ascorbic acid[27,28], it is interesting to note that the inability of the guinea pig to synthesize this vitamin could well be a deficiency of UDPGlcUA pyrophosphatase, as demonstrated in this study, rather than/or in addition to the absence of L-gulonic oxidase[29].

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