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Abstract

The effect of the boiled liver extract on the velocity of 4-methyl umbelliferone lucuronide formation by mouse liver homogenate was studied. The results were as follows: 1. Addition of mouse or rat liver boiled extract to the complete system for the glucuronide formation produced an increase in the velocity of the glucuronide formation. 2. The boiled liver extract produced the increase in the velocity of the glucuronide formation not as a substrate but as an activator. 3, The activator in the boiled liver extract was relatively heat stable, acid labile, and precipitated as a fairly ethanol-soluble barium salt. The solution of the activator partially purified by ethanol fractionation of the barium salt indicated its absorption maximum at 262 mμ. These results suggested that the most poible substance in the boiled liver extract responsible for the activation of 4methyl umbelliferone glucuronide formation might be a sugar nucleotide.

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ENZYMATIC STUDIES OF GLUCURONIDE FORMATION IN IMPAIRED LIVER

II. ACTIVATING EFFECT OF BOILED LIVER EXTRACT ON LIVER GLUCURONIDE FORMATION

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DUTTON and STOREY[1], in their earlier work on the glucuronide synthesis in liver suspensions, observed that the glucuronide formation could not be demonstrated unless a boiled extract of liver was added to the system. They suggested that an unknown compound in the boiled liver extract might be responsible for the glucuronide synthesis. In their subsequent work they indentified the compound as uridine diphosphate glucuronic acid (UDPGA)[2]. It is generally accepted that glucuronic acid is transferred from UDPGA to various receptors to form glucuronides and that this reaction involves a liver microsomal enzyme, glucuronyl transferase (GT)[2,3,4,5]. On the basis of these observations, the boiled liver extract has been used merely as a UDPGA source for glucuronide synthesis[5,6,7].

In the present work, studies were made of an activating effect of the boiled liver extract on the glucuronide formation in the presence of sufficient amounts of UDPGA, and an attempt was made to clarify the nature of the activating effect.

MATERIALS AND METHODS

Boiled extract of mouse or rat liver was prepared according to the method described by DUTTON and STOREY[1]. Usual concentration of the extract was 3 g. liver in 10 ml. 0.5 per cent potassium chloride and, when necessary, a higher concentration, 5 g. liver in 10 ml. water, was also employed. There was no significant difference in the activating effect between the boiled extract of mouse liver and that of rat liver; hence mouse or rat liver boiled extract was used in a series of experiment.

Glucuronide formation was estimated by means of 4-methyl umbelliferone (4-MU), a highly fluorescent substance, as a glucuronide receptor according to a modification[8] of the method of ARIAS[9]. As a GT source mouse liver

homogenate, 2.5 per cent in alkaline, isotonic, potassium chloride[10] as a suspending medium, was used. The incubation system for 4-MU glucuronide formation consisted of 0.05 μ mole of 4-MU, 0.1 μ mole of UDPGA (90 per cent pure as the ammonium salt, Sigma Chemical Company), 10 μ moles of magnesium chloride, 25 μ moles of Tris buffer, pH 7.4, and 0.4 ml. of the homogenate (equivalent to 10 mg. liver weight). Moreover, 0.4 ml. of boiled liver extract or 0.4 ml. of the solution to be tested for the activating effect was added to the system, except control, and the final volume was adjusted to 1.6 ml. by adding water. The mixture was incubated for 10 minutes at 37°C. The 4-MU glucuronide, a non-fluorescent substance, formed following the incubation was hydrolyzed with bacterial β-glucuronidase. From the increase in fluorescence following the enzymatic hydrolysis, the amount of 4-MU glucuronide formed in the initial incubation was estimated. The velocity of 4-MU glucuronide formation was expressed in mμ moles 4-MU glucuronide formed per 10 minutes of incubation under the above conditions. The results of preliminary experiments indicated that the activating effect of the homogenate added to the system as GT source was negligible; thus, the velocity of 4-MU glucuronide formation by the homogenate in the control mixture was used as a control in comparison with the activating effect.

The ratio of the velocity of 4-MU glucuronide formation in the system containing the solution to be tested for the activating effect to the velocity in the control system was referred to as an activation factor of the solution. This factor was used as a reference of the degree of the activating effect. Since the velocity of 4-MU glucuronide formation varied to some extent in individual liver tissue, the velocities obtained in a series of experiment using the same liver homogenate as the enzyme source were compared with each other.

RESULTS

Velocities of 4-MU glucuronide formation in the presence of varying amounts of UDPGA or boiled liver extract were determined (Fig. 1). The increase in the velocity of 4-MU glucuronide formation due to the increase in the amount of added boiled liver extract was far greater than that due to the increase in the amount of added UDPGA. The addition of a boiled extract of mouse skeletal muscles, similarly prepared as in the case of boiled liver extract, failed to present any increase in the velocity of 4-MU glucuronide formation. The fluorescence of the boiled liver extract itself was almost negligible even after hydrolysis of the extract with β-glucuronidase.

Since another pathway for 4-MU glucuronide formation without involving UDPGA and the microsomal GT had been suggested by ARIAS[11], an experiment was performed in order to clarify whether the boiled liver extract was respon-

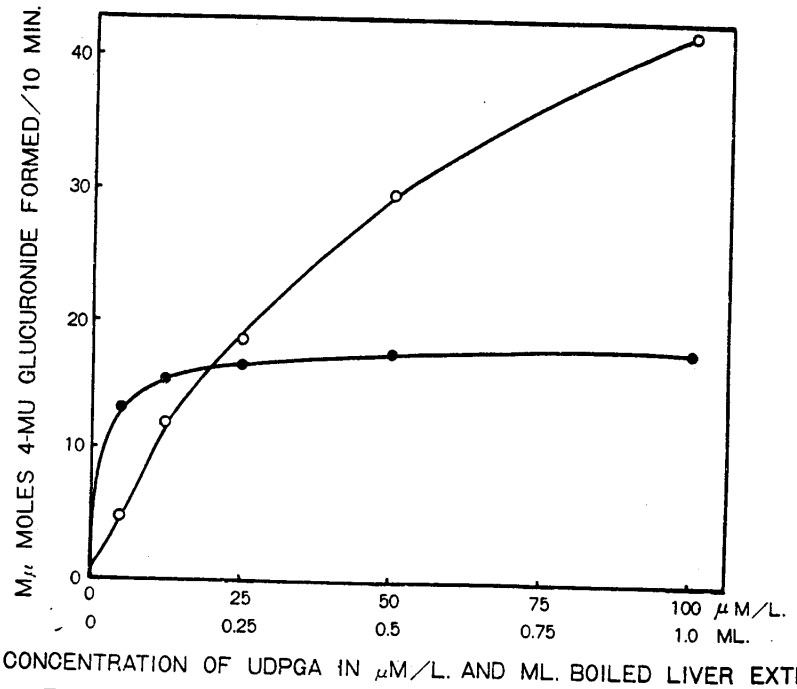

Fig. 1. Velocities of 4-MU glucuronide formation in the presence of varying amounts of UDPGA or boiled liver extract. ●——●, in the case of addition of UDPGA; ○——○, in the case of addition of boiled extract of rat liver, addition of UDPGA was omitted.

sible for the increased formation of 4-MU glucuronide as an activator or as a substrate. When a boiled liver extract prepared from the rat liver homogenate preincubated for 20 minutes at 37°C was incubated with the mouse liver homogenate in the presence of 4-MU, 4-MU glucuronide formation was not observed in an appreciable amount; hence it was apparent that the extract did not contain any substrate for the glucuronide formation. Nevertheless, in the presence of a sufficient amount of UDPGA, the extract also caused an additional increase in the velocity of 4-MU glucuronide formation as compared with the velocity in the system with a sufficient amount of UDPGA and without the extract. These results are summarized in Fig. 2.

As indicated in the previous report[8], the GT of mouse liver homogenate was slightly inactivated in the course of incubation, and this inactivation of the enzyme, although in a negligible amount in the present system, was avoided to some extent by a greater amount of the homogenate added to the system. In this connection, it was necessary to examine whether the increased formation of 4-MU glucuronide by the boiled liver extracts added was due to the elimination of the enzyme inactivation by the protein contained in the added extract, although the protein content was in a trace quantity. In order to avoid the effect

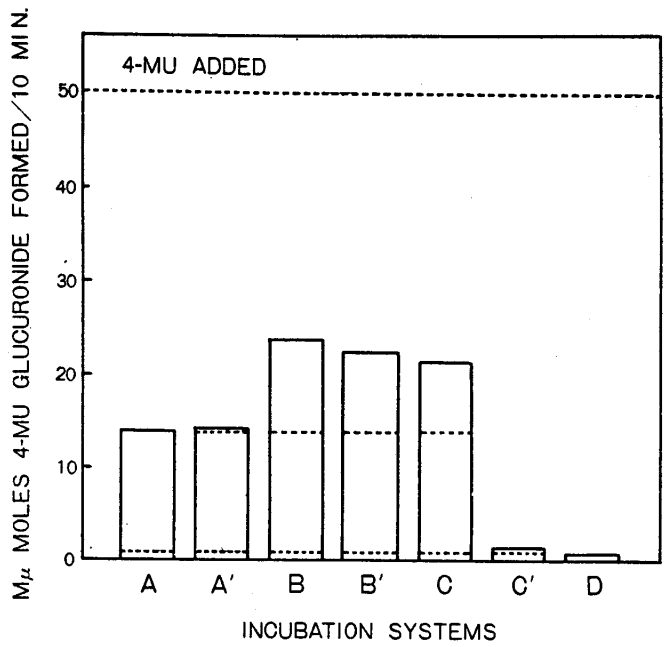

Fig. 2. Effects of various boiled extracts of rat liver on the velocity of 4-MU glucuronide formation. In the following combinations, UDPGA and the boiled liver extract were added to the incubation system to give a final volume of 1.6 ml. **A**, 0.1 μ mole of UDPGA (control); A', 0.2 μ mole of UDPGA; **B**, 0.1 μ mole of UDPGA and 0.4 ml. of boiled liver extract; **B'**, 0.4 ml. of boiled liver extract alone; **C**, 0.1 μ mole of UDPGA and 0.4 ml. of preincubated boiled liver extract; **C'**, 0.4 ml. of preincubated boiled liver extract alone; **D**, without both UDPGA and boiled liver extract (control for the 'endogenous' 4-MU glucuronide formation by the liver homogenate used as GT source).

of the protein, 5 ml. aliquot of the boiled mouse liver extract of the higher concentration was dialyzed against distilled water, 5 ml., for 24 hours at a temperature of 0 to 4°C. Each 0.4 ml. aliquot of the dialyzate, diffusate, and original boiled liver extract was assayed for the activating effect. Activation factors of the dialyzate, diffusate, and original extract were 1.90, 1.85, and 2.44 respectively. The activating effect of the dialyzed boiled liver extract on the 4-MU glucuronide formation was reduced considerably as compared with that of the original extract, while the activating effect was recovered from the diffusate to an appreciable degree. A similar experiment was also performed by means of charcoal adsorption. To 15 ml. aliquot of the boiled extract prepared from 10 g. rat liver in 20 ml. of water, 0.5 g. of the acid-washed charcoal was added and the mixture was allowed to stand for 15 minutes at 15°C, and then the charcoal was separated by filtration. After the charcoal was washed with water, the first elution from the charcoal was performed with 50 ml. of 50 per cent

ethanol followed by the second elution with 20 ml. of 20 per cent pyridine in water. Each of these two elutes was concentrated to 3 ml. *in vacuo* at 45°C and the pH was adjusted to 7.0 with sodium hydroxide. The elute with ethanol, elute with pyridine, filtrate, and original boiled liver extract were assayed for their activating effect; the activation factors of these four solutions were 2.88, 2.33, 1.19, and 2.41 respectively. Although the total recovery of the activating effect of the original extract from the filtrate and the elutes was not sufficient, the fact that the filtrate lost the activating effect and the effect was recovered from the elutes indicated evidently that an activator responsible for the activating effect was adsorbed on the charcoal and eluted with these agents. From these properties of the activator, it was suggested that inorganic phosphate, sugar phosphate, salt, and protein might be excluded from the probable activator. Thereupon, nucleotides remained as a most possible activator of the glucuronide formation.

On the basis of these resutls, an attempt was further made to concentrate the activator as a barium salt according to the procedures described by STOREY and DUTTON[2] for the isolation of UDPGA. In the experiment for this purpose, some modifications of the method were made because of fairly ethanol-soluble nature of the barium salt of the activator. 20 ml. aliquot of the boiled liver extract prepared from 10 g. mouse liver in 20 ml. of water was brought to a pH 2.0 with 20 per cent trichloroacetic acid followed by the addition of an equal volume of ethanol, and then the precipitate of glycogen and protein was centrifuged down. The supernatant solution was immediately brought to a pH 7.0 with sodium hydroxide and further to a pH 10.0 with saturated barium hydroxide. The barium salts were precipitated by adding ethanol to a total concentration of 80 per cent. The mixture was centrifuged and the supernatant almost free from barium salt was obtained (fraction I). The precipitate was dissolved in dilute hydrochloric acid and the solution was readjusted to a pH 8.2. The precipitate of the water-insoluble barium salts (fraction II) thus obtained was separated by centrifugation. The barium salts in the supernatant solution of pH 8.2 were precipitated by adding four volumes of ethanol. The precipitate of the water-soluble ethanol-insoluble barium salts (fraction III) was separated by centrifugation. Then, the pH of the supernatant solution decreased to 5.0. By readjusting the decreased pH to 8.2 with sodium hydroxide a small amount of precipitate of a fairly ethanol-soluble barium salt (fraction IV) was obtained, and the supernatant was almost free from barium salt (fraction V). All these procedures were carried out at a temperature of 0 to 4°C. After removing barium as barium sulfate from these fractions, the volume and the pH of each solution were adjusted to 1.0 ml. and to 7.0 respectively. The effects of these solutions and the original boiled extract on the velocity of 4-MU glucuronide

formation were studied (Fig. 3). The activation of 4-MU glucuronide formation was the largest in the fraction IV, being slight in the fractions I and III. Even

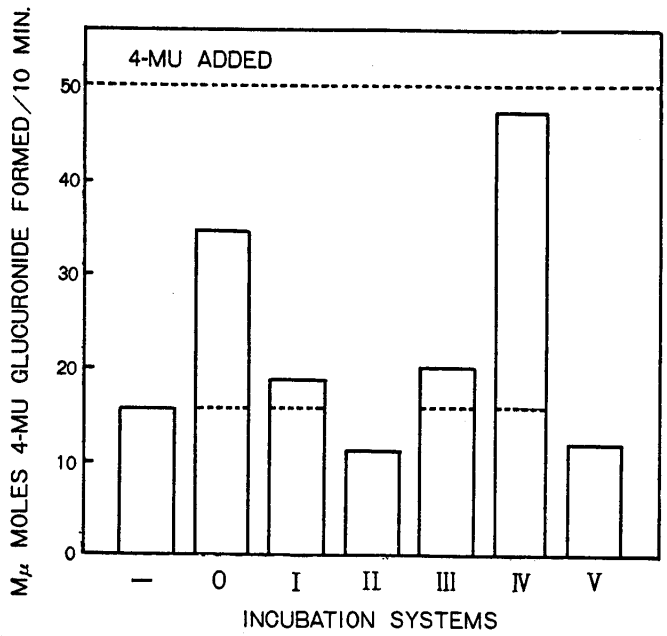

Fig. 3. Effects of the solutions obtained from boiled mouse liver extract by ethanol fractionations of the barium salts on the velocity of 4-MU glucuronide formation. The complete system with a sufficient amount of UDPGA was used, and the other additions were as follows: —, distilled water; **O**, original boiled liver extract; **I**, the initial fraction free from barium salt; **II**, the fraction of water-insoluble barium salt; **III**, the fraction of water-soluble ethanol-insoluble barium salt; **IV**, the fraction of fairly ethanol-soluble barium salt; **V**, the last fraction free from barium salt.

depressed glucuronide formation was observed in the fractions II and V. Although these decreases in 4-MU glucuronide formation were considered in part to be due to the presence of excess amount of the salt resulting from the repeated neutralizations, it could not be neglected that either substances with a quenching effect on the fluorescence of 4-MU or inhibitors of 4-MU glucuronide formation or of the hydrolysis of the thus formed glucuronide with β-glucuronidase might also be present in those solutions in a relatively high concentration. Incidentally, these solutions were analyzed for UDPGA[8], and the results indicated that UDPGA was almost exclusively detected in the fraction III. At any rate, it was apparent that the substance responsible for the activation of 4-MU glucuronide formation was relatively concentrated in the solution from the fraction IV. The absorption maximum of this solution was observed at 262 mμ. At this wave

length the optical density of the 200-fold diluted solution was 0.035 and that of the similarly diluted original boiled liver extract was 0.674. Hence the activation of 4-MU glucuronide formation per unit optical density by the solution from the fraction IV was calculated to be approximately thirty times that by the original boiled liver extract.

One of the three aliquots of the solution from the fraction IV was hydrolyzed in 1 N hydrochloric acid at 100°C for 15 minutes and other one was kept in 1 N hydrochloric acid at 0°C for 15 minutes, and then both were neutralyzed with 1 N sodium hydroxide. These three aliquots, after adjusting each volume to be equal with water, were assayed for the activation of 4-MU glucuronide formation. Activation factors of the solutions, hydrolyzed in hydrochloric acid at 100°C, kept in hydrochloric acid at 0°C, and treated without hydrochloric acid, were 0.23, 1.18, and 1.83 respectively. Consequently, by hydrolyzing the solution in 1 N hydrochloric acid at 100°C the solution lost the activating effect and even inhibited the 4-MU glucuronide formation. Inorganic phosphate was not detected[12] in these solutions before or after the hydrolysis.

DISCUSSION

Addition of the boiled extract of mouse or rat liver to the complete system for 4-MU glucuronide formation by mouse liver homogenate as GT source produced a remarkable increase in the glucuronide formation. It was confirmed that the boiled liver extract was responsible for this increase not as a substrate but as an activator.

The activator in the boiled liver extract was easily diffusable through a cellophane membrane, adsorbed on the acid-washed charcoal and eluted with 50 per cent ethanol or 20 per cent pyridine in water, precipitated as a fairly ethanol-soluble barium salt, and deprived of its activating effect by hydrolysis in 1 N hydrochloric acid at 100°C for 15 minutes. The maximum absorption of the solution containing the activator in a relatively high concentration was obtained at 262 mμ. From these results, nucleotides were considered to be the most possible activator of the glucuronide formation. Adenosine triphosphate and adenosine diphosphate were generally considered to be precipitated as the barium salts in the fraction II, and mononucleotides, diphosphopyridine nucleotide, and triphosphopyridine nucleotide were in the fraction III; therefore, these nucleotides might be also excluded from the possible activator. This was also supported in part by the observation that the boiled extract prepard from muscle homogenate failed to produce any increase in 4-MU glucuronide formation. From these results and considerations, it was suggested that sugar nucleotides might be the most possible activator. However, substances other than nucleotide could not be entirely excluded from the possibility as the activator because of the fact

that inorganic phosphate could not be detected even after the treatment of the solution containing the activator in a relatively high concentration with hydrochloric acid, although this might be in part attributed to an excessively small amount of the activator contained in the solution.

From a different point of view, POGELL and LELOIR[13] studied the activation of glucuronide formation and reported that uridine diphosphate N-acetylglucosamine and adenosine triphosphate activated rat and guinea-pig liver microsomal glucuronidation and other nucleotides were not effective. In this connection, it was strongly suggested that the activator of glucuronide formation in the boiled liver extract might be uridine diphosphate N-acetylglucosamine, although it is a problem to be solved in the future whether the activator in the boiled liver extract is identical with uridine diphosphate N-acetylglucosamine or not.

SUMMARY

The effect of the boiled liver extract on the velocity of 4-methyl umbelliferone glucuronide formation by mouse liver homogenate was studied. The results were as follows:

1. Addition of mouse or rat liver boiled extract to the complete system for the glucuronide formation produced an increase in the velocity of the glucuronide formation.

2. The boiled liver extract produced the increase in the velocity of the glucuronide formation not as a substrate but as an activator.

3, The activator in the boiled liver extract was relatively heat stable, acid labile, and precipitated as a fairly ethanol-soluble barium salt. The solution of the activator partially purified by ethanol fractionation of the barium salt indicated its absorption maximum at 262 mμ. These results suggested that the most possible substance in the boiled liver extract responsible for the activation of 4-methyl umbelliferone glucuronide formation might be a sugar nucleotide.

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