Enzymatic Studies of Glucuronide Formation in Impaired Liver I. Assay Methods For the Determination of Glucuronyl Transferase Activity and Uridine Diphosphate Glucuronic Acid Content of Liver Tissue Using 4-Methyl Umbelliferone as a Glucuronide Receptor; Its Application to Needle Liver Biopsy Tissues

Kazuhisa Taketa*

*Okayama University,

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Kazuhisa Taketa

Abstract

An attempt was made to apply the method devised by ARIAS for the determination of liver glucuronyl transferase activity using 4-methyl umbelliferone as a glucuronide receptor to the small amounts of liver tissue obtained by needle biopsy. This was accomplished by studying the kinetics of enzymatic 4-methyl umbelliferone glucuronide formation by mean(of mouse liver homogenates. The improved method was proved to be applicable to human liver and gave a satisfactory result. In addition, an assay method for the estimation of liver uridine diphosphate glucuronic acid content from the amount of 4-methyl umbelliferone glucuronide formed from the uridine diphosphate glucuronic acid contained in the liver homogenate used as a source of glucuronyl transferase was studied, and as a result it was proved to be also applicable to the small amounts of human liver tissue.

ENZYMATIC STUDIES OF GLUCURONIDE FORMATION IN IMPAIRED LIVER

I. ASSAY METHODS FOR THE DETERMINATION OF GLUCURONYL TRANSFERASE ACTIVITY AND URIDINE DIPHOSPHATE GLUCURONIC ACID CONTENT OF LIVER TISSUE USING 4-METHYL UMBELLIFERONE AS A GLUCURONIDE RECEPTOR; ITS APPLICATION TO NEEDLE LIVER BIOPSY TISSUES

Kazuhisa TAKETA

Department of Internal Medicine, Okayama University Medical School Okayama (Director: Prof. K. Kosaka)

Increasing interest has been focused on the enzyme, glucuronyl transferase (GT),[1,2,3,4] transferring glucuronic acid from uridine diphosphate glucuronic acid (UDPGA)[1] to its various receptors, and more sensitive and specific assay methods of glucuronide formation have been sought. As for the determination of *in vitro* glucuronide formation, several methods by means of various glucuronide receptors, such as o-aminophenol[5], p-nitrophenol [2], phenolphthalein[6,7], or bilirubin[8], have been reported. ARIAS[9] devised an excellent method for this purpose using 4-methy umbelliferone (4-MU), 4-methyl-7-hydroxy coumarin, as a glucuronide receptor. Although WAKISAKA[10] and others reported a similar method also using 4-MU, this method was considered inadequate for the assay using liver homogenate, because the assay in their method was all indirect and in fact the exact activity of GT was not obtained; namely, the activity was expressed in per cent fluorescence decrease of a given 4-MU concentration, ranging from 0.002 to 0.005 μ mole per 2.0 ml. ARIAS employed, in his method using 4-MU, reincubation by means of β-glucuronidase to identify the decrease in fluorescence of 4-MU in the initial incubation as a formation of 4-MU glucuronide. He suggested, however, that his method was inconvenient in that it was not applicable to such small amounts of liver tissue obtained by needle biopsy.

In the present study, an attempt was made to apply the method of ARIAS to small amounts of human liver tissue by studying the kinetics of enzymatic 4-MU glucuronide formation by means of homogenates, as GT source, prepared from small amount of mouse liver tissue. Incidentally, an effort was made to establish an assay method for the determination of UDPGA content of small

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amounts of liver tissue also by means of 4-MU.

EXPERIMENTAL

Outline of Assay Procedure : In the following experiments, estimations of 4-MU glucuronide formation were performed, unless otherwise noted, according to the standard assay procedure simultaneously using the standard incubation system (Table 1) as described hereafter in this paper. The glucuronide

Table 1. Standard incubation system for determination of liver GT activity

	ml.	(μ moles)
0.1 mM 4-MU	0.5	(0.05)
0.5 mM UDPGA*	0.2	(0.1)
0.2 M MgCl$_2$	0.05	(10)
0.5 M Tris buffer (pH 7.4)	0.05	(25)
2.5% Liver homogenate	0.4	10 mg.
H$_2$O**	0.4	
total volume	1.6	

In the direct determination of liver UDPGA content, 0.2 ml. of distilled water was added in place of UDPGA*.

Note. In the indirect determination of liver UDPGA content, 0.2 ml. of distilled water and 0.4 ml. of boiled liver extract prepared from 2.5 per cent liver homogenate were added in place of UDPGA* and H$_2$O** respectively, and 0.4 ml. of 2.5 per cent homogenate of normal mouse liver was used as GT source. On the other hand, the 'endogenous' 4-MU glucuronide formation by the normal mouse liver homogenate used as the enzyme source was determined by the direct method and correction was made for this value.

receptor, 4-MU, is a highly fluorescent substance, and its etherial glucuronide is not a fluorescent[11]. After incubation of 4-MU, UDPGA, and liver homogenate, the mixture was washed with chloroform for deproteinization and extraction of unconjugated 4-MU. The supernatant solution obtained from the mixture by centrifugation contained thus formed 4-MU glucuronide. This non-fluorescent solution was subjected to reincubation with β-glucuronidase. The restored fluorescence following this enzymatic hydrolysis was measured fluorometrically.

Materials : Adult male mice, each weighing 15 to 20 g., were selected. Each animal was killed by decapitation immediately before use, and a small piece of the liver was used for preparing homogenates. Human liver homogenates were prepared from the liver obtained by needle biopsy under direct vision on peritoneoscopy. These liver homogenates were used as a source of GT. UDPGA as the ammonium salt, 98 to 100 per cent purity, was obtained from Sigma Chemical Company. Bacterial β-glucuronidase, 12500 units per 288 mg., was obtained from Daiichi Pure Chemical Company. 4-MU glucuronide was prepared

from 4-MU, rat liver boiled extract as a UDPGA source, and mouse liver homogenate by an appropriate incubation of these substances. By washing the incubated mixture containing thus formed 4-MU glucuronide with chloroform, deproteinization and extraction of unconjugated 4-MU were performed. After determination of the concentration of 4-MU glucuronide by means of enzymatic hydrolysis with β-glucuronidase, the crude solution of 4-MU glucuronide thus obtained was used for the assay of β-glucuronidase.

Preparation of Liver Homogenate: The loss of the GT activity in minor liver tissues, each weighing approximately 50 mg., with the passage of time at room temperature was determined. At higher temperature the decrease in the GT activity with the passage of time was more rapid as shown in Table 2. In

Table 2. GT activity of mouse liver tissue following removal of the tissue

Time after Removal of Liver	GT Activity (%)	
	room temperature	
	15°C	25°C
min.		
0. 5	100	100
5	98.7	95.0
10	91.0	89.0
15	76.9	75.2

GT activity is expressed as per cent of the activity of the liver homogenized 30 seconds after removal of the tissue.

actual performance of the assay, exposure of liver tissues to the room temperature (below 15 °C) was minimized to within one minute; hence the loss of the GT activity in liver tissues before homogenization was negligible.

On the other hand, there was also a loss of the GT activity, to some extent, during the course of homogenization, even under an ice-cold condition. The smaller was the amount of the homogenate to be prepared and the lower was the concentration of the homogenate, the greater was the loss of the activity with time. Since the amount of liver tissue and the volume of the homogenizer available in the experiment were limited, the homogenate with a concentration of 2.5 per cent was found to be the most suitable and convenient one. The loss of the GT activity in course of homogenization at this concentration was determined on varied amounts of liver tissue. As a suspending medium ice-cold, alkaline, isotonic potassium chloride[12] was used. Each amount of liver tissue was homogenized for varied periods of time at 600 r. p. m. with a 5 ml. teflon homogenizer in the ice water. At the end of each homogenization period, the GT activity of the homogenate was determined (Table 3). From the results in this table, the

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Table 3. Effects of duration period of homogenization and amount of liver tissue
homogenized on GT activity of mouse liver

Duration Period of Time for Homogenization	GT Activity (%)		
	amount of liver homogenized (mg.)		
	10	25	75
sec.			
10	100.0	100.0	92.9
20	84.5	99.5	93.6
30	76.0	93.6	96.6
40	68.0	91.6	100.0
50			94.4
60			93.6

GT activity is experessed as per cent of the maximum activity in each liver tissue.
Suspending medium was added to a given amount of liver tissue to yield homogenate in a concentration of 2.5 per cent.

time-period of homogenization to give the maximum GT activity in each amount of liver tissue was connected with a smooth curve, and a graph related to the liver amount to be homogenized and the suitable duration period of time for homogenization was prepared. According to the graph thus obtained the liver tissues were homogenized. Twenty-five to seventy-five mg. liver weight was the suitable amount for obtaining a consistent result under the present condition of homogenization.

Effect of Magnesium Ion : When magnesium chloride was added to the incubation mixture to give a concentration of 10 μ moles per 1.6 ml., the maximum GT activity was obtained. This concentration of magnesium chloride did not form turbidity to interfere with the reading of the fluorescence of 4-MU when an alkali was added on measurement of the fluorescence.

Effect of pH of Incubation Mixture : Incubation mixtures for 4-MU glucuronide synthesis with various pH ranging from 4.5 to 8.5 were prepared using Tris (hydroxy methyl) aminomethene (Tris) and hydrochloric acid to give a final concentration of 0.1 M. Then, 4-MU glucuronide formations at these pH were estimated. On the other hand, incubation mixtures containing a sufficient amount of 4-MU glucuronide in place of 4-MU and UDPGA and each having a different pH were similarly prepared. Hydrolyses of 4-MU glucuronide at these pH by the β-glucuronidase in the mouse liver homogenate were also investigated. Both results are illustrated in Fig. 1. At pH 7.4 the velocity of 4-MU glucuronide synthesis was close to its maximum, whereas that of the hydrolysis was negligible at the same pH. Hence, by selecting 7.4 as the pH of the incubation mixture, the interfering effect of the β-glucuronidase in the homogenate on the formation
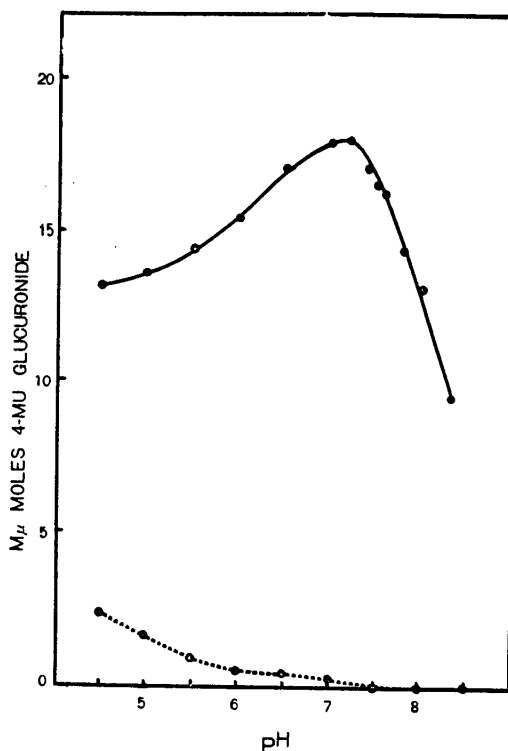
Fig. 1. Effects of pH on the velocity of 4-MU glucuronide synthesis and hydrolysis by mouse liver homogenate. ●———●, 4-MU glucuronide synthesized/10 mg. wet liver wt. /10 min. ●-------●, 4-MU glucuronide hydrolyzed/10 mg. wet liver wt. /60 min.

of 4-MU glucuronide could be eliminated even if saccharic acid, an inhibitor of β-glucuronidase, was not added to the mixture. In order to further confirm this fact, the amount of 4-MU glucuronide formed at pH 7.4 in 10 minutes of incubation in the presence of saccharic acid in 0.01 M was estimated indirectly from the decrease in fluorescence of 4-MU following the incubation. The amount of 4-MU glucuronide formed in the similar mixture without saccharic acid was also determined indirectly and served as a control. This result indicated that both incubation mixtures with or without saccharic acid produced an almost equal amount of 4-MU glucuronide. Accordingly, it was concluded that the possible effect of the β-glucuronidase in the homogenate on the formation of 4-MU glucuronide was negligible in the present assay.

Substrate Concentration and Reaction Velocity of 4-MU Glucuronide Formation : Kinetic studies of enzymatic 4-MU glucuronide formation related to the concentration of substrates, 4-MU and UDPGA, were made in order to determine

the optimum substrate concentrations so that the reaction could proceed linearly at its maximum velocity. The amount of 4-MU glucuronide formed in 10 minutes of incubation under the conditions of this experiment was regarded as an initial velocity as described hereafter. The reaction velocities of 4-MU glucuronide formation at varying concentrations of 4-MU in the presence of a sufficient amount of UDPGA and those at varying concentrations of UDPGA in the presence of a sufficient amount of 4-MU were determined (Figs. 2 and 3). Incidentally, the Michaelis-Menten constant was calculated to be 6.0×10^{-6}M for
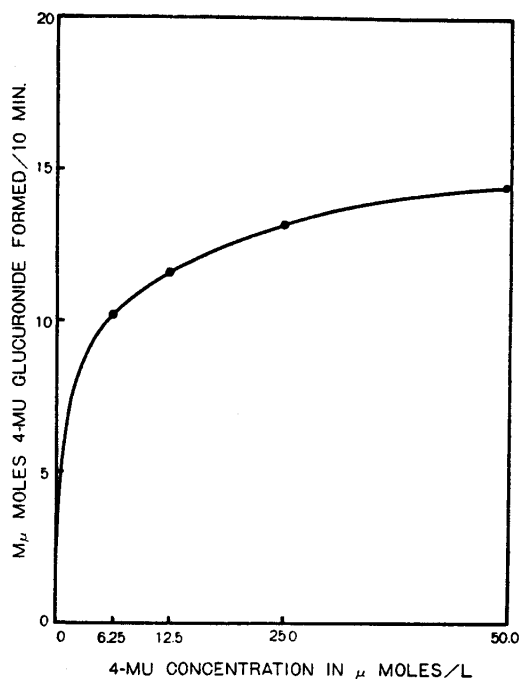
Fig. 2. Effect of 4-MU concentration on the velocity of 4-MU glucuronide formation by mouse liver homogenate. The velocity is expressed in mμ moles 4-MU glucuronide formed by 10 mg. liver per 10 minutes of incubation at 37°C. The incubation systems consisted of 0.1 μ mole of UDPGA, 50 μ moles of Tris buffer (pH 7.4), 10 μ moles of magnesium chloride, homogenate equivalent to 10 mg. liver weight, and each of varying amounts of 4-MU, 0.0125 to 0.1 μ mole, in a total volume of 2.0 ml.

4-MU and 2.5×10^{-6}M for UDPGA by a graphical work plotting the reciprocal of velocity against the reciprocal of the substrate concentration giving the velocity. As a result of this experiment, adequate concentrations of these substrates were selected for the standardization of the incubation system (Table 1). In this regard, it was necessary to add an excess amount of UDPGA compared with that of 4-MU, because varying degrees of decomposition of UDPGA during
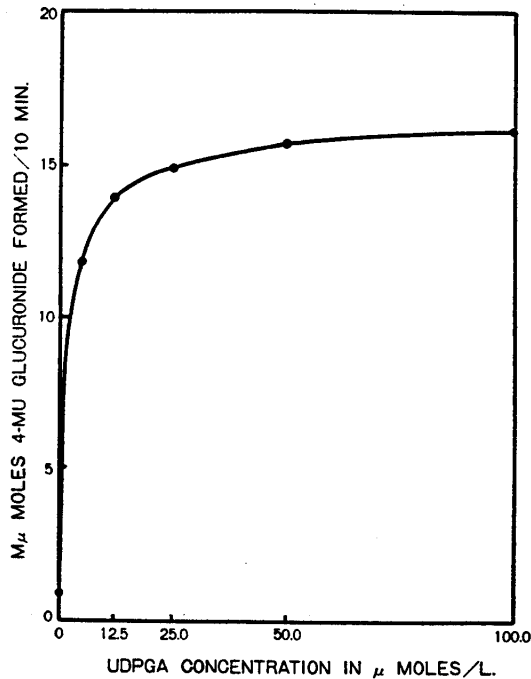
Fig. 3. Effect of UDPGA concentration on the velocity of 4-MU glucuronide formation by mouse liver homogenate. The velocity is expressed in mμ moles 4-MU glucuronide formed by 10 mg. liver per 10 minutes of incubation at 37°C. The incubation systems consisted of 0.05 μ mole of 4-MU, 50 μ moles of Tris buffer (pH 7.4), 10 μ moles of magnesium chloride, homogenate equivalent to 10 mg. liver weight, and each of varying amounts of UDPGA, 0.01 to 0.2 μ mole, in a total volume of 2.0 ml.

the incubation with the homogenate were observed, depending on the nature of the homogenate used.

Time Course of 4-MU Glucuronide Formation: Time courses of 4-MU glucuronide formation in the mixtures, each with a different amount of liver homogenate, were estimated. Two typical time courses of them are indicated in Fig. 4. In the case of Curve A observed in the system containing a relatively large amount of liver homogenate compared with that in the system for Curve B, the reaction proceeded almost linearly, maintaining almost its maximum velocity, for at least 10 minutes, whereas in the case of Curve B the velocity decreased with time in course of incubation. In order to clarify the cause of this decrease, incubation mixtures, each containing the same amount of liver homogenate as that in the system for Curve B, were preincubated respectively for 5, 10, 15, and 20 minutes adding neither 4-MU nor UDPGA to the mixtures. At the end of each preincubation period, a sufficient amount of 4-MU and
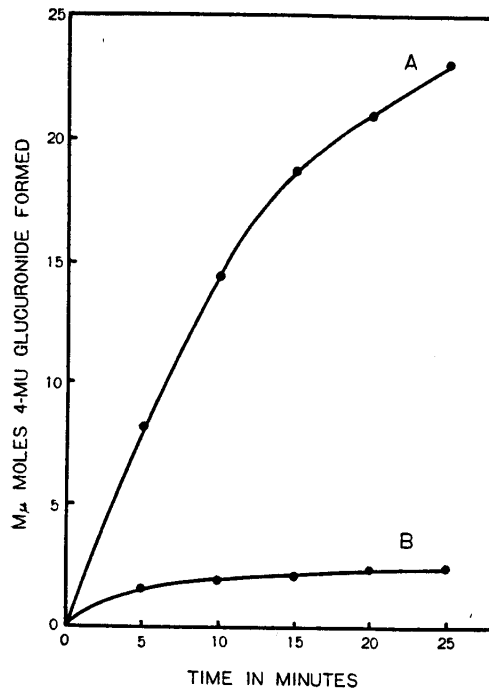
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Fig. 4. Time courses of 4-MU glucuronide formation by mouse liver homogenates. **A**, homogenate equivalent to 10 mg. liver weight in 1.6 ml. incubation mixture; **B**, homogenate equivalent to 3.6 mg. liver weight in 1.6 ml. incubation mixture. Other additions as in Table 1. Incubation at 37°C.

UDPGA was added to the mixture, which was then incubated for 5 minutes. The results of this experiment indicated that the amount of 4-MU glucuronide formed decreased with the time of preincubation at a rate of approximately fifty per cent per 5 minutes of preincubation. In view of these results, it was considered that the decrease in the velocity with time in the case of Curve B was attributed to an inactivation of the enzyme in course of the incubation and that the inactivation could be eliminated to some extent, as in the case of Curve A, by adding a larger amount of the liver homogenate as GT source. For the purpose of standardizing the present assay, an incubation system containing an equivalent amount of the homogenate to 10 mg. liver weight in a total volume of 1.6 ml. was defined as a standard system (Table 1), being 10 minutes at 37°C as a standard incubation period.

Enzyme Concentration and Reaction Velocity of 4-MU Glucuronide Formation: The reaction velocity of 4-MU glucuronide formation which was related to the amount of liver homogenate added to the system as GT source was determined. As shown in Fig. 5, an S-shaped curve was obtained. The reduction in reaction velocity, indicated with the arrow sign in the figure, was considerd to
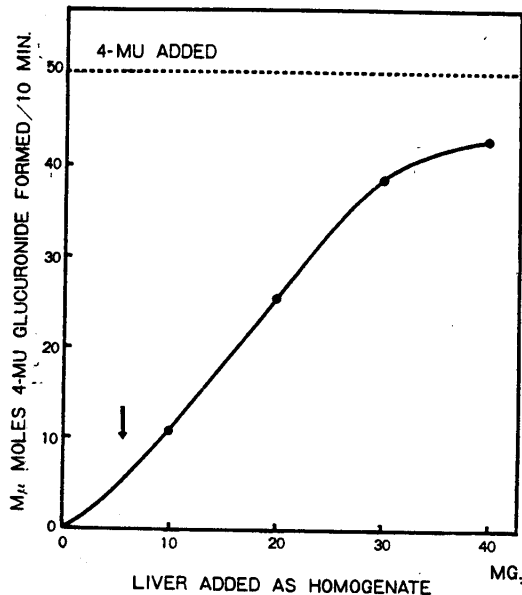
Fig. 5. The velocity of 4-MU glucuronide formation related to the amount of mouse liver added as homogenate. The velocity is expressed in mμ moles 4-MU glucuronide formed per 10 minutes of incubation at 37°C. Incubation systems consisted of 0.05 μ mole of 4-MU, 0.1 μ mole of UDPGA, 50 μ moles of Tris buffer (pH 7.4), 10 μ moles of magnesium chloride, and homogenate equivalent to each of varying amounts of liver, 10 to 40 mg., in a total volume of 2.0 ml.

be due to the inactivation of the enzyme during the incubation because of excess dilution of the homogenate. As it was indicated above that the inactivation was not involved in the incubation of the standard system containing homogenate equivalent to 10 mg. liver weight in a volume of 1.6 ml., it was reasonable that the reaction velocity of 4-MU glucuronide formation in the standard mixture would actually increase in direct proportion to the enzyme activity of the homo-genate added until approximately seventy per cent of the added 4-MU was converted to its glucuronide. Further experiments performed according to the standard assay indicated that the GT activity of 10 mg. of mouse liver was within the range of a linear relationship between the enzyme activity and the velocity of 4-MU glucuronide formation. In addition, this amount of mouse live tissue contained very little 'activator' of glucuronide formation[13], and thus it was possible, by using the standard system, to estimate GT activity itself without an appreciable effect of the 'activator' on the GT activity.

Effect of Protein Precipitation with Chloroform on the Recovery of 4-MU Glucuronide as 4-MU: Mixtures, each containing a certain amount of 4-MU glucuronide and a different amount of liver homogenate, were washed with

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incubation was noted. Other two aliquots of the solution, one after extraction of the unconjugated 4-MU with chloroform and the other as it was, were subjected to the hydrolysis with β-glucuronidase, and the increase in fluorescence following the hydrolysis of thus formed 4-MU glucuronide was determined. The restoration of fluorescence following the enzymatic hydrolysis of the 4-MU glucuronide in different concentrations of total 4-MU was thus obtained. From these two kinds of restoration of fluorescence, simultaneous equations were set up, and by solving the equations the degree of hydrolysis of 4-MU glucuronide with β-glucuronidase was calculated to be 98 per cent. These complicated manipulations were necessary, because other conjugates of 4-MU might be formed in the mixture following the initial incubation and they would interfere with the restoration of fluorescence by the hydrolysis with β-glucuronidase, although the formation of other 4-MU conjugates was actually to a lesser extent.

When 4-MU and the mouse liver homogenate in which the endogenous UDPGA was entirely decomposed by the preincubation without an addition of substrate were incubated with potassium sulfate and adenosine triphosphate using the system synthesizing sulfuric acid esters of phenols described by DE MEIO and others[14], a reduction in the fluorescence of 4-MU, presumably resulting from the formation of 4-MU sulfate[11], was observed. This reduction in fluorescence failed to be restored by the hydrolysis with the β-glucuronidase used in the present study; hence it was considered that the β-glucuronidase had no sulfatase activity.

The 4-MU used in the present study was incubated with β-glucuronidase and the fluorescence was determined before and after the incubation. No increase in the fluorescence following this incubation was observed, and thus it was confirmed that the 4-MU was free from its glucuronide.

Effect of Glycine Buffer on Fluorescence of 4-MU : When 3.0 ml. of 0.2 M glycine buffer, pH 10.42, was added to 2.0 ml. of 4-MU solution at pH 7.0, the fluorescence was fairly stabilized almost at the maximum. Since, however, quenching of the fluorescence was observed to some extent following irradiation of ultraviolet light, the fluorescence was measured in every determination within one minute after the addition of the glycine buffer and immediately after irradiating ultraviolet light to the solution.

Standardization of Fluorometry : The fluorescence of 4-MU activated by ultraviolet light (365 mμ) was measured at a wave length of 460 mμ with the Beckmann fluorescence spectrophotometer against the fluorescence of a standard quinine sulfate solution. In each determination of the fluorescence of 4-MU, 3.45 μg. quinine sulfate per ml. 0.1 N sulfuric acid was used as the standard for setting the fluorometer at 50 per cent for convenience. This standard quinine sulfate solution was equivalent in fluorescence to 1 mμ mole 4-MU per ml.

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0.2 M glycine buffer, pH 10.42. There was a linear relationship between the concentration and the fluorescence of 4-MU over the range of 0 to 2 mμ moles 4-MU per ml. similar glycine buffer.

Estimation of UDPGA Content of Liver Tissue : When 4-MU was incubated with mouse liver homogenate without adding UDPGA to the system, a slight decrease in the fluorescence was observed. This decrease in the fluorescence was restored by the hydrolysis with β-glucuronidase; hence it was apparent that the restored fluorescence was attributed to an 'endogenous' formation of 4-MU glucuronide. It was necessary to confirm whether the glucuronic acid moiety of thus formed 4-MU glucuronide was derived from UDPGA, since another pathway for 4-MU glucuronide formation without involving UDPGA and the microsomal GT had been suggested by Arias[16]. When the two kinds of incubation mixture for the synthesis of 4-MU glucuronide, with and without UDPGA in the system, were each incubated with glucuronic acid, an inhibitor of microsomal glucuronide formation[2], the glucuronic acid slightly but equally inhibited the 4-MU glucuronide formation in both cases. Therefore, the 'endogenous' formation of 4-MU glucuronide was considered to be attributed to the UDPGA which was contained in the homogenate used as GT source. On the basis of these observations, it was considered to be reasonable to estimate the amount of UDPGA contained in the homogenate used as the enzyme source directly from the amount of 4-MU glucuronide formed 'endogenously' by using the standard GT assay system in which the addition of UDPGA was omitted (Table 1). In this case, the results of tentative and preliminary experiments indicated that other conditions of the assay, such as those of homogenate preparation, incubation, and fluorometry, were permitted to be the same as those in the GT assay.

As shown in Fig. 6, the time course of the 'endogenous' 4-MU glucuronide formation indicated that the glucuronide formation was so rapid as to reach up to 98.5 per cent of the maximum in 5 minutes and completed in 10 minutes of incubation, and neither increase nor decrease in the amount of the glucuronide formed was demonstrated thereafter. This indicated that the successive formation of UDPGA from the uridine diphosphate glucose which was supposed to be contained in the homogenate used was not significant in the present *in vitro* experiment. It was also indicated that the formed glucuronide was not hydrolyzed in an appreciable amount by the β-glucuronidase in the homogenate in course of the incubation. These two results were further supported by the fact that a similar curve was traced even when the incubation was performed in the presence of saccharic acid. However, since the breakdown of endogenous UDPGA in course of the incubation of mouse liver homogenate was observed as described previously in this paper, recoveries of added UDPGA as 4-MU
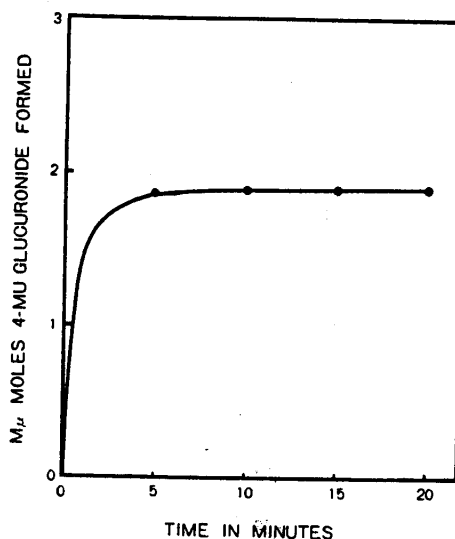
Fig. 6. Time course of 'endogenous' 4-MU glucuronide formation by mouse liver homogenate. Incubation mixtures without an addition of UDPGA, each containing homogenate equivalent to 10 mg. liver weight, were incubated for varying periods of time at 37°C; other additions were as in Table 1. The GT activity of the homogenate used was 14.8 mμ moles 4-MU glucuronide formed per 10 mg. wet liver weight per 10 minutes.

glucuronide were examined by using various mouse liver homogenates. In addition, the effect of the GT activity of each homogenate on the recovery of added UDPGA was studied (Fig. 7). In all cases of A, B, and C, the amount of 4-MU glucuronide formed increased in proportion to the amount of UDPGA added, provided the amount (mμ moles) of UDPGA added did not exceed fifty per cent of the corresponding GT activity (mμ moles). In this range of a linear relationship between the amount of UDPGA added and that of 4-MU glucuronide formed, the recovery of added UDPGA as 4-MU glucuronide by normal mouse liver homogenate, Cases A and B in Fig. 7, was almost constant and not affected by the GT activity of the homogenate used. The mean recovery was calculated to be 96.9 per cent. However, when the homogenate prepared from the liver of carbon tetrachloride-injured mouse was used, Case C in Fig. 7, the recovery of added UDPGA was rather small irrespective of high GT activity of the homogenate. Since the same result was obtained in a similar system with saccharic acid, this decrease in the recovery of added UDPGA was attributed to the enhanced decomposition of UDPGA, during the incubation, by the homogenate prepared from the impaired liver.

Applicabilities of These Methods to Human Liver: The time course of 4-MU glucuronide formation obtained by using the standard incubation system
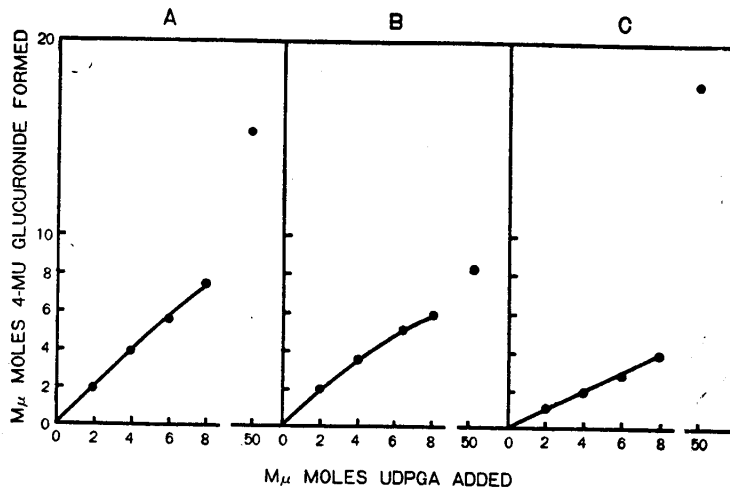
Fig. 7. Recoveries of UDPGA as 4-MU glucuronide by mouse liver homogenates with varying GT activities. **A**, normal mouse liver homogenate; **B**, normal mouse liver homogenate preincubated without adding the substrates for 15 minutes at 37°C; **C**, liver homogenate from the mouse in 24 hours after a subcutaneous injection of carbon tetrachloride (0.01 ml. per g. body weight). All other additions, except UDPGA, were as in Table 1. Incubation for 10 minutes at 37°C. GT activity of the homogenate used is indicated by the isolated solid circle at the position corresponding to the amount of 4-MU glucuronide formed in 50 mμ moles of UDPGA. The values were corrected for the 'endogenous' 4-MU glucuronide formation by the homogenate used.

with human liver homogenate indicated that the reaction proceeded linearly with time over 10 minutes of incubation. Namely, human liver GT was as stable as mouse liver GT. Moreover, it was indicated that the activity of normal human liver GT was similar to that of normal mouse liver GT. In this connection, it was considered that the procedures and conditions of GT assay established by the experiments using mouse liver were also applicable to the determination of human liver GT activity. Although the UDPGA content of normal human liver tissue was approximately three times that of normal mouse liver tissue, the UDPGA content (mμ moles) of human liver tissue was, actually in most cases, less than fifty per cent of the GT activity (mμ moles) of the tissue. From this fact it was considered to be reasonable to estimate the UDPGA amount in human liver homogenate directly from the amount of 4-MU glucuronide formed 'endogenously' by the homogenate used as GT source. The time course of 'endogenous' 4-MU glucuronide formation by human liver homogenates was indicated as similar to that by mouse liver homogenates; the glucuronide formation reached up to 92 per cent of the maximum in 5 minutes and completed in 10 minutes of incubation. This apparently indicated that the reaction of the 'endogenous'

glucuronide formation by human liver homogenate was completed as rapidly as that by mouse liver homogenate; thus, the method accomplished by the experiments by means of mouse liver was also applicable to the estimation of UDPGA content of human liver. On the other hand, even when an impaired human liver gave a result of considerably small amount of UDPGA compared with that of normal human liver in the determination according to the present direct method, the velocity of UDPGA decomposition by the homogenate of the impaired human liver was smaller than that by normal mouse liver homogenates. Accordingly, in the direct estimation of UDPGA content of human liver, the amount (mμ moles) of the 4-MU glucuronide formed 'endogenously' by the homogenate was regarded as representing the amount (mμ moles) of the UDPGA contained in the homogenate whether or not liver injuries were present.

Final Standardization of the Present Methods and Errors in the Results: The incubation system for the determination of liver GT activity was standardized (Table 1) from the results of the present experiments. By means of this standard system the measurement of liver GT activity was performed as follows. Immediately after obtaining a liver tissue by percutaneous needle biosy under direct vision on peritoneoscopy, a portion of it, weighing 25 to 40 mg., was placed on a piece of filter paper in a well-iced small glass vessel and brought to the experimental room. The blood attached to the tissue was removed on the filter paper and the tissue was weighed accurately by a torsion balance within 30 seconds. The tissue was quickly transferred into an ice-cold 5 ml. teflon homogenizer, to which then the ice-cold, alkaline, isotonic potassium chloride was added to yield a 2.5 per cent homogenate. Homogenization was performed in the ice water at a speed of 600 r. p. m. until giving the maximum GT activity. The time required for the homogenization depended on the amount of the liver tissue. 0.4 ml. aliquot of the homogenate was added to the substrate solution (Table 1) in a glass-stoppered 10 ml. test tube which had been warmed at 37 °C beforehand, and then the mixture was incubated for 10 minutes at 37 °C. In order to obtain a consistent result, the incubation had to be started within 5 minutes after obtaining the tissue. At the end of this incubation period, the mixture was washed twice with 5 ml. of chloroform by shaking vigorously with hand, and then centrifuged at 1500 r. p. m. for 10 minutes. 0.4 ml. aliquot of the supernatant was transferred to a test tube, and further 1.6 ml. of 0.1 M Tris buffer, pH 7.0, containing 32 units of β-glucuronidase was added. A similar sample without β-glucuronidase served as a control. Both samples were incubated for 60 minutes at 37 °C. After the incubation, 3.0 ml. of 0.2 M glycine buffer, pH 10.42, was added and the fluorescence was measured. When the β-glucuronidase was fluorescent, if any, correction was made for the fluorescence. From the difference in fluorescence between the control and the enzymatically hydroly-

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zed mixture, the amount of 4-MU liberated by the hydrolysis was obtained. The amount (mμ moles) of the liberated 4-MU was used as an expression of the amount (mμ moles) of the 4-MU glucuronide formed in the initial incubation, because it was established in the present experiment that the hydrolysis of 4-MU glucuronide with β-glucuronidase was almost complete. Hence GT activity was expressed as mμ moles of 4-MU glucuronide formed per 10 mg. wet liver weight per 10 minutes under the above conditions. The standard deviation from the mean activity of GT in determinations on eight aliquots of the same homogenate was 2.0 per cent, which was smaller than that, 3.1 per cent, determined on seven pieces of the same liver tissue.

UDPGA content of liver tissue was determined directly from the amount of 4-MU glucuronide formed 'endogenously' by the liver homogenate according to the same procedures as those for the determination of GT activity and by using the standard incubation system for GT assay except the addition of UDPGA as shown in Table 1. UDPGA content of liver tissue was expressed as mμ moles of 4-MU glucuronide formed per 10 mg. wet liver weight in 10 minutes of incubation. This direct assay method of UDPGA determination was not adequate, however, when the amount (mμ moles) of 'endogenously' formed 4-MU glucuronide by liver homogenate was more than fifty per cent of the GT activity (mμ moles) of the homogenate and also when the velocity of UDPGA decomposition by the liver homogenate was greater than that by normal mouse liver homogenate; thus, the assay in these cases was necessary to be performed according to the indirect assay method described in the foot note to Table 1.

In 15 normal male adult mice the mean GT activity of liver tissue and the standard deviation therefrom were 15.38 and 0.96 mμ moles respectively, and those values for liver UDPGA content were 1.28 and 0.68 mμ moles. In 5 normal human subjects those values for liver GT activity were 13.52 and 0.91 mμ moles and those for liver UDPGA content were 3.89 and 0.78 mμ moles.

DISCUSSION

Because of an unstable nature of the GT in the liver tissue removed or in the homogenate prepared therefrom, loss of GT activity was observed in several processes of the assay. ARIAS[9] remarked that the expression of GT activity in terms of wet liver weight was unreasonable because of the probable loss of the activity in weighing process and of inaccuracy of weighing itself. However, it was considered to be rather unfavorable and not advisable to subject an estimated amount of liver tissue to the assay as in his method, because the results of the present experiments indicated that the inactivation of GT during homogenization and incubation, the loss of formed 4-MU glucuronide into precipitated protein, and the effect of the 'activator' of glucuronide formation, contained in

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the liver homogenate, on the GT activity of the homogenate varied to a considerable degree, depending on the amount of the liver tissue subjected to the assay. Since it was established to be reasonable to determine accurate amount of small liver tissue without an appreciable loss of the enzyme activity under the conditions described in the preceding chapter, it was reasonable to perform the assay by using the liver tissue of which the exact amount was determined.

Since in the present study the volume of the homogenizer available was limited, more than 25 mg. of liver tissue was necessary for the preparation of homogenate in order to obtain a consistent result of the assay without an appreciable loss of the enzyme activity in the process of homogenization. However, it was suggested that a satisfactory result would be also obtained by using a smaller amount of liver tissue, less than 25 mg., if a teflon homogenizer with a volume of less than 5 ml. was available.

The addition of saccharic acid to the initial incubation mixture in order to inhibit the β-glucuronidase activity in the homogenate as GT source should be avoided, because the present method involved a second incubation with β-glucuronidase. Fortunately, the possible effect of the β-glucuronidase in the homogenate on the formation of 4-MU glucuronide could be eliminated to a satisfactory degree by reducing the incubation period of time to 10 minutes at pH 7.4 even if saccharic acid was not added.

ARIAS[9] reported that there was never more than 80 per cent restoration of fluorescence after hydrolysis of 4-MU glucuronide with β-glucuronidase. In the present study, however, the hydrolysis of 4-MU glucuronide with β-glucuronidase was almost complete, 98 per cent. The difference between these two results could not be well explained.

The present method for the direct estimation of UDPGA in liver homogenate from the amount of the 'endogenously' formed 4-MU glucuronide by the homogenate was convenient in that it involved less manipulation and was more sensitive than that by means of phenolphthalein as a glucuronide receptor[16]. However, this method also had a limitation in that it could not be clarified in each determination to what degree the loss of UDPGA in the homogenate, arising from its decomposition by the homogenate during incubation, was involved, although it was indicated that in cases of human liver the loss was not in an appreciable degree. In this connection, when the amount of liver tissue available is limited as in case of the human liver tissue obtained by needle biopsy, the present direct method is considered as being a satisfactory one.

The GT activity of normal human liver determined according to the present method was close to that determined by ARIAS according to his original method[9], although the expression of the activity between these two methods was somewhat different.

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SUMMARY

An attempt was made to apply the method devised by ARIAS for the determi-
nation of liver glucuronyl transferase activity using 4-methyl umbelliferone as
a glucuronide receptor to the small amounts of liver tissue obtained by needle
biopsy. This was accomplished by studying the kinetics of enzymatic 4-methyl
umbelliferone glucuronide formation by means of mouse liver homogenates. The
improved method was proved to be applicable to human liver and gave a satis-
factory result.

In addition, an assay method for the estimation of liver uridine diphosphate
glucuronic acid content from the amount of 4-methyl umbelliferone glucuronide
formed from the uridine diphosphate glucuronic acid contained in the liver homo-
genate used as a source of glucuronyl transferase was studied, and as a result
it was proved to be also applicable to the small amounts of human liver tissue.

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