Acta Medica Okayama

Volume 16, *Issue* 5 1962 *Article* 5

OCTOBER 1962

Isolation of Endogenous Activator of Glucuronide For-mation in Liver

Kazuhisa Taketa*

*Okayama University,

Isolation of Endogenous Activator of Glucuronide For-mation in Liver*

Kazuhisa Taketa

Abstract

A purified compound of the activator of glucuronide formation was isolated from a boiled extract of rat liver by charcoal adsorption, ethanol fractionation of barium salts, and finally paper and Dowex-l column chromatographies. The analytical data and the chemical properties of the compound sugggested that the endogenous activator of glucuronide formation in rat liver might be uridine diphosphate N-acetylglucosamine.

Acta Med. Okayama **16**, 275—281 (1962)

ISOLATION OF ENDOGENOUS ACTIVATOR OF GLUCURONIDE FORMATION IN LIVER

Kazuhisa TAKETA

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

Received for publication, December 14, 1962

In the study of POGELL and LELOIR[1] on the nucleotide activation of liver glucuronide formation *in vitro*, uridine diphosphate N-acetylglucosamine (UDPAG) and adenosine triphosphate were proved to be effective in the glucuronide formation with rat liver microsomes. From a different point of view, the activating effect of boiled liver extracts on the glucuronide formation with mouse liver homogenate and uridine diphosphate glucuronic acid (UDPGA) was studied by the author, and the presence of an endogenous activator of glucuronide formation in liver was suggested[2]. In the present study, an attempt was made to isolate the activator from a boiled extract of rat liver and to clarify the chemical properties thereof.

MATERIALS AND METHODS

Boiled extract of rat liver was prepared according to the method of DUTTON and STOREY[3], substituting 0.5 per cent potassium chloride for distilled water. Glucuronide formation was determined according to the modification[4] of the method of ARIAS using 4-methyl umbelliferone (4-MU) as a glucuronide receptor. As a source of glucuronyl transferase, 1.0 per cent mouse liver homogenate suspended in alkaline, isotonic, potassium chloride solution[5] was used. The incubation system consisted of 0.03 μmole of 4-MU, 0.05 μmole of UDPGA (90 per cent pure as the ammonium salt, Sigma Chemical Co.), 5 μmoles of magnesium chloride, 25 μmoles of Tris buffer (pH 7.4), 0.1 ml. of the homogenate, and 0.1 ml. of a solution to be tested for the activation, being substituted for distilled water in control system. The final volume was adjusted to 0.5 ml. with water. After incubation of the mixture for 10 minutes at 37°C, 1.0 ml. of ice-cold water was added, and the mixture was washed twice with 5 ml. of chloroform followed by centrifugation. Two 0.5 ml. aliquots of the supernatant solution containing 4-MU glucuronide thus formed were subjected to the assay with β-glucuronidase according to the method described in the previous paper[4]. From the velocity of 4-MU glucuronide formation thus obtained, the ratio of the velocity

in the system containing the solution to be tested for the activation to the velocity in the control system was calculated and referred to as an activation factor of the solution.

A charcoal column was prepared by pouring 1g. of acid-washed charcoal (Shirasagi R-78, 50 to 100 mesh) suspended in water into a column measuring 0.8 cm. in inside diameter. A Dowex 1-×10 (200 to 400 mesh) column (0.8 cm. in inside diameter and 2.0 cm. in hight) in the chloride form was used for the chromatography of ultraviolet-absorbing substances, and the simplified elution procedure of CABIB, LELOIR and CARDINI[6] was employed.

For paper chromatography Schleicher and Schüll No. 2043-a filter paper was used throughout. The paper was washed in 2 *N* acetic acid for quantitative use. Ultraviolet-absorbing substances were chromatographed in the following solvent systems : ethanol-neutral or acid (pH 3.8) ammonium acetate (5 : 2)[7]; n-propanol-concentrated ammonia-water (6 : 3 : 1)[8] or the same solvent except for substituting n-propanol for iso-propanol. Adenosine was run in every paper chromatography as a standard, and the position of ultraviolet-absorbing substances was given relative to the position of adenosine. The ultraviolet-absorbing substances on the paper were located by visual inspection or contact printing by using an ultraviolet lamp with Riken ultraviolet filter-2537. Amino sugars were chromatographed with ethyl acetate-pyridine-concentrated ammonia-water (10 : 5 : 3 : 3)[6] or n-butanol-pyridine-water (6 : 4 : 3)[9] and detected with aniline hydrogen phthalate[10] or ninhydrin (0.4 per cent in n-butanol saturated with water). Glucose and glucosamine were used as reference compounds.

N-acetylamino sugar was determined by the method of REISSIG, STROMINGER and LELOIR[11]. Phosphate was determined by the method of FISKE and SUBBAROW[12]. Absorption spectra of ultraviolet-absorbing compounds were obtained by using Beckmann spectrophotometer Model EPU-2.

RESULTS

The boiled liver extract prepared from 20 g. of rat liver was brought to a pH 3.0 with hydrochloric acid at 0 to 4°C, and the resulting precipitate was removed by centrifugation. The supernatant solution was passed through the charcoal column at room temperature. The column was washed with water until the pH of the effluent had risen to 5.0 and eluted first with 50 ml. of 50 per cent ethanol followed by 50 ml. of 50 per cent ethanol containing 0.1 per cent of concentrated ammonia at a flow rate of approximately one ml. per minute. The elution was followed by measurements of the absorbancy at 260 mμ. Following the elution with 50 per cent ethanol containing ammonia, a slight turbidity was observed in eluate followed by a yellow color and a rise in pH. Appropriate eluates were tentatively combined, and aliquots of the pooled frac-

tions were each concentrated *in vacuo* at 45°C to one tenth in volume. The concentrated fractions were analyzed for the activation of glucuronide formation and the absorption at 260 mμ (Fig. 1). It was indicated that the activator was

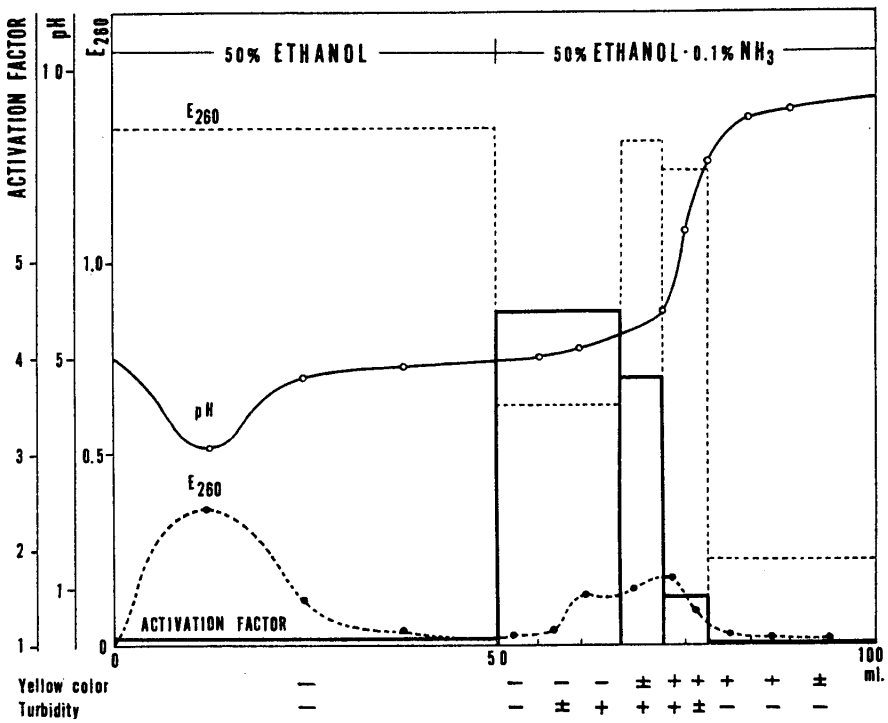

Fig. 1. Fractionation of boiled liver extract on charcoal column. The results in pooled and 10-fold concentrated fractions are indicated with ▭ or ⋯. The values for the absorbancy at 260 mμ are those determined on 50-fold diluted solutions.

eluted in a relatively high concentration in the first colorless ethanol-ammonia fraction with slight turbidity. Another aliquot of this original alcohol solution was used for further fractionation as barium salts.

Approximately fifteen ml. aliquot of the solution was brought to a pH 12.0 with sodium hydroxide. After addition of 0.2 ml. of 1 *M* barium acetate, the barium salts were precipitated by adding ethanol to a total concentration of 80 per cent. The precipitate was separated by centrifugation and dissolved in dilute hydrochloric acid. The pH of the solution was adjusted to 8.2 and four volumes of ethanol was added. The precipitate of water-soluble ethanol-insoluble barium salts thus obtained was separated by centrifugation. By readjusting the pH of the supernatant solution to 12.0 with 80 per cent ethanol contaiuing sodium hydoxide, a small amount of precipitate of fairly ethanol-soluble barium salts

was obtained. All these procedures for ethanol fractionation of barium salts were performed at a temperature of 0 to 4°C. The barium salts of these two fractions were each dissolved in a small amount of dilute hydrochloric acid, and the barium was removed as the sulfate. The solutions were neutralized and tested for the activation of glucuronide formation. The activating effect was recovered mainly in the solution from the fairly ethanol-soluble barium salts. The results of paper chromatography of these two solutions were indicated in Fig. 2. In ethanol-neutral ammonium acetate and iso-propanol-ammonia-water,

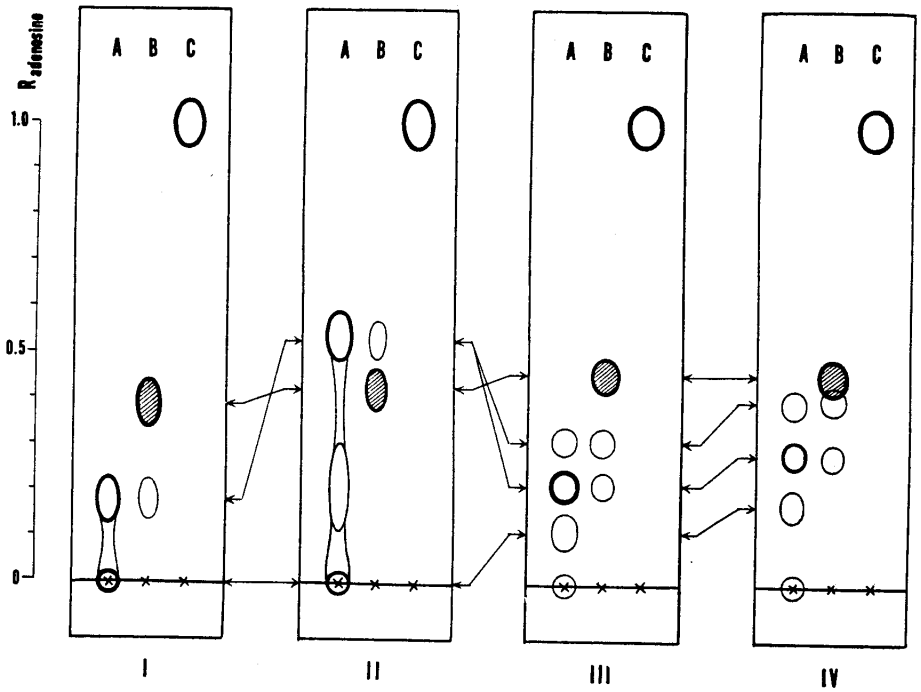

Fig. 2. Paper chromatography of ultraviolet-absorbing substances. A, sample from the water-solubleet hanol-insoluble barium salts; B, sample from the fairly ethanol-soluble barium salts; C, adenosine as a standard. The shaded areas corresponding to ultraviolet-absorbing spot indicate the presence of the activator. Solvent system I, ethanol-ammonium acetate (pH 7.0); II, ethanol-ammonium acetate (pH 3.8); III, iso-propanol-ammonia-water; IV, n-propanol-ammonia-water. Ascending development for 10 hours at 20±1°C.

satisfactory resolutions of ultraviolet-absorbing substances were obtained. The interrelations of the ultraviolet-absorbing spots were examined by alternative rechromatography with either solvent I or III and indicated with arrows in the same figure. Each spot was eluted with water and tested for the activation of glucuronide formation. The activation was demonstrated with the eluate from

the shaded areas corresponding to ultraviolet-absorbing spot. Other areas with or without ultraviolet absorption failed to demonstrate the activation. Ninhydrin reaction of the shaded area was negligible.

The eluate from the spot responsible for the activation was brought to a pH 9.0 with ammonia and chromatographed on the Dowex-1 chloride column by stepwise elution[6]. A single ultraviolet-absorbing peak was demonstrated following the elution with 0.03 *N* sodium chloride in 0.01 *N* hydrochloric acid. The fraction corresponding to this peak was concentrated by adsorbing on a small charcoal column followed by an elution with 50 per cent ethanol-0.1 per cent ammonia aud analyzed for the activation, ultraviolet absorption spectrum, phosphate, and N-acetylamino sugar.

This sample gave a positive result in the activation. The absorption spectra of the sample were identical with those of uridine, giving the absorption maximum at 262 mμ and the minimum at 232 mμ in neutral medium and at 243 mμ in alkaline medium (pH 11.0). The absorption spectrum of the sample hydrolyzed in 0.01 *N* hydrochloric acid for 15 minutes at 100°C and treated with borate buffer followed by p-dimethylaminobenzaldehyde reagent[11] was identical with that of N-acetylglucosamine treated similarly. The sample was hydrolyzed in 2 *N* hydrochloric acid for 3 hours at 100°C and subjected to the paper chromatography with ethyl acetate-pyridine-ammonia-water or n-butanol-pyridine-water. The single spot positive for both ninhydrin aud aniline hydrogen phthalate and corresponding to the position of reference glucosamine was obtained in either solvent system. The analytical data for the sample of activator were summarized in Table 1 and compared with the values calculated from UDPAG, a possible compound as the activator as revealed by the found component ratio in the Table.

Tabld 1. Analytical data for sample of activator

Component	μmoles per μmole uridine	
	found	calculated from UDPAG
Urdidine	1.00	1
Total phosphate	1.98	2
Acid-labile phosphate	1.05	1
N-Acetylamino sugar	0.97* 2.64**	1

* calculated as N-acetylglucosamine

** calculated as N-acetylgalactosamine

DISCUSSION

In the isolation procedures of the activator of glucuronide formation from a boiled extract of rat liver, the step with a charcoal column and the ethanol

fractionation of the barium salts were considered as valuable means in that a satisfactory separation of the activator could be obtained on the successive paper chromatography. However, a considerable loss of the activator was involved in these preliminary purification steps and the results were less reproducible.

Analyses of the purified sample of activator, obtained by the chromatographies on paper and Dowex-1 chloride column indicated the properties of an amino sugar derivative of uridine nucleotides; the absorption spectra identical with those of uridine and liberations of inorganic phosphate and N-acetylamino sugar in mild acid hydrolyses. The amino sugar moiety was identified as glucosamine by paper chromatography of the deacetylated sample. This was also supported by the result that the ratio of N-acetylamino sugar to the component uridine was calculated to be approximately one in case of that the N-acetylamino sugar in the sample was assumed to be N-acetylglucosamine, whereas the ratio gave an incompatible value in case of the calculation as N-acetylgalactosamine. Therefore, it was considered that the nucleotide responsible for the activator might be UDPAG. This was consistent with the fact that the activator was eluted from Dowex-1 chloride column in the fraction supposed to be corresponding to that of UDPAG[6].

From these results it was concluded that the activator of glucuronide formation isolated from the boiled extract of rat liver might be identical with UDPAG, which was indicated by POGELL and LELOIR[1] to be an activator of liver microsomal glucuronide formation. Comparison of the present activator with the authentic sample of UDPAG is a further problem for identification.

SUMMARY

A purified compound of the activator of glucuronide formation was isolated from a boiled extract of rat liver by charcoal adsorption, ethanol fractionation of barium salts, and finally paper and Dowex-1 column chromatographies.

The analytical data and the chemical properties of the compound sugggested that the endogenous activator of glucuronide formation in rat liver might be uridine diphosphate N-acetylglucosamine.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the Tokyo Biochemical Research Institute.

REFERENCES

1. POGELL, B. M. and LELOIR, L. F.: Nucleotide activation of liver microsomal glucuronidation. *J. Biol. Chem.* 236, 293, 1961
2. TAKETA, K.: Enzymatic studies of glucuronide formation in impaired liver. II. Activating effect of boiled liver extract on liver glucuronide formation. *Acta Med. Okayama* 16, 90, 1962

3. DUTTON, G. J. and STOREY, I. D. E.: Uridine compouds in glucuronic acid metabolism. 1. The formation of glucuronides in liver suspensions. *Biochem. J.* 57, 275, 1954
4. TAKETA, K.: 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. *Acta Med. Okayama*, 16, 71, 1962
5. POTTER, V. R.: The homogenate technique. In *Methods in Medical Research*, Year Book Publishers, Inc., Chicago, Vol. 1, p. 317, 1948
6. CABIB, E., LELOIR, L. F. and CARDINI, C. E.: Uridine diphosphate acetylglucosamine. *J. Biol. Chem.* 203, 1055, 1953
7. PALADINI, A. C. and LELOIR, L. F.: Studies on uridine-diphosphate-glucose. *Biochem. J.* 51, 426, 1952
8. HANES, C. S. and ISHERWOOD, F. A.: Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* 164, 1107, 1949
9. PONTIS, H. G.: Uridine diphosphate acetylgalactosamine in liver. *J. Biol. Chem.* 216, 195, 1955
10. PARTRIDGE, S. M.: Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* 164, 443, 1949
11. REISSIG, J. L., STROMINGER, J. L. and LELOIR, L. F.: A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.* 217, 959, 1955
12. FISKE, C. H. and SUBBAROW, Y.: The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375, 1925