MAGNESIUM-INDUCED INNER MEMBRANE AGGREGATION IN HEART MITOCHONDRIA

Prevention and Reversal by Carboxyatractyloside and Bongkrekic Acid

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ABSTRACT

Mg\(^{2+}\) at an optimal concentration of 2 mM (pH 6.5) induces large increases (up to 30\%) in the optical density of bovine heart mitochondria incubated under conditions of low ionic strength (< approx. 0.01). The increases are associated with aggregation (sticking together) of the inner membranes and are little affected by changes in the energy status of the mitochondria. Virtually all of a number of other polyvalent cations tested and Ag\(^+\) induce increases in mitochondrial optical density similar to those induced by Mg\(^{2+}\), their approximate order of concentration effectiveness in respect to Mg\(^{2+}\) being: La\(^{3+}\) > Pb\(^{2+}\) = Cu\(^{2+}\) > Cd\(^{2+}\) > Zn\(^{2+}\) > Ag\(^+\) > Mn\(^{2+}\) > Ca\(^{2+}\) > Mg\(^{2+}\). With the exception of Mg\(^{2+}\), all these cations appear to induce swelling of the mitochondria concomitant with inner membrane aggregation. The inhibitors of the adenine nucleotide transport reaction carboxyatractyloside and bongkrekic acid are capable of preventing and reversing Mg\(^{2+}\)-induced aggregation at the same low concentration required for complete inhibition of phosphorylating respiration, suggesting that they inhibit the aggregation by binding to the adenine nucleotide carrier. The findings are interpreted to indicate (a) that the inner mitochondrial membrane is normally prevented from aggregating by virtue of its net negative outer surface charge, (b) that the cations induce the membrane to aggregate by binding at its outer surface, decreasing the net negative charge, and (c) that carboxyatractyloside and bongkrekic acid inhibit the aggregation by binding to the outer surface of the membrane, increasing the net negative charge.

KEY WORDS: heart mitochondria • inner membrane • aggregation • polyvalent cations • carboxyatractyloside • bongkrekic acid • surface charge

In the course of studies on the effects of polyvalent metal cations on adenine nucleotide-induced inner membrane contraction in heart mitochondria by the optical density (OD) method (28), we encountered considerable interference from rapid and extensive increases in mitochondrial OD induced by the cations. Increases of up to 40\% were observed and appeared to be largely reversed upon induction of inner membrane contraction. The principal objective of this study was to characterize this phenomenon so that interference from
it may be recognized or avoided in determinations of changes in mitochondrial volume and ultrastructure by the OD method.

Although virtually all of a number of polyvalent metal cations tested were found capable of inducing at low concentration marked increases in the OD of heart mitochondria, most of the studies reported here were carried out with Mg²⁺, the reason being that the other cations appeared to induce concomitant swelling of the mitochondria. The Mg²⁺-induced increase is shown to be due to aggregation (sticking together) of the inner membranes and thus to be unrelated to the increases in mitochondrial OD associated with adenine nucleotide-induced inner membrane contraction (26, 28) and energy-dependent contraction of the mitochondrial matrix (7, 19, 27). The aggregation is shown to be strongly influenced by the ionic strength and composition of the medium and by extremely small amounts of the inhibitors of the adenine nucleotide transport reaction carboxyatractyloside and bongkrekic acid.

MATERIALS AND METHODS

Bovine heart mitochondria were isolated according to the Nagarse procedure previously described (28). Respiratory activity, mitochondrial OD (800 nm), and pH were monitored simultaneously in rapidly stirred suspensions (8 ml) contained in the closed, thermostated (30°C) reaction chamber of the apparatus previously described (28). The concentration of mitochondrial protein in the incubation mixtures was 0.25 mg/ml. Other conditions of incubation are given with the results of the individual experiments reported. Mitochondria were prepared for electron microscopy exactly as previously described (28). Bongkrekic acid was generously donated by W. Berends (Technological University of Delft, The Netherlands), and carboxyatractyloside by A. Bonati (Inverni della Beffa, Milan, Italy) and S. Luciani (University of Padua, Italy).

RESULTS

Comparison of Changes in Mitochondrial OD and Ultrastructure

Fig. 1 shows the effect of 2 mM Mg²⁺ on the OD of heart mitochondria suspended in weakly buffered sucrose at pH 6.5. The cation induces a rapid, fairly large increase in the OD which can be largely prevented or reversed by adding carboxyatractyloside. The mitochondria of Fig. 1 were fixed for electron microscopy at the time indicated. As shown in Fig. 2, the large increase in OD induced by Mg²⁺ in mitochondria incubated in the absence of carboxyatractyloside is associated with aggregation of the inner membranes (Fig. 2b), and the reversal of the OD change brought about by carboxyatractyloside is associated with reversal of the aggregation (Fig. 2c). Electron micrographs of the mitochondria exposed only to carboxyatractyloside (not shown) were indistinguishable from those of the control (Fig. 2a), and electron micrographs of mitochondria treated with Mg²⁺ subsequent to carboxyatractyloside (not shown) were indistinguishable from those of mitochondria treated with Mg²⁺ before carboxyatractyloside (Fig. 2c). It is thus evident that the disorganized appearance of mitochondria treated with Mg²⁺ and then carboxyatractyloside (Fig. 2c) relative to that of the control mitochondria (Fig. 2a) was due to the presence of Mg²⁺ rather than to the mitochondria having been exposed to carboxyatractyloside or having undergone aggregation-deaggregation. This small change in ultrastructure presumably was responsible for the slight increase in OD observed upon exposure of carboxyatractyloside-treated mitochondria to Mg²⁺ (Fig. 1).

In previous reports (28, 29), we described a change in the ultrastructure of heart mitochondria associated with the binding of adenine nucleotides to the adenine nucleotide carrier. Like Mg²⁺-induced inner membrane aggregation, this change results in a substantial increase in mitochondrial OD and is strongly inhibited by carboxyatractyloside. To avoid confusion between the Mg²⁺-in-
FIGURE 2  Electron micrographs of the mitochondrial preparations of Fig. 1. Differential conditions were: (a) no additions; (b) 2 mM MgCl₂ added after 2 min of incubation; and (c) MgCl₂ addition followed after 3 min by 5 μM carboxyatractyloside. × 40,000.
duced change and this adenine nucleotide-induced change, we will briefly point out the distinguishing features of each. The adenine nucleotide-induced change involves a marked rounding up of the intracristal spaces (the clear spaces within the mitochondria of Fig. 2) and a marked decrease in the amount of this space, resulting in a substantial decrease in the overall intracristal + matrix space and in the formation of a large space between the inner and outer membranes on one side of the mitochondria. It involves, in addition, a pooling of the matrix space with no apparent change in the density of the matrix. Although no quantitative measurements have been made, the nature and magnitude of these changes are such as to indicate clearly that the changes result from contraction (decrease in expanse) of the inner membrane. In contrast, Mg²⁺-induced inner membrane aggregation involves simply a sticking together of the outer surfaces of the inner membranes, resulting in the virtual disappearance of some of the intracristal spaces and in a great expansion of others (Fig. 2). There is no indication of a pooling of the matrix space and, ordinarily, little or no indication of retraction of the overall intracristal + matrix space from the outer membrane. The outer membranes are usually observed to be very closely associated with the inner membranes regardless of whether or not the mitochondria are in the aggregated state, and, where clearly visible, are usually seen to be somewhat battered and broken as a result of the mitochondria having been fixed before sedimentation (28).

The adenine nucleotide- and Mg²⁺-induced changes differ also in regard to the effect of bongkrekic acid, which, like carboxyatractyloside, is a potent inactivator of the adenine nucleotide carrier (9, 16, 18, 32). Whereas this agent strongly inhibits the Mg²⁺-induced change, it enhances adenine nucleotide-induced inner membrane contraction when the concentration of the nucleotide is too low to saturate the adenine nucleotide carrier (26, 29), and it induces inner membrane contraction in the absence of added adenine nucleotides when supplied at concentrations greater than that needed for complete inactivation of the carrier (29).

**Comparison of Mg²⁺ with Other Cations**

As was noted in the introductory paragraph, a number of cations other than Mg²⁺ induce inner membrane aggregation in heart mitochondria. For most of the cations tested, the relationships between cation concentration and the maximum increase in mitochondrial OD occurring within 2 min after addition of the cation are presented in Fig. 3. The observed order of effectiveness in respect to concentration was La³⁺ > Pb²⁺ = Cu²⁺ > Cd²⁺ > Zn²⁺ > Ag⁺ > Mn²⁺ > Ca²⁺ > Mg²⁺. Fe³⁺ was also tested and was found to cause such rapid swelling of the mitochondria at the lowest concentration eliciting a response (20 μM) that little aggregation could be detected.

With the exception of Mg²⁺, all the cations tested in the experiment of Fig. 3 appeared to induce swelling as well as aggregation at the concentrations indicated to give maximum aggregation. This is demonstrated in the case of La³⁺ in Fig. 4, which presents the Mg²⁺ and La³⁺ recorder tracings of the experiment of Fig. 3. It may be seen that La³⁺ at a concentration of 3 μM induces a slow increase in mitochondrial OD similar to that induced by 1 or 2 mM Mg²⁺, and that increasing the concentration of La³⁺ to 10 μM or beyond results in a very rapid increase in mitochondrial OD which is followed within a few seconds by a rapid and extensive decrease, indicating rapid and extensive swelling of the mitochondria. Although, like the maximum extents of the increases in OD induced by La³⁺ and most of the other cations that cause aggregation, the maximum extent of the Mg²⁺-induced increase was observed to increase up to a certain concentration of the cation and then to decrease (Figs. 3 and 4),
there was no indication of the decrease being due to swelling of the mitochondria. As may be seen shortly, the decrease in this case likely resulted from the accompanying increase in ionic strength of the medium.

Some of the cations caused aggregation of the particles as well as aggregation of the inner membranes and swelling. This was evidenced by the appearance of clumps of mitochondria in the suspensions and by apparent decreases in mitochondrial OD. To distinguish between decreases in OD due to particle aggregation and those due to mitochondrial swelling, both types of aggregation were reversed by inactivating the cations by chelating them with ethylenediaminetetraacetic acid (EDTA). In several cases, the simultaneous occurrence of swelling and aggregation was observed to result in very complex OD responses. For example, addition of 0.1 mM Cu²⁺ was observed to result first in a rapid increase in mitochondrial OD, followed in turn by a rapid decrease, another rapid increase, a slower decrease, and finally a slow increase, all within 2 min.

The monovalent cations, K⁺, Na⁺, NH₄⁺, and imidazole-H⁺ at concentrations comparable to those used in the experiment of Fig. 3, have little or no effect on the OD of heart mitochondria. However, virtually all salts of these cations inhibit Mg²⁺-induced aggregation at fairly low concentration. This is shown in the experiment of Fig. 5, in which the buffers imidazole-HCl, K-MES, and potassium piperazine-N,N'-bis(2-ethanesulfonate) (K-PIPES) and a number of salts in media buffered with 5 mM imidazole-HCl were compared at pH 6.5. It is evident that although simply increasing the ionic strength of the medium inhibits Mg²⁺-induced aggregation, the degree of inhibition elicited by a given increase is quite dependent on the nature of the anion. Thus, Pi, sulfate, and oxidizable substrate anions were observed to be particularly inhibitory. The inhibitory effect of the oxidizable substrates seems not to have occurred as a result of stimulation of respiration or energization of the mitochondria, because inhibitors of the respiratory chain and uncouplers of oxidative phosphorylation have little or no effect on either the aggregation or the inhibition of the aggregation by the substrates. It thus appears that the aggregation does not depend on active accumulation of Mg²⁺ or on the formation of an electrical potential difference across the inner membrane.

**Effect of pH**

The effect of pH on Mg²⁺-induced inner membrane aggregation over the range 5.5-8.0 was determined, using Mg²⁺ at five concentrations ranging from 0.5 to 8 mM. The results (Fig. 6) show that aggregation occurs over a wide pH

![Figure 4](image4.png)

**Figure 4** Recorder tracings from which the Mg²⁺ and La³⁺ data of Fig. 3 were obtained.

![Figure 5](image5.png)

**Figure 5** Effects of various monovalent cation salts on Mg²⁺-induced inner membrane aggregation. The media contained 150 mM sucrose and were buffered at pH 6.5. The buffers imidazole-HCl (Imi Cl), K-MES, and K-PIPES were added alone before the mitochondria. All other salts were added 1 min after the mitochondria to media buffered with 5 mM imidazole-HCl. In the cases of salts having significant buffering capacity at pH 6.5, the concentrations indicated refer to the species that buffer. The mitochondria were preincubated for 2 min, and aggregation was induced by adding 3 mM MgCl₂. The extents of aggregation were obtained from the maximum increases in mitochondrial OD occurring within 1 min after MgCl₂ addition and are presented relative to the extent of aggregation observed in the 5 mM imidazole-HCl medium. Abbreviations not previously identified are: Ac, acetate; Pyr, pyruvate; Pi, inorganic phosphate; and Succ, succinate.
range and that the concentration of Mg\(^{2+}\) that gives maximum aggregation shifts upward as the pH is increased. At pH 5.5, addition of Mg\(^{2+}\) results in a decrease in mitochondrial OD rather than an increase. The decrease was preceded during the 2-min preincubation period by a marked increase in mitochondrial OD, suggesting that it was due to reversal of a spontaneous type of aggregation or of some other change that increases mitochondrial OD. No attempt was made to determine what the change might be.

**Effects of Carboxyatractyloside and Bongkrekic Acid**

These inhibitors of the mitochondrial adenine nucleotide transport reaction (9, 16, 18, 32) were found to be exceptionally effective inhibitors of Mg\(^{2+}\)-induced aggregation. As shown in Fig. 7, carboxyatractyloside inhibits aggregation maximally at the same low concentration needed for complete inhibition of phosphorylating respiration and adenosine diphosphate (ADP)-induced inner membrane contraction, indicating that the inhibitor affects aggregation primarily through binding to the adenine nucleotide carrier or to an inner membrane component closely associated with the carrier. Atractyloside is equally as effective as carboxyatractyloside as an inhibitor of aggregation, but is slightly less effective as an inhibitor of phosphorylating respiration and inner membrane contraction, because the ADP used in the measurement of these activities competitively displaces some of the bound atractyloside under conditions of low atractyloside concentration (1).

Bongkrekic acid is somewhat less effective than the atractylosides in regard to maximum inhibition of Mg\(^{2+}\)-induced inner membrane aggregation. However, as shown in Fig. 8, it is approximately
equally as effective as the atractylosides in regard to half-maximal inhibition of the aggregation. Although, like the atractylosides, bongkrekic acid apparently binds tightly and specifically to the adenine nucleotide carrier or to a component closely associated with it (5, 15-17), the rate of binding, as indicated by direct binding studies (17) and studies on a number of activities involving the adenine nucleotide carrier (5, 9, 12-17, 26, 29), is slow relative to that of the atractylosides and is strongly dependent on the pH of the incubation mixture. To find out whether this pH dependence is indicated also by inner membrane aggregation, parallel determinations of the effects of the inhibitor on phosphorylating respiration and inner membrane aggregation were carried out at pH 6.5 and 7.5. The results show that whereas, in accord with previous observations (12, 29), increasing the pH from 6.5 to 7.5 markedly decreases the effectiveness of bongkrekic acid as an inhibitor of phosphorylating respiration, changing the pH over this range has little effect on bongkrekic acid inhibition of aggregation.

Because of the differing requirements for the assay of phosphorylating respiration and aggregation, it was not possible to carry out the determinations under identical conditions. Thus, it was necessary to include respiratory substrates, ADP, and Pi, in the assay of phosphorylating respiration and to exclude these agents in the assay of inner membrane aggregation. Previous studies on the effects of bongkrekic acid on activities involving the adenine nucleotide carrier indicate that Pi slows the binding of the inhibitor slightly (13) and that ADP accelerates it (5, 13, 17). In the experiment of Fig. 8, which included determination of the effect of bongkrekic acid on phosphorylating respiration at pH 5.5 as well as at pH 6.5 and 7.5, an accelerating effect of ADP on the binding was clearly evident at pH 6.5 but not at pH 5.5 and 7.5. Thus, under conditions of pH 6.5 and bongkrekic acid concentration less than that required for maximum inhibition, the rate of phosphorylating respiration, initiated by the addition of ADP, decreased within 1 min to the extent that the level of inhibition at pH 6.5 corresponded to that indicated by the initial rate of phosphorylating respiration at pH 5.5 (Fig. 8). This and the observation that there was no similar decrease in the rate under conditions of pH 5.5 indicate that under the pH 5.5 conditions the binding of bongkrekic acid was complete or very nearly complete by the time the phosphorylating respiration was initiated. This was confirmed in separate experiments in which the period of time for which the mitochondria were preincubated in the presence of the inhibitor was varied. The results (not shown) indicate that binding equilibrium is reached within 30 s under these conditions. Since mitochondria bind bongkrekic acid and carboxyatractyloside with approximately equal affinity (17, 31, 32), the results of Fig. 8, showing that the minimum amount of bongkrekic acid required for complete inhibition of phosphorylating respiration is essentially the same as that observed in the case of carboxyatractyloside (Fig. 7), are thus in accord with previous observations (16, 17) indicating that the number of high affinity binding sites in mitochondria is the same for bongkrekic acid as for the atractylosides. These observations and the results of direct (14, 15) and indirect (29) adenine nucleotide binding studies indicate that the inhibitors bind in 1:1 stoichiometry with the adenine nucleotide carrier.

DISCUSSION
Mechanism of Inner Membrane Aggregation
Aggregation phenomena in biological membrane systems have been studied extensively (3, 11, 20, 22, 33-37) and a number of theories have been proposed to account for the observations. The most successful theories have been based on the theory of lyophobic colloid stability (23, 30), the fundamental premise of which is that the total
energy of interaction between two similarly charged surfaces consists of an electrostatic repulsive energy and a van der Waals attractive energy. The repulsive energy depends on conditions in the electrical double layers at the surfaces and can be lowered either by increasing the ionic strength of the medium or by decreasing the net charge on the surfaces by specific ion binding. Thus, on reduction of the repulsive energy, the surfaces come together as a result of van der Waals attraction. Once they have made contact, more specific chemical and physical forces may come into operation (25).

Electrophoretic studies on mitochondrial membrane fractions (8) indicate that the outer surfaces of the inner membranes of isolated mitochondria are negatively charged at physiological pH. According to the colloid stability theory, it is this charge and the consequent diffuse layer of counter ions that prevent the membrane surfaces from coming together. Binding of cations at the membrane surface would decrease the charge and probably was responsible for the cation-induced inner membrane aggregation observed in this study. Thus, all the cations found here to induce aggregation are known to bind to biological membranes (24), and the observed order of their effectiveness (Fig. 3) is generally consistent with binding affinity data (10, 24, 36, 37).

According to the colloid stability theory, an increase in ionic strength of the medium should contract the diffuse layers of counter ions at the membrane surfaces and thereby tend to promote aggregation. Contrary to this prediction, it was found in the case of Mg<sup>2+</sup>-induced aggregation that increasing the ionic strength even slightly results in marked inhibition, and that the degree of inhibition for a given increase varies considerably depending on the anion used. Of the anions tested sulfate, phosphate, pyruvate, and succinate were observed to be the most effective inhibitors (Fig. 5). These anions are known to penetrate the inner membranes of mitochondria by carrier mechanisms, and the characteristics of the transport processes are such as to indicate the unbound carriers to be positively charged (2, 21). Binding of these carriers with their substrates could increase the net negative charge at the outer surface of the inner membrane and thus could inhibit aggregation.

The observed general inhibitory effect of salts on Mg<sup>2+</sup>-induced aggregation (Fig. 5) appears not to be consistent with the colloid stability theory. However, account must be taken of the fact that biological membrane surfaces are much more complex than the simple charged surfaces with which this theory was meant to deal. For example, in contrast to the surfaces of simple colloids, the surfaces of biological membranes have an abundance of proton-binding groups with pK's in the physiological pH range. In consequence, the net surface charge depends on the pH at the membrane surface. Under conditions of low ionic strength, the pH at the surface of negatively charged membranes in aqueous media can be expected to be considerably lower than the pH of the bulk medium, because the scarcity of counterions for the negatively charged groups under these conditions results in a concentration of protons near the membrane surface (6). Increasing the ionic strength of the medium allows the protons to move away from the surface and thus results in an increase in the surface pH. In consequence, protons dissociate from neutral and positively charged groups at the membrane surface, increasing its net negative charge. Although it seems unlikely in view of the observed effects of changing the pH of the bulk medium on Mg<sup>2+</sup>-induced aggregation (Fig. 6), it is nevertheless conceivable that, in the case of the inner mitochondrial membrane, increasing the ionic strength of the medium decreases the tendency for aggregation more by this mechanism than it increases the tendency for aggregation by contracting the diffuse layer.

It seems more likely that the observed general inhibitory effect of salts occurred as a result of the salts inhibiting the binding of Mg<sup>2+</sup> to the membrane surface, such as by increasing the desolvation energies of Mg<sup>2+</sup> and the surface groups to which it binds. A mechanism of this general type seems consistent with the results of preliminary studies suggesting that aggregation induced by cations that appear to bind more tightly than Mg<sup>2+</sup> (Fig. 3) is considerably less sensitive to changes in ionic strength. Because of uncertainties arising from the induction of mitochondrial swelling by polyvalent cations other than Mg<sup>2+</sup>, no detailed studies of this type were conducted.

**Mechanism of Inhibition by Carboxyatractyloside and Bongkrekic Acid**

Bongkrekic acid has three carboxyl groups (4) and carboxyatractyloside two sulfonic acid and two carboxyl groups (18, 32). In consequence, these inhibitors are negatively charged at physiological pH. Their inhibitory effect on Mg<sup>2+</sup>-in-
duced aggregation (Figs. 7 and 8) can be explained satisfactorily by assuming them to bind at or near the outer surface of the inner membrane and thereby to increase its net negative charge. Both inhibitors are known to bind tightly to the inner membrane, and, in the case of carboxyatractylate, present evidence indicates the binding site to be located at or near the outer surface (31).

Previous studies have suggested that bongkrekic acid binds either within the inner membrane or at its inner surface, the principal evidence being the marked pH dependence of the binding. The inhibitory effect of high pH has been interpreted to mean that the inhibitor must enter either the inner membrane or the matrix phase to gain access to its site of binding, and that only the fully protonated (uncharged) form of the inhibitor can do so (5, 12, 14, 15, 17, 26, 29).

Inner membrane contraction and direct adenine nucleotide binding studies suggest that bongkrekic acid decreases the activity of the adenine nucleotide carrier by preventing the movement of its nucleotide binding site from the inner surface of the inner membrane to the outer surface without preventing the binding and release of adenine nucleotides. Thus, adenine nucleotide-induced inner membrane contraction occurs in bongkrekic acid-treated mitochondria whether the nucleotide binding site of the carrier is located at the outer surface of the inner membrane or at the inner surface (29), and addition of an adenine nucleotide to bongkrekic acid-treated mitochondria in which the nucleotide binding site of the carrier is located at the outer surface results in an amount of the nucleotide equal to the amount of carrier being irreversibly bound in the mitochondria and being rapidly mixed with the adenine nucleotides of the matrix (14).

The observation of the present study that bongkrekic acid at a concentration comparable to the concentration of adenine nucleotide carrier markedly inhibits Mg²⁺-induced inner membrane aggregation (Fig. 8) suggests that the inhibitor prevents the outward movement of the nucleotide binding site of the carrier by binding at or near the outer surface of the inner membrane either directly to the carrier or to an inner membrane component present at the same concentration as the carrier. That the inhibitor does not inhibit aggregation by causing a shift in the position of the nucleotide binding site of the carrier from an outer location to an inner location is shown by previous studies indicating that this transition occurs only upon addition of adenine nucleotides (14, 26, 29).

The observation that, in contrast to what is observed in other types of studies on the inhibitory action of bongkrekic acid, increasing the pH of the medium from 6.5 to 7.5 has little effect on the inhibition of aggregation (Fig. 8) is difficult to understand at this point. Additional studies are needed to determine whether this discrepancy can be explained by the differences in the conditions of incubation used. It is conceivable, for example, that, under the relatively low ionic strength conditions used in the aggregation study, bongkrekic acid binds rapidly at pH 7.5 by virtue of the relatively low pH that might be expected to exist at the outer surface of the inner membrane under these conditions (6).

We are grateful to Dr. H. R. Blackwell for generously providing us with laboratory facilities in the Institute for Research in Vision, and to Miss Karen Willardson for secretarial assistance.

This work was supported by grants from the Rosenstiel Foundation, the Central Ohio Heart Chapter, Inc., and the United States Public Health Service (Research grant HL 18038 from the National Institutes of Health).

Received for publication 4 April 1977, and in revised form 7 October 1977.

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