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COMPARISON OF THE PROPERTIES OF GLUCOSAMINEPHOSPHATE ISOMERASE (GLUTAMINE-FORMING) FROM RAT LIVER AND A HEPATOMA

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SUMMARY

Properties of glucosaminephosphate isomerase (glutamine-forming (EC 5.3.1.19)) partially purified from a rat ascites hepatoma have been compared with properties of rat liver enzyme. Hepatoma glucosaminephosphate isomerase is more unstable, more sensitive to inhibition by UDP-*N*-acetylglucosamine and more tightly bound to DEAE-Sephadex gel than the liver enzyme. The two enzymes can also be distinguished immunologically.

Isoelectric focusing studies have demonstrated that the major form of hepatoma glucosaminephosphate isomerase has a pI at 4.5 and is distinctly different from the major liver form, with a pI of 5.0. The tumor in addition contains a minor component having a pI at 4.1. A small amount of the major tumor form has also been found in liver.

The possible significance of the difference in the glucosaminephosphate isomerase forms from liver and hepatoma is discussed.

INTRODUCTION

Glucosaminephosphate isomerase (glutamine-forming) (EC 5.3.1.19; formerly EC 2.6.1.16) catalyzes the formation of glucosamine 6-phosphate (GlcN-6-*P*) from fructose 6-phosphate (Fru-6-*P*) and glutamine. The enzyme is subject to feedback inhibition by UDP-*N*-acetylglucosamine (UDP-GlcNAc) [1, 2] and believed to be the rate-limiting and regulatory enzyme in the pathway specific for the formation of UDP-GlcNAc. The role of glucosaminephosphate isomerase is therefore to provide *N*-acetylaminosugars for biosynthesis of blood glycoproteins, epithelial mucins, mesenchymal proteoglycans, membrane glycoproteins and still other groups of glycoproteins. Since each group of glycoproteins have discrete tissue distribution and biological function, it may be worth speculating that different isozymic forms of glucosaminephosphate isomerase exist which are specifically linked to the biosynthesis of different groups of glycoproteins.

Recently, evidence has accumulated that cell surface glycoproteins play a signi-

ficant role in malignant transformation as well as proliferation of the cell [3–5]. It is therefore desirable to examine if any characteristic changes may occur in glycoprotein-biosynthesizing systems upon cancerization. In view of the functional importance of glucosaminephosphate isomerase to glycoprotein biosynthesis, we attempted to compare the properties of this enzyme from normal and neoplastic tissues.

We have already reported that rat hepatoma glucosaminephosphate isomerase is more sensitive to UDP-GlcNAc inhibition than rat liver enzyme [6]. The present communication deals with further studies on rat liver and hepatoma glucosaminephosphate isomerases, which suggest that they represent different isozymic forms of glucosaminephosphate isomerase.

MATERIALS AND METHODS

Animals and tumor

Male Donryu rats weighing 200–250 g and fed ad libitum were used. The tumor studied was a strain of rat ascites hepatoma, Yoshida sarcoma, maintained by serial intraperitoneal transplantation into animals. Yoshida sarcoma had long been misregarded as a sarcoma, but was recently found to be of hepatoparenchymal origin [7, 8]. Tumor cells were harvested from the peritoneal cavity 4 or 5 days after implantation and washed twice with cold physiological saline.

Partial purification of glucosaminephosphate isomerase

All preparative experiments were performed at 0–4 °C. Livers were homogenized in 2 vol. of 0.154 M KCl–1 mM EDTA–12 mM Glc-6-*P* adjusted to pH 7.5 and the homogenate was centrifuged at 105 000 × *g* for 1 h. The resulting supernatant was found to contain glucosaminephosphate isomerase, which was purified by $(NH_4)_2SO_4$ fractionation followed by DEAE-Sephadex chromatography. When indicated, the enzyme was further purified on a hydroxyapatite column. Details of the procedure and typical purification data have been given previously [9].

Saline-washed hepatoma cells were washed twice with 0.154 M KCl–1 mM EDTA adjusted to pH 7.5, resuspended in the same medium and centrifuged at 1000 × *g* for 5 min. An equal volume of cold distilled water was added to these packed cells and the mixture was homogenized for 1 min using a glass-Teflon homogenizer. The homogenate was then made isotonic by addition of 0.308 M KCl–2 mM EDTA adjusted to pH 7.5 and centrifuged at 105 000 × *g* for 1 h. Glucosaminephosphate isomerase was purified from the resulting supernatant by the same procedure as described above except that the DEAE-Sephadex chromatography was conducted in the presence of 20 mM glutamine and 0.05 mM GclN-6-*P*. A more than 30-fold purification was achieved at the DEAE-Sephadex stage.

Assay of glucosaminephosphate isomerase

Glucosaminephosphate isomerase was assayed by measuring the formation of GlcN-6-*P* according to the method described previously [9]. The standard assay mixture contained 55 mM sodium phosphate (pH 7.5), 15 mM glutamine, 10 mM Fru-6-*P*, 0.2 mM EDTA, 4 mM dithiothreitol and enzyme in a final volume of 1 ml. Protein concentration was determined by the method of Lowry et al. [10].

Immunological analysis

An antibody against rat liver glucosaminephosphate isomerase was prepared as follows. The enzyme purified by a hydroxyapatite column (10 mg) [9] was mixed with an equal volume of Freund's Complete Adjuvant and injected subcutaneously into an adult albino rabbit. After 5 injections at weekly intervals, 3 more intravenous injections of the enzyme (2–4 mg each) were given at the same intervals. 2 weeks after the last injection the rabbit was bled; γ-globulin was precipitated from the serum between 20 and 33 % saturation of $(NH_4)_2SO_4$ and purified further by DEAE-Sephadex chromatography [11]. Control γ-globulin was prepared by the same procedure, but the rabbit had received physiological saline instead of the enzyme. Variable amounts of the antibody were mixed with a constant amount of glucosaminephosphate isomerase and after incubation at 37 °C for 10 min, the residual enzyme activity was determined. Centrifugation of the incubated sample prior to assay had no effect on the results.

Isoelectric focusing

Isoelectric focusing was conducted according to the method of Vesterburg and Svensson [12] using the LKB 8101 column with a volume of 110 ml and Ampholine carrier ampholytes of the pH range 3–6. A 0–46 % (w/v) sucrose density gradient containing 1 % Ampholine was formed and a 1.5 ml sample was applied. The voltage was set initially at 200–300 V and increased to 600–700 V after 12 h. The focusing was then conducted for 28 h while the column was kept at 2 °C. Fractions of 1.5 ml were collected and pH (12 °C) and enzyme were assayed in each fraction.

Chemicals and commercial enzymes

Glc-6-*P* and Fru-6-*P* were purchased from Boehringer and GlcN-6-*P* and UDP-GlcNAc were from Sigma. Carrier ampholytes was obtained from LKB Corp. and Freund's Complete Adjuvant was from Iatron Laboratories in Tokyo. Urease (soy bean), catalase (beef liver) and lactate dehydrogenase (rabbit muscle) used as markers for molecular weight determination were the products of Boehringer.

RESULTS

Stability

When glucosaminephosphate isomerase was purified from hepatoma by the method developed for liver [9], more than half of the activity was lost during a single stage of DEAE-Sephadex chromatography. The recovery, however, was improved greatly by conducting the chromatography in the presence of glutamine and GlcN-6-*P*. The data presented in Table I suggest that the tumor enzyme is more labile than the liver enzyme and that only the stability of the former is enhanced by glutamine and GlcN-6-*P*.

Effect of thiol

We have previously reported that thiols such as dithiothreitol activate glucosaminephosphate isomerase, the degree of activation being increased with the progress of purification [6]. Thus, the activity of both liver and hepatoma enzymes at the DEAE-Sephadex stage was almost totally dependent on dithiothreitol. Fig. 1, how-

TABLE I

EFFECT OF GLUTAMINE AND GlcN-6-*P* ON THE PURIFICATION OF RAT LIVER AND HEPATOMA GLUCOSAMINEPHOSPHATE ISOMERASES BY DEAE-SEPHADEX CHROMATOGRAPHY

The $(NH_4)_2SO_4$ precipitate fraction was purified by DEAE-Sephadex column according to the procedure described in the legend to Fig. 2. In (B) all the buffers used contained 20 mM glutamine and 0.05 mM GlcN-6-*P*. Active eluates were combined and assayed for glucosaminephosphate isomerase and protein in order to obtain the data for the purification and recovery from the $(NH_4)_2SO_4$ precipitate. The figures in parenthesis are the specific activities of the purified enzymes in terms of nmoles of GlcN-6-*P* formed per h/mg of protein.

	A. No addition		B. Glutamine + GlcN-6-*P*	
	Purification (X)	Recovery (%)	Purification (X)	Recovery (%)
Liver	11.03 (772)	85	11.26 (566)	90
Hepatoma	1.97 (279)	43	4.57 (813)	78

ever, shows that there is a marked difference in the concentrations of dithiothreitol required. The liver enzyme required 1 mM dithiothreitol for maximum activity while the tumor enzyme exhibited maximum activity with 3 mM dithiothreitol.

DEAE-Sephadex chromatography

Fig. 2 shows the elution profiles of liver and hepatoma glucosaminephosphate isomerase from a DEAE-Sephadex column. A linear gradient of KCl from 50–500 mM in 50 mM sodium phosphate buffer (pH 7.5) was used for elution. It should be noted that the figure represents a composite of two different chromatographic separations.

The tumor enzyme was bound more tightly to the column and under our experimental conditions, the two enzymes were eluted with a difference of 100–200 $\mu\Omega^{-1}$ in conductivity. In liver glucosaminephosphate isomerase, however, there was a small fraction of activity, bound to the column as tightly as the tumor enzyme. As mentioned above, the chromatography of hepatoma enzyme was performed in the presence of

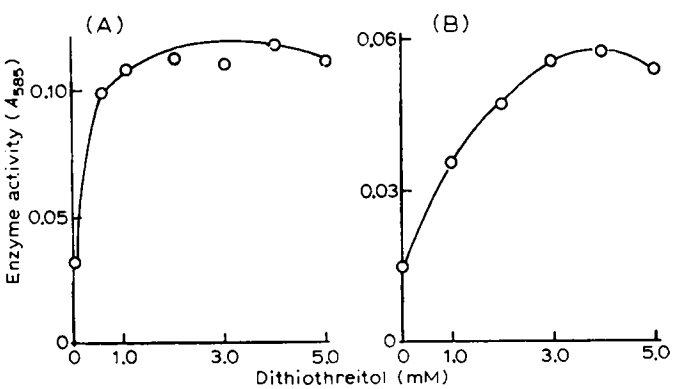

Fig. 1. Effect of dithiothreitol on the activity of glucosaminephosphate isomerases from rat liver (A) and hepatoma (B). The enzymes at the DEAE-Sephadex stage were assayed in the presence of varying concentrations of dithiothreitol.

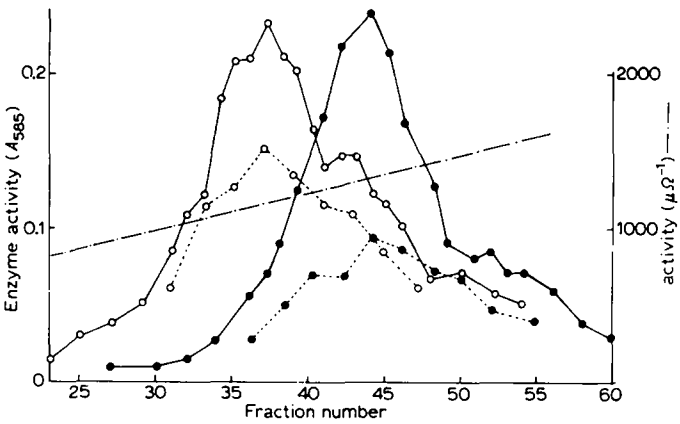

Fig. 2. Elution profiles of glucosaminephosphate isomerases from rat liver (○) and hepatoma (●) after DEAE-Sephadex chromatography. The enzyme at the $(NH_4)_2SO_4$ stage (approx. 60 (liver) or 40 (tumor) mg in protein) was dissolved in 50 mM sodium phosphate (pH 7.5)–50 mM KCl–1 mM EDTA, passed through a desalting column of Sephadex G-25 and applied to a column (1.5 cm × 18 cm) of DEAE-Sephadex previously equilibrated with the above buffer. After washing with the same buffer, a linear gradient of KCl from 50–500 mM was applied. When the glucosaminephosphate isomerase from hepatoma was chromatographed, all the buffers used contained 20 mM glutamine and 0.05 mM GlcN-6-*P*. Fractions of 2 ml were collected at a flow rate of 15 ml/h. Enzyme activity in the absence (———) and presence (·····) of 0.2 mM UDP-GlcNAc plus 7 mM Glc-6-*P* and conductivity (· —) were assayed in each fraction.

glutamine and GlcN-6-*P*. The omission of these stabilizing agents resulted in a marked reduction in recovery, but did not alter the eluting position of the tumor enzyme.

Inhibition by UDP-GlcNAc

Previous work from this laboratory has shown that UDP-GlcNAc inhibits hepatoma glucosaminephosphate isomerase more profoundly than the liver enzyme [6]. These studies, however, were performed with the enzyme at the $(NH_4)_2SO_4$ stage and we have subsequently found that the enzyme at the DEAE-Sephadex stage is no longer inhibited effectively by UDP-GlcNAc unless Glc-6-*P* is deliberately added [9]. Glc-6-*P* is not inhibitory by itself, but potentiates greatly the inhibitory action of UDP-GlcNAc [9]. Similar observation was made by Winterburn and Phelps [13].

In the experiments shown in Fig. 2, the effect of UDP-GlcNAc (0.02 mM) on the glucosaminephosphate isomerase fractions eluted from the DEAE-Sephadex column was examined in the presence of Glc-6-*P* (7 mM). It is clear that the tumor enzyme is inhibited more profoundly than the liver enzyme, thereby confirming the previous results obtained with the less purified preparations.

Immunological studies

An antibody to liver glucosaminephosphate isomerase was prepared by injection of the hydroxyapatite-purified enzyme into rabbits and its effect on activities of liver and hepatoma enzymes was determined (Fig. 3). When the liver enzyme was treated with the antibody, more than 60% of the activity was lost and the extent of inactivation was independent of the degree of purification. On the other hand, there was only a slight effect on the tumor enzyme, suggesting that the two enzymes may be

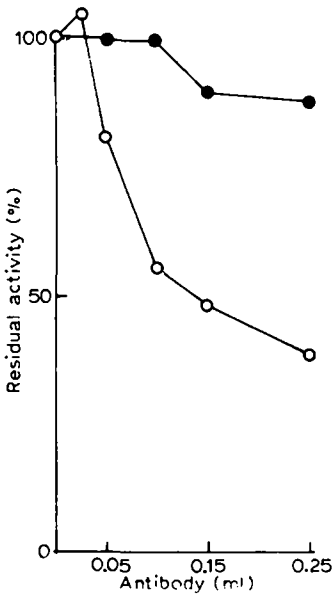

Fig. 3. Inhibition of rat liver (○) and hepatoma (●) glucosaminephosphate isomerases by antibody to liver glucosaminephosphate isomerases. The enzyme at the DEAE-Sephadex stage was incubated with the antibody and assayed as described in the text.

different proteins. Parallel titrations were carried out with control γ-globulin and corrections were made for any loss of activity.

γ-Globulin isolated from rabbits injected with the purified hepatoma glucosaminephosphate isomerase was found to inactivate the tumor enzyme but had little effect on the liver enzyme. Although the data are compatible with the above finding that the two enzymes are immunologically different, the antigenicity of tumor glucosaminephosphate isomerase is extremely low and several trials to obtain a potent antibody have been unsuccessful. Attempts are continuing to further potentiate the antigenicity of the tumor enzyme in order to more clearly determine its immunological specificity.

Isoelectric focusing

The isoelectric focusing patterns of liver and hepatoma glucosaminephosphate isomerase are shown in Fig. 4. The enzymes assayed were those at the $(NH_4)_2SO_4$ stage. In some instances they were further purified by a column of Sephadex G-200 prior to subjecting to isoelectric focusing, but the patterns obtained were identical to those with the less purified preparations.

The major peak of the tumor pattern had a pI at 4.5 and was clearly different from the major liver peak, with a pI of 5.0. As predicted from the chromatographic work (Fig. 2), there was an additional peak of activity in the liver pattern which had a pI identical to that of the major tumor peak. The tumor pattern also had an additional peak at pI 4.1, which was not found in the liver pattern. In the experiment shown in Fig. 4C, a mixture of liver and tumor enzymes was subjected to isoelectric focusing. A pattern emerged which was additive for the two enzymes, thereby showing

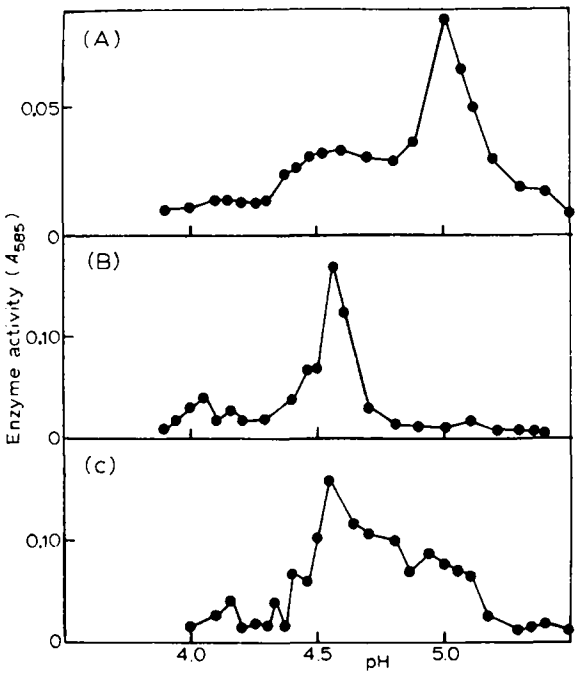

Fig. 4. Isoelectric focusing patterns of glucosaminephosphate isomerases from rat liver and hepatoma. The enzyme at the $(NH_4)_2SO_4$ stage was dissolved in 10 mM sodium phosphate (pH 7.5), passed through a desalting column of Sephadex G-25 and 1.5 ml of the resulting solution was subjected to isoelectric focusing as described in text. (A) 30 mg of liver enzyme; (B) 20 mg of hepatoma enzyme; and C, a mixture of 30 mg of liver and 15 mg of hepatoma enzymes.

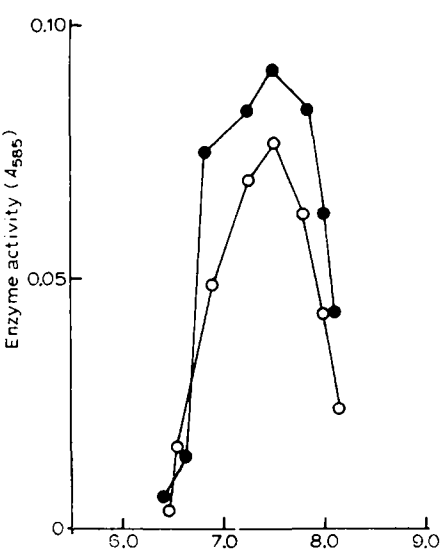

Fig. 5. Effect of pH on the activity of rat liver (○) and hepatoma (●) glucosaminephosphate isomerases. The enzyme at the $(NH_4)_2SO_4$ stage was assayed under the standard conditions except that potassium phosphate buffers of varying pH values were used.

TABLE II

THE APPARENT K_m VALUES OF RAT LIVER AND HEPATOMA GLUCOSAMINEPHOSPHATE ISOMERASE FOR SUBSTRATES

The enzymes at the DEAE-Sephadex stage were used.

	K_m (mM) for	
	Glutamine	Fru-6-*P*
Liver	0.69	0.31
Hepatoma	0.71	0.42

that their particular patterns were properties of the enzymes themselves and not due to their environments.

Other properties

Except for sensitivity to feedback inhibition, there are marked similarities in the kinetic properties of liver and hepatoma glucosaminephosphate isomerase. Fig. 5 shows the effect of pH on enzyme activity: a single peak of maximum activity was observed at pH 7.5 for both enzymes. Both enzymes exhibited normal Michaelis–Menten kinetics towards Fru-6-*P* and glutamine and gave apparent K_m values similar to each other (Table II).

The molecular weights of liver and hepatoma glucosaminephosphate isomerase were estimated by gel filtration chromatography on Sephadex G-200 (Fig. 6) according to the method of Andrews [14]. The values obtained were 380 000 for liver and 410 000 for tumor enzyme.

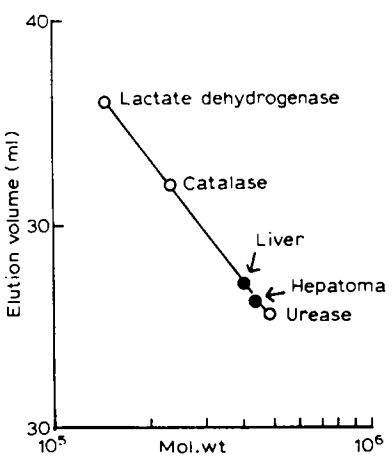

Fig. 6. Estimation of molecular weight of rat liver and hepatoma glucosaminephosphate isomerases by Sephadex G-200 chromatography. The enzyme at the $(NH_4)_2SO_4$ stage was dissolved in 50 mM sodium phosphate (pH 7.5)–1 mM EDTA (approx. 20 mg protein/ml) and applied to a column (1.5 cm × 43 cm) of Sephadex G-200 previously equilibrated with the same buffer. Fractions of 0.5 ml were collected at a flow rate of 5 ml/h. Rabbit muscle lactate dehydrogenase (mol. wt 130 000), beef liver catalase (230 000) and soy bean urease (480 000) were used as markers.

DISCUSSION

Liver synthesizes large amounts of blood glycoproteins; reasonably high levels of glucosaminephosphate isomerase activity found in this tissue [6, 15] probably reflect high demands for the supply of *N*-acetylaminosugars. The capacity of liver to synthesize blood proteins is known to be lost upon cancerization, but under the same condition, glucosaminephosphate isomerase increases to a great extent as judged from the markedly high levels of this enzyme in hepatomas as compared to liver [6]. These findings suggested that hepatoma glucosaminephosphate isomerase might be different from the liver enzyme in molecular form as well as in biological function.

This hypothesis has gained support from our finding that the glucosaminephosphate isomerase from hepatoma can be distinguished from the liver enzyme not only in sensitivity to feedback inhibition, as previously reported [6], but also chromatographically, in isoelectric point (as determined by isoelectric focusing) and immunologically. The two enzymes were also different with respect to their stability and response to added thiol. The differences observed are sufficient to suggest that the two enzymes represent different isozymic forms of glucosaminephosphate isomerase, although their molecular relationship has not yet been elucidated. We have also studied several properties, such as UDP-GlcNAc inhibition and affinity towards DEAE Sephadex gel, of glucosaminephosphate isomerase partially purified from other rat ascites hepatomas. They were AH-66F and AH-130 [16]. The results obtained were the same as those for the hepatoma enzyme studied in the present work.

Isoelectric focusing as well as chromatographic studies have further demonstrated that liver glucosaminephosphate isomerase is not isozymically homogeneous. The major form having a pI at 5.0 is not found in hepatoma, whereas the minor component, with a pI of 4.5, increases upon cancerization so as to become the major glucosaminephosphate isomerase form of the hepatoma. Thus, the alteration of glucosaminephosphate isomerase upon hepatocarcinogenesis appears to follow the general pattern of "loss of those isozymes that play a functional role in liver metabolism and their replacement by isozymes that are low or absent in the adult differentiated liver cells" [17].

The pattern of glucosaminephosphate isomerase, however, has been complicated by the finding that hepatoma possesses the third form of glucosaminephosphate isomerase, with a pI of 4.1, though in a minute amount. The form must have arisen upon cancerization, since it is not found in liver. On the other hand, the occurrence of more than two forms of glucosaminephosphate isomerase in rat tissues tends to support our supposition that there may be multi-isozymic forms of glucosaminephosphate isomerase, functionally differentiated from each other (see Introduction). To clarify this point further, it is desirable to examine the molecular forms of glucosaminephosphate isomerase in various tissues under various conditions. These studies will also provide answers to the important question of what is the significance of the two isozymic forms, with pI values of 4.5 and 4.1, appearing in hepatomas.

Perhaps the most useful means to distinguish different forms of the enzyme in various tissues is isoelectric focusing. Work currently in progress (unpublished observations) has indicated the presence of the form with a pI of 4.1 in early embryo (12 day) and adult brain and of the form with a pI of 4.5 in late fetal (19 day) and regenerating liver (48 h).

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