BIOCHEMICAL MEDICINE 5, 285–296 (1971)

Enzymatic Determination of Cardiac Glycosides[1]

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Received March 9, 1971

The frequent clinical use of cardiac glycosides (CG) and their narrow range of therapeutic effectiveness emphasize the need for a reliable and simple method of determination performable in an average clinical laboratory. In addition to specificity, an acceptable assay procedure for CG has to provide high sensitivity: average plasma levels of digoxin and digitoxin (the two most commonly used CG) in fully digitalized patients were reported to be 1.5 and 25 ng/ml, respectively (1, 2). Various attempts have been made to fulfill these analytical requirements (3–6). The more recently applied methods involve the use of radioactively labeled compounds (7–11).

An enzymatic assay based on the inhibition of the sodium potassium-activated adenosinetriphosphatase (Na,K-ATPase) was described by Burnett and Conklin (12). The procedure allowed the determination of 20 ng of digitoxin with a standard deviation of 19%. The authors were unable to estimate digoxin. Since the enzymatic assay provides the required specificity, involves a relatively simple technique and requires a minimum of laboratory equipment, we attempted to develop a highly sensitive enzymatic procedure applicable for the determination of both digoxin and digitoxin in plasma.

The present work is based on the specific inhibition by CG of the Na,K-ATPase. This enzyme was first described by Skou in crab nerve (13), and since then was found in membrane preparations from a variety of tissues (14). Particularly high enzymatic activity is found in the microsomal fraction from cerebral cortex. An extensive survey of prepa-

[1] This work was supported in part by PHS Grants 5 P11 GM 15559 and 5 T01 HE05526.

[2] Portions of this work have been included in a thesis submitted by U. K. in partial fulfillment of the requirements for the degree of Master of Science at the University of Michigan, Ann Arbor, Mich.

rations of Na,K-ATPase from brains of several species showed the overall advantage of using beef brain microsomes as a source of this enzyme (15, 16). The preparation contains both the Na,K-ATPase and a magnesium-activated adenosinetriphosphate (ATP) hydrolyzing activity. CG, including ouabain, specifically inhibit the Na,K-ATPase, not affecting the magnesium-stimulated activity. Using the total inhibition of the Na,K-ATPase by an excess of ouabain as a reference it is possible to establish a relationship between various amounts of CG (digoxin, digitoxin) and the degree of their inhibition of this enzyme.

MATERIALS AND EQUIPMENT

All common chemicals used were of reagent grade. Ouabain, Tris-base, imidazole and ATP were obtained from Sigma Chemical Company, St. Louis, Mo. Digoxin and digitoxin were obtained from the Burroughs Wellcome and Co., Tucahoe, N. Y. and the Nutritional Biochemical Laboratories, Cleveland, Ohio, respectively. The beef brains were generously supplied by the Standard Beef Company, Detroit, Mich. Human blood and plasma were obtained from the Blood Bank, The University Hospital, Ann Arbor, Mich. Throughout the experiment deionized water produced by a multibed cartridge (Barnstead 0803) was used.

The equipment used included: refrigerated centrifuge (Sorvall RC2-B), table-top centrifuge (Adams Dynac CT1300), shaking water bath (Lab-Line Instruments, Inc.), mechanical shaker (Eberbach), mixer with vortex action (Lab-Line Instruments, Inc.), spectrophotometer (Gilford, model 240), and a deep-freeze chest (Revco, ULT 659).

All the glassware used in the present work was washed with special care, primarily to avoid contamination by inorganic phosphate. The tubes used were subjected to a washing procedure involving boiling with 0.1 N NaOH and 50% HNO_3. Prior to drying, the glassware was repeatedly rinsed with deionized water.

EXPERIMENTAL AND RESULTS

Preparation of a Microsomal Fraction from Beef Brain

The brains of two 2-year-old calves were transported to the laboratory in crushed ice immediately after slaughter. All further steps in the preparation of the microsomal fraction were performed at 0–4°. After removing the pia and blood vessels, the gray matter was snipped off with scissors (a total of 120 gm) and homogenized with 1000 ml of 0.32 M sucrose–2 mM EDTA (pH 7.4) in a Waring Blendor, which was run at full speed for 60 seconds. The homogenate was centrifuged

at 650*g* for 10 minutes (Sorvall GSA rotor).[3] The supernatant fluid (approximately 850 ml) was carefully decanted from the loose sediment, centrifuged at 10,500*g* for 15 minutes (Sorvall GSA rotor), and the collected supernatant fluid (approximately 700 ml) was centrifuged at 22,500*g* for 60 minutes (Sorval GSA rotor). The pellet, representing the "heavy" microsomal fraction, was washed by suspending in 250 ml of 0.32 M sucrose–2 mM EDTA (pH 7.4), using a Potter–Elvehjem glass homogenizer, and then centrifuged at 35,000*g* for 60 minutes (Sorvall SS-34 rotor). The resulting pellet was evenly suspended in the sucrose–EDTA solution to give a total of approximately 50 ml, and aliquots of the suspension were stored at −70°. The protein concentration of the final suspension, as determined by the method of Lowry *et al.* (17) with bovine serum albumin as a standard was 12.2 mg/ml.

For the assay of the Na,K-ATPase the microsomal preparation was diluted 1:80 with cold 50 mM imidazole. HCl (pH 7.2). Two hundred microliters of this suspension was pipetted into 70 × 12-mm tubes, which were kept in ice. To four of the tubes were added 200 μl of an incubation medium containing NaCl, KCl, $MgCl_2$, ATP, and imidazole to give concentrations of 180, 40, 6, 6, and 50 mmoles/liter, respectively. Four additional tubes received 200 μl each of an incubation medium which in addition contained 0.2 mmoles/liter ouabain. Appropriate blanks were prepared by mixing 200 μl of the biological material with 200 μl of the incubation medium without ouabain, followed by the immediate addition of 1.6 ml of cold 0.6 M $HClO_4$. Whereas, the blanks were retained in ice, the other tubes were incubated in a shaking water bath at 37° for 40 minutes, then returned to ice and the protein precipitated with $HClO_4$ as described above. After mixing, the precipitate was removed by centrifugation at 1700*g* for 10 minutes in the cold (4°).

The inorganic phosphate (P_i) liberated was measured by a modification of the method of Fiske and SubbaRow (18). To 1 ml of the supernatant fluid was added 1 ml of a freshly prepared solution of 144 mM $FeSO_4$ in 8.15 mM NH_4–molybdate–0.58 M H_2SO_4 (the latter solution was stocked at room temperature). After 5 minutes at room temperature, the absorbance at 700 nm was determined against distilled water. The amount of P_i liberated was quantitated from a standard curve obtained by adding 1 ml of color reagent to known amounts of inorganic phosphate (1–15 μg P_i as KH_2PO_4) contained in 1 ml distilled water.

In the present study, two batches of the microsomal fraction were prepared. The specific activities of the Na,K-ATPase in these prepara-

[3] Throughout this work, the given centrifugal forces refer to the middle of the tubes (R_{av}).

tions were 16.6 and 15.9 μmoles P_i liberated/mg protein per hour. These values agree closely with the specific activities of numerous microsomal preparations obtained previously from beef cerebral cortex by the same procedure (16).

Inhibition of the Microsomal Na,K-ATPase by Cardioactive Glycosides

Potassium is essential for the activity of the Na,K-ATPase. On the other hand, it has been shown that high concentrations of potassium (K) prevent the inhibitory effect of CG on this enzyme (19). In order to achieve maximum inhibition of the Na,K-ATPase by CG, the optimum concentration of K during the incubation was investigated. Figure 1 illustrates the considerable effect of varying concentrations of K on the inhibition of the enzyme by CG. Maximum inhibition was obtained in the presence of 0.1 mM K. However, at lower concentrations of K the increase in the extent of inhibition was accompanied by a decrease in the activity of Na,K-ATPase, resulting in low absorbancies for P_i[4]. Since low values for the total, uninhibited Na,K-ATPase would adversely

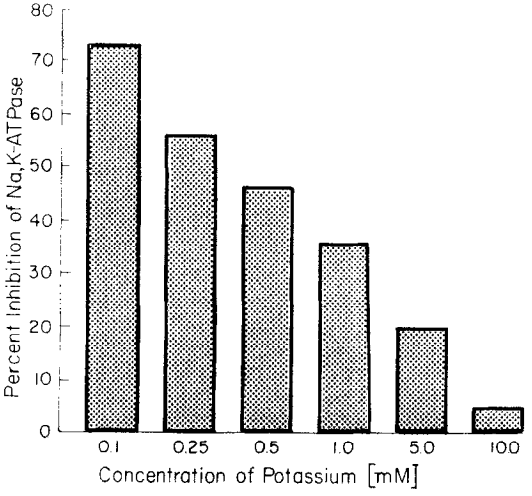

FIG. 1. Effect of potassium on the inhibition of Na,K-ATPase by cardiac glycosides. The inhibition was achieved by 10 ng digoxin which was included in the incubation medium (Table 1) containing different amounts of KCl to give final concentrations of potassium during the incubation as indicated.

[4] Higher concentrations of the enzyme suspension (i.e., a dilution of 1:40 instead of the used 1:80) proved to be less advantageous.

affect the precision of the method, a compromise between maximum sensitivity and acceptable precision was made: the concentration of K during the incubation was selected to be 1 mM.

Since the enzymatic reaction involved in the assay was found to be linear with time up to 60 minutes, the incubation time was accordingly increased.

Total inhibition of the Na,K-ATPase was used as a reference for the inhibition by various amounts of CG and was achieved by 0.1 mM ouabain. Higher concentrations up to 0.5 mM did not cause further inhibition. Ouabain was chosen for convenience because of its greater solubility in aqueous media as compared to the other CG investigated (digoxin, digitoxin).

An aliquot of the microsomal fraction was thawed and diluted 1:80 with cold 50 mM imidazole. HCl (pH 7.0)[5]. Using a Lang–Levy micropipet or, more conveniently, an Eppendorf pipet, 200 μl of the diluted microsomal fraction was pipetted into a series of 70 × 12-mm tubes which were kept in ice. To these tubes 200 μl of an incubation medium was then added. The various media were prepared in bulk and stored in aliquots needed for one set of analysis. In addition to the media with and without ouabain, five media containing various concentrations of the CG (digoxin or digitoxin) were prepared. Their composition is shown in Table 1. The blanks were prepared and all the tubes treated as described in the previous section.

The inhibition of the Na,K-ATPase by CG was expressed as per-

TABLE 1

COMPOSITION OF THE INCUBATION MEDIUM[a]

Component	Stock sol.	Concentration in incubation medium	μl stock sol./ml incub. medium
NaCl	1000	180	180
KCl	50	2	40
$MgCl_2$	100	6	60
ATP[b]	100	6	60
Imidazole	100	50	500
H_2O			160

[a] All concentrations are given in millimoles per liter. The incubation media with ouabain, digoxin, or digitoxin were prepared by adding appropriate aliquots of the aqueous solutions of cardiac glycosides and adjusting the amount of water accordingly.

[b] The ATP solution was adjusted to pH 7 with NaOH and was kept at −20°.

[5] The biological material should be kept frozen in aliquots corresponding to the daily requirement, thus avoiding repeated freezing and thawing.

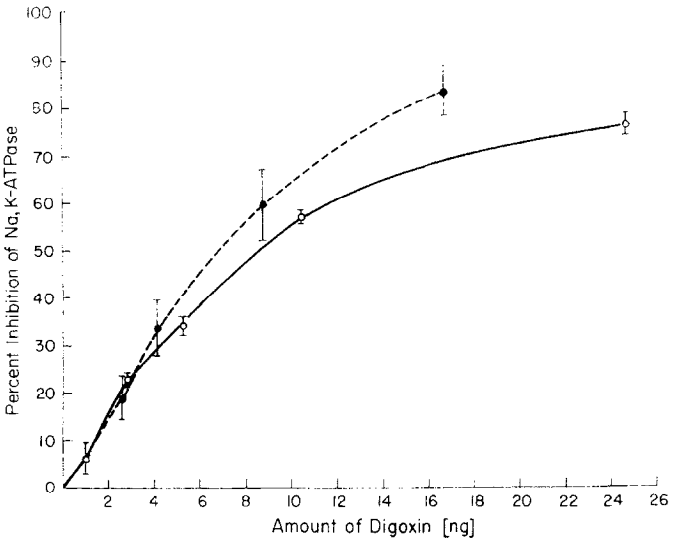

FIG. 2. Inhibition of Na,K-ATPase by digoxin: (○—○) incubation medium containing the indicated amonts of digoxin was added to diluted suspensions of a microsomal preparation of Na,K-ATPase. After incubation, the enzymatic activity was determined and the extent of inhibition calculated as described in the text; (●---●) indicated amounts of digoxin were added to 5-ml aliquots of plasma.[6] After an extraction procedure the drug was determined by the extent of its inhibition of the Na,K-ATPase. Each point of the inhibition represents the mean ± SD of at least 24 determinations.

centage of the activity obtained in the absence of cardiac glycosides. The latter was obtained by subtracting the activity in the presence of ouabain (total inhibition of the Na,K-ATPase) from the activity in the absence of this compound (total ATP-hydrolyzing activity). Figures 2 and 3 show the inhibition curves obtained with digoxin and digitoxin, respectively. There was a difference in the extent of inhibition of the Na,K-ATPase by the two CG. Whereas 25 ng of digitoxin produced 88% inhibition, the same amount of digoxin inhibited the enzyme only 77%. Ten percent inhibition was obtained by 0.7 ng of digitoxin as compared to 1.3 ng digoxin. The standard deviations for digitoxin ranged from ± 2 to ± 4 and for digoxin from ± 1.5 to ± 3%.

Determination of Cardiac Glycosides in Plasma

1. *Extraction.* At each level of CG the analysis was performed in quadruplicate. To 5 ml plasma obtained from citrated human blood

[6] Plotted are the amounts determined in 8-ml aliquots of a total of 10 ml methylene chloride used for the extraction: actually 1.25 times the amounts indicated were added to plasma.

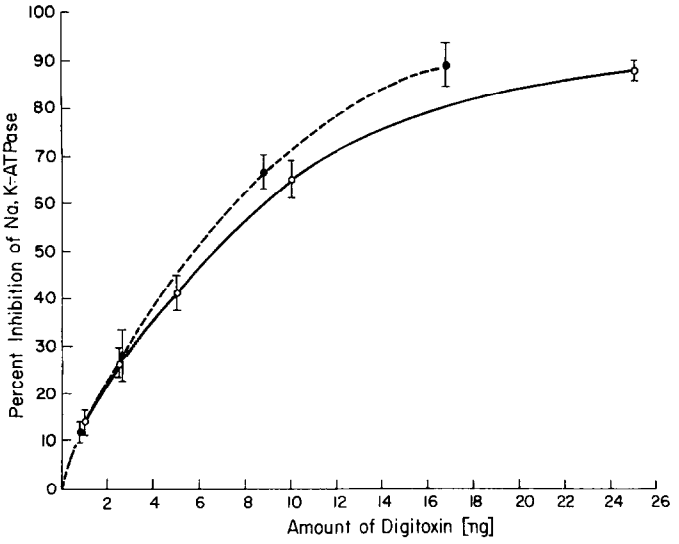

FIG. 3. Inhibition of Na,K-ATPase by digitoxin: (○—○) incubation medium containing the indicated amounts of digitoxin was added to diluted suspensions of a microsomal preparation of Na,K-ATPase. After incubation, the enzymatic activity was determined and the extent of inhibition calculated as described in the text; (●---●) indicated amounts of digitoxin were added to 5-ml aliquots of plasma.[6] After an extraction procedure the drug was determined by the extent of its inhibition of the Na,K-ATPase. Each point of the inhibition curves represents the mean ± SD of at least 24 determinations.

were added, in 50-ml centrifuge tubes (120 × 27 mm), known amounts of the CC in aliquots of 1–10 μl aqueous solution.[7] After careful mixing, 10 ml of methylene chloride was added, the tubes capped (polyethylene inner lining), and shaken horizontally for 5 minutes at medium speed in an Eberbach shaker. The tubes were then centrifuged in the cold (4°) at 1700g for 10 minutes and the upper aqueous layer aspirated. After a "hole" was made through the protein layer at the interface, 8 ml of the methylene chloride was pipetted into 100 × 13-mm tubes.[8] The organic solvent was evaporated at 40° under a stream of nitrogen (time requirement: 2.5–3 hours). After the solvent was evaporated, the tubes were left for an additional 15 minutes in the water bath to assure

[7] As an alternative, the CG were added to a larger volume of plasma from which aliquots of 5 ml were taken, using a volumetric pipet.

[8] These tubes were previously siliconized by applying a thin coat of Dow-Corning High Vacuum Grease with a cotton-tipped applicator, followed by overnight heating at 150°. Special care was taken in this step to achieve a uniform coat.

complete drying. Finally, the tubes were placed in a vacuum desiccator over $CaSO_4$ until the time of analysis.

2. *Analysis.* One set of analyses consisted of plasma extracts containing given amounts of the CG (Figs. 2 and 3) and of extracts of plasma to which no drug was added. All the tubes were placed in ice-water and 200 μl of the cold incubation medium without ouabain (Table 1) was added to the extracts containing CG and to four tubes of blank plasma extracts. Four additional tubes of the blanks received 200 μl of the incubation medium containing excess ouabain (Table 1). The tubes were gently mixed by tapping and incubated for 15 minutes at 37° in a shaking water bath. This step was included to achieve complete solubilization of the CG prior to the enzymatic assay. The tubes were then returned to the ice-water bath and 200 μl of a 1:80 dilution (with 50 mM imidazole. HCl, pH 7.0) of the microsomal fraction were added. After gentle mixing, the tubes were incubated for 60 minutes at 37° with shaking, then placed in ice and the protein precipitated with 1.6 ml of cold 0.6 M $HClO_4$. Mixing at this step, which did not cause separa-

TABLE 2

ILLUSTRATIVE DATA ON THE INHIBITION OF Na,K-ATPase BY CARDIAC GLYCOSIDES[a]

Sample	Absorbancy at 700 nm	Average	Absorbancy corresp. to Na,K-ATPase	Percent inhibition
	0.586			
Absence of CG	0.580	0.583		
	0.582			
	0.587			
	0.424			
Presence of excess ouabain	0.426	0.424	0.159	
	0.423			
	0.426			
	0.540			
	0.541			
Presence of 5.12 ng digitoxin[b]	0.540	0.539	0.044	28.04
	0.537			
	0.545			
	0.532			

[a] In this experiment 5.12 ng digitoxin were added to 5-ml aliquots of plasma. After an extraction procedure the extent inhibition of Na,K-ATPase was determined as described in the text.

[b] The inhibition indicated was actually caused by $5.12 \times 0.8 = 4.09$ ng. The factor reflects the ratio of methylene chloride evaporated (8 ml) to the original volume used for the extraction (10 ml).

tion of the yellow pellet from the bottom of the tube, was accomplished by a vortex mixer adjusted to medium speed. The supernatant fluids were decanted into 70 × 12-mm tubes kept in an ice-water bath and centrifuged in the cold at 1700*g* for 10 minutes. One milliliter each of the clear supernatant fluid was pipetted into a new set of 70 × 12-mm tubes, 1 ml of the freshly prepared color reagent added and after 5 minutes at room temperature the absorbance of the samples at 700 nm measured against distilled water.

The results were expressed as percentage of inhibition by CG of the total Na,K-ATPase activity, as described above. A numerical example of the calculation is given in Table 2. In Figs. 2 and 3, the inhibition of the Na,K-ATPase is plotted against various amounts of digoxin and digitoxin. As in the experiments in which CG were directly added to the incubation mixture, digitoxin inhibited the enzyme more strongly. Whereas, 1 ng of this compound caused an inhibition of 14%, almost 2 ng of digoxin were needed to produce the same effect. The standard deviations ranged from ± 2.3 to ± 5.5% for digitoxin and from ± 4.5 to ± 7.6% for digoxin.

DISCUSSION

The enzymatic assay described here provides a specific and highly sensitive method for the determination of cardiac glycosides. The analytical procedure is applicable with good precision to the estimation of both digoxin and digitoxin in plasma. One to 15 ng of the two CG were determined in plasma with standard deviations ranging from ± 7.6 to ± 2.5%.

Although plasma separated from citrated blood (75 ml ACE-Formula 1 per 500 ml) was used in this work, control experiments with heparinized plasma were performed. Furthermore, for each experiment plasma from a different donor was obtained. These variations revealed no difference in the extent of enzyme inhibition by CG. Plasma extracts obtained from blood containing a variety of drugs, including ethacrynic acid, were shown to have no effect on the Na,K-ATPase system (20). This finding is substantiated by the successful clinical application of methods based on the inhibition of the Na,K-ATPase (2, 9). The procedure described here is presently applied to estimate plasma levels of CG in man in a study on drug equivalency. However, because of the sensitivity of the enzyme preparation toward both digoxin and digitoxin and due to the different extent of inhibition of the Na,K-ATPase by these CG, the analysis of plasma from patients receiving both drugs would give conflicting results.

The sensitivity of the assay is illustrated by the determination of

1 ng each of digoxin or digitoxin. In the course of this study even amounts of 0.7 ng of digitoxin were successfully quantitated in plasma. In the present work 5 ml plasma were used for each determination. The sensitivity of the method being 1 ng, this amount of plasma would allow the estimation of 0.2 ng of CG per ml. In the case of fully digitalized patients the requirement for plasma would be considerably reduced: assuming levels of 1.5 ng digoxin and 25 ng digitoxin per ml plasma (1, 2), 1 ml and 0.04 ml, respectively, would be needed.

The method offers the advantage of simplicity over the more elaborate and expensive procedures providing similar sensitivity. These involve the use of radioactively labeled compounds and/or specific antibodies (7–11). Owing to its requirement of relatively simple laboratory techniques and equipment, the method should prove valuable for providing data for the control of CG therapy in hospitals equipped with an average clinical laboratory. The preparation and characterization of the microsomal fraction used in the described method requires 2 working days and the material can be conveniently stored at low temperatures without loss of biological activity: after 1 year at −70°, the specific activity was determined as 16.2 μmoles P_i/mg protein per hour as compared to the initial value of 16.6. Assuming the analysis of 40 samples (tubes) per day, the microsmal fraction prepared from two beef brains should last for more than 400 days. In this study two batches of the microsomal Na,K-ATPase were prepared according to the described procedure. Both preparations contained the same level of enzymatic activity and exhibited similar sensitivity toward digoxin and digitoxin.

In applying the method to plasma several steps are of particular importance. If the plasma extract is not thoroughly dry, the pellet shows a tendency to separate from the bottom of the tube. This floating residue, which cannot be removed by centrifugation then interfers with the measurement of the absorbance. By following the procedure described, including the use of siliconized tubes, this possible interference is completely avoided. In addition, the siliconizing of the tubes in which the organic solvent was evaporated considerably improved the precision of the method. The latter is illustrated by the standard deviations which for 1–15 ng of CG ranged from ± 7.6 to ± 2.3%.

The inhibition curves for digoxin and digitoxin obtained both after direct addition of the CG to the incubation medium and after preceding extraction from plasma agree closely (Figs. 2 and 3), showing high recovery of the drugs from the biological material. A slightly stronger inhibition by CG was obtained in the presence of plasma extracts.

The reasons for the considerably lower sensitivity toward digitoxin (20 ng were estimated with a standard deviation of 19%) and for the

unsuccessful application of the previously reported method, applying the same principle (12), to determine digoxin could be manifold. One possible explanation might be a different sensitivity of the two enzyme preparations towards CG. Burnett and Conklin (12) used preparations from pork brain as a source of the Na,K-ATPase. The preparation included a NaI treatment of the microsomal fraction as described by Nakao *et al.* (21). In addition to the species difference (beef cerebral cortex was used in the method described here), it is possible that the salt treatment unfavorably affected the sensitivity of the enzyme preparation toward CG. An additional difference between the two enzyme preparations was accentuated in their stability during storage at low temperatures. Burnett and Conklin reported a loss of up to 20% enzymatic activity over a period of 2 months in comparison to the complete stability of our preparation for more than 1 year. Furthermore, in the latter work the recovery of digitoxin at the level of 20 ng dropped to 90% and at 10 ng to 60%. Finally, in view of the considerable effects of K on the inhibition of the Na,K-ATPase by CG (Fig. 1) the final concentration of 3 mM used by Burnett and Conklin does not represent an optimal condition for the inhibition.

SUMMARY

A specific and highly sensitive enzymatic method for the determination of cardiac glycosides has been developed. The method is based on the inhibition of the Na,K-ATPase in a microsomal preparation from beef cerebral cortex. The procedure, which involves simple laboratory techniques and equipment, has been applied to biological material. One to 20 ng of digoxin or digitoxin were analyzed in human plasma with a standard deviation of ±7.5% to ±2%.

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