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THE ROLE OF MEMBRANE-BOUND MAGNESIUM IN THE PERMEABILITY OF GHOSTS TO K^+

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SUMMARY

Mg^{2+} is required for restoring a low cation permeability of erythrocyte membranes after osmotic lysis. These ions promote binding of haemoglobin to the ghost membrane. Because the optimal binding occurs at a pH where the K^+ retention by ghosts is maximal, the possibility exists that Mg^{2+} exerts an indirect effect by facilitating the adsorption of haemoglobin, which may be essential for the maintenance of a low permeability. In order to investigate this possibility, a systematic study was done of the effects of pH and hypotonic washing on the retention of haemoglobin, Mg^{2+} and K^+ by human erythrocyte ghosts.

Between pH 5.5 and 7.5, the retention of haemoglobin paralleled that of K^+ and Mg^{2+} on resealing immediately after lysis. Such a parallelism was not observed if ghosts were both washed with fresh haemolytic media and resuspended in a medium of lower osmolarity before reversal.

No correspondence was found between ghost K^+ content and membrane-bound haemoglobin, indicating that the latter is not involved in the maintenance of a low permeability. By contrast, Mg^{2+} binding was altered by pH in the same way as ghost K^+ and a strict relationship between membrane-bound Mg^{2+} and K^+ retention was obtained.

The results suggest that K^+ permeability is mainly determined by the amount of Mg^{2+} associated with the ghost membrane.

INTRODUCTION

Mg^{2+} has been implicity accepted as a requirement for restoring a low permeability of erythrocyte membranes. Thus, conventional techniques always have made use of this ion for resealing the ghosts [1–5]. However, a strict correspondence between membrane Mg^{2+} content and ghost permeability has not been established.

Mg^{2+} promotes the binding to erythrocyte membranes of haemoglobin as well as other proteins [6, 7] and there is a Mg^{2+}-dependent relationship between ghost haemoglobin and K^+ permeability under certain conditions (Romero, P. J., unpublished). Moreover, optimal resealing to K^+ occurs at pH values where haemo-

globin binding is nearly maximal [4]. The question then arises as to whether the lowering of permeability can be entirely accounted for by a direct interaction of Mg^{2+} with the red cell membrane. Alternatively, Mg^{2+} may have an indirect effect promoting the adsorption of haemoglobin or other protein(s), which may be essential for the maintenance of a low cation permeability.

The purpose of the present work was to investigate these possibilities in human erythrocyte ghosts. A tight relationship between K^+ retention and membrane-bound Mg^{2+} was found, suggesting that K^+ permeability is mainly determined by the Mg^{2+} content of the ghost membrane.

MATERIALS AND METHODS

Whenever possible analytical grade reagents were obtained from BDH Chemicals, England. All solutions were prepared in glass-distilled water and the pH was adjusted at room temperature within a range of $\pm$ 0.02 units, using a Radiometer TTT-1c pH-meter with scale expander.

Preparation of ghosts

Packed cells from human blood which had been stored for 3–4 weeks at 4 °C in acid–citrate–dextrose solution were prepared as described previously [8]. One volume of these cells was lysed under vigorous stirring at 4 °C in 30 vol. of a medium containing 2 mM $MgCl_2$ and 10 mM Tris–acetate buffer at the required pH. The lysate was divided in two lots.

The isotonicity of the first portion was restored 1 min after lysis by adding a sufficient amount of a 3 M KCl solution. Resealing was completed by a 30-min incubation at 37 °C in a water-bath shaking incubator [9]. The ghosts were centrifuged for 10 min at 20 000 $\times g$ in an International HR-1 refrigerated centrifuge at 4 °C and washed twice by resuspension and centrifugation in 30 vol. of a 160 mM NaCl+20 mM Tris–HCl medium, buffered at pH 7.6. They were finally lysed in 100 vol. of distilled water for chemical analysis. This lysate is referred to as the original lysate.

Ghosts from the remaining portion were washed 3 times with 30 vol. of fresh haemolytic medium and resuspended in a similar volume of a 2 mM $MgCl_2$+1 mM Tris–acetate medium, at the same pH. After stirring for 1 min, the isotonicity was restored as described above and ghosts were further treated as those from the first portion.

Determinations of ghost dry weight

Ghosts were dried overnight at 140 °C and the dry weights determined.

Measurements of haemoglobin

Ghost haemoglobin was estimated by the pyridine haemochromogen method [10] in the presence of 0.2 vol. of 10% Triton X-100 in 8 M urea. In some experiments it was referred to non-haemoglobin ghost dry weight, which was obtained by deducting the amount of haemoglobin from the corresponding ghost dry weight. Duplicate samples usually agreed within 10%.

Estimations of ghost cation content

K^+ and Mg^{2+} were determined by flame emission at 766.3 nm and atomic

absorption at 285.1 nm, respectively, using a Varian Techtron 1000 spectrophotometer. The error associated with these determinations was always below 10%.

Measurements of haemoglobin and Mg^{2+} bound to membranes

A known volume of the original lysate was centrifuged at 40 000 $\times g$ for 30 min at 4 °C. The supernatant solution was removed by suction and carefully replaced by an identical volume of glass-distilled water in order to eliminate trapped fluid without disturbing the sedimented membranes. These were later solubilized with 0.2 vol. of 10% Triton X-100 in 8 M urea and aliquots were withdrawn for analysis of haemoglobin and Mg^{2+}.

Membrane haemoglobin and Mg^{2+} contents were referred to non-haemoglobin ghost dry weight. Triplicates were always run and their agreement was usually above 90%. The recovery obtained with the above procedure was never lower than 90%.

RESULTS

Influence of pH on ghost composition

The entrapment of haemoglobin by erythrocytes ghosts is affected by pH [10, 11] and by the presence of divalent cations [12]. It was of interest to investigate

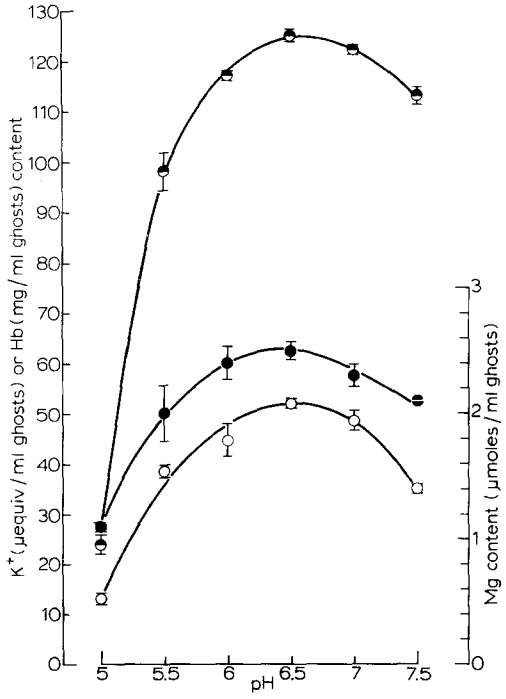

Fig. 1. Parallel changes with pH of haemoglobin, K^+ and Mg^{2+} contents of ghosts resealed immediately after lysis. Human red cells were lysed in the presence of 2 mM Mg^{2+} at the various pH values indicated above. After resealing with K^+, the ghosts were washed twice with a Na^+ medium and the amount of haemoglobin (○), K^+ (◓) and Mg^{2+} (●) retained was estimated. Results are given as mean values ±S.D. of at least three experiments.

whether a change in pH at a constant Mg^{2+} concentration, had a similar effect on the permeability to K^+ and Mg^{2+}. In addition, to test if the lowering of haemoglobin is associated with a proportional decrease in the retention of these ions, ghosts were repeatedly washed with fresh haemolytic media. They were then resuspended in a medium of lower osmolarity before reversal since it is known that a second lysis occurs under this condition [3].

Fig. 1 shows that in unwashed ghosts, the retention of haemoglobin is paralleled by a concomitant capture of cations Thus, varying the pH from 5 to 6.5 doubled the ghost Mg^{2+} concentration and increased 5-fold the contents of haemoglobin and K^+.

Similarly, after hypotonic washing the contents of K^+ (in μequiv/ml ghosts) and Mg^{2+} (in μmoles/ml ghosts) are simultaneously increased to a maximum of 80 and 2, respectively, and decreased in somewhat parallel fashion by further altering the pH to 7.5 (Fig. 2). Although the retentions of haemoglobin and K^+ are considerably reduced after washing, ghost haemoglobin is only slightly affected by pH.

These results show that the changes in cation content are not mirror images of those in haemoglobin, suggesting cation permeability is not related to ghost haemoglobin. On the other hand, the parallelism found in both types of ghosts between Mg^{2+} and K^+ retention as a function of pH suggests that the permeability depends on internal Mg^{2+}. The results also show that there is an optimal pH for cation retention, where a low K^+ permeability seems to be partially recovered after hypotonic washing. Mg^{2+} permeability, by contrast, appears completely restored to low levels at this pH.

The alteration by pH of haemoglobin and Mg^{2+} binding to membranes

As an optimal pH for cation retention was found, Mg^{2+} and haemoglobin

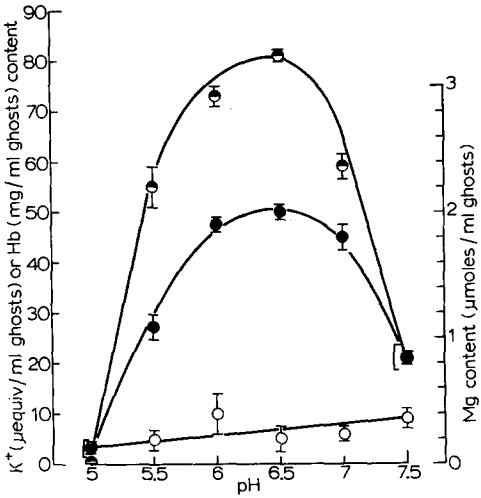

Fig. 2. The alteration of haemoglobin, K^+ and Mg^{2+} retention by hypotonic washing. Red cells were both lysed and washed with hypotonic media at the pH values given above and in the presence of 2 mM Mg^{2+}. They were resuspended in a low osmolarity medium before reversal and treated as described in Fig. 1. The concentrations of haemoglobin (○), K^+ (◓) and Mg^{2+} (●) are shown as mean values ±S.D. of three experiments.

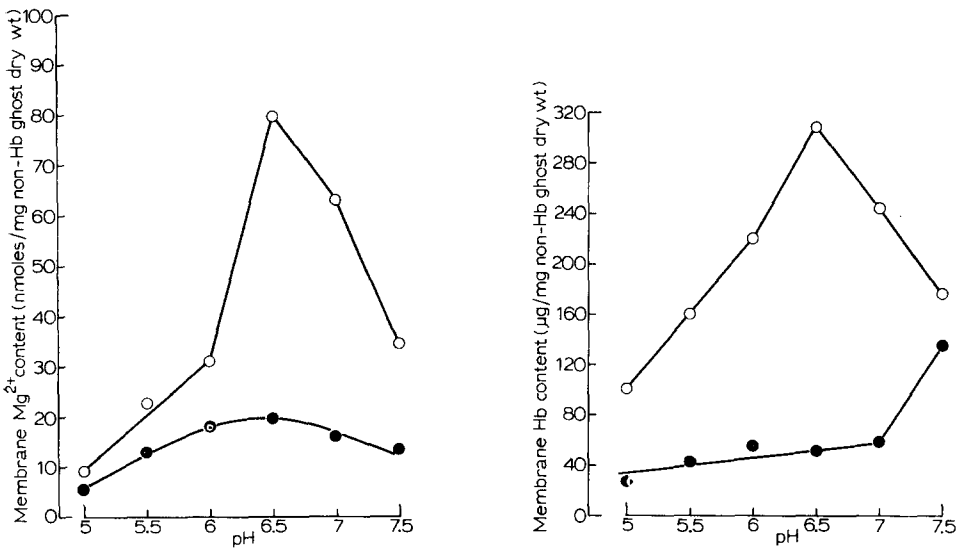

Fig. 3. The influence of pH on Mg^{2+} and haemoglobin binding to ghosts. The membranes of ghosts resealed immediately after lysis (○) and ghosts washed with hypotonic media before reversal (●) were solubilised and analysed for Mg^{2+} (Part a) and haemoglobin (Part b). The results shown are the mean values of at least two experiments.

were estimated in membranes from washed and unwashed ghosts to ascertain if their bindings were also optimal at the same pH.

Fig. 3a shows that in unwashed ghosts, membrane Mg^{2+} increases 8-fold by varying the pH from 5 to 6.5 and becomes halved at pH 7.5. Lesser amounts are found after washing and the content (in nmoles/mg non-haemoglobin ghost dry wt) only increases 4-fold at pH 6.5 and is slightly diminished from about 20 to 14 by further raising the pH. These results indicate that there is an optimal pH for Mg^{2+} binding which is identical to that for cation retention.

The associated changes in haemoglobin were markedly different for both types of ghosts. Thus, as pH varies from 5 to 7.5, the membrane haemoglobin of unwashed ghosts (in μg/mg non-haemoglobin ghost dry wt) sharply passes through a maximum value of about 310 at pH 6.5 (Fig. 3b). After washing and resuspending in a low osmolarity media, by contrast, it is more or less steadily raised from about 20 to 60 as the pH alters from 5 to 7. However, it abruptly increases to nearly 140 at pH 7.5. These findings demonstrate that the binding pattern of haemoglobin as a function of pH is considerably modified by hypotonic washing followed by resuspension in low osmolarity media.

The dependence of K^+ retention on membrane Mg^{2+}

In order to determine if there is a close correspondence between Mg^{2+} binding and the extent of K^+ retention, the K^+ contents of unwashed ghosts and ghosts resuspended in low osmolarity media after washing, were related to the corresponding membrane-bound Mg^{2+} from different experiments between pH 5 and 7.5.

A strict relationship was obtained (Fig. 4). Thus, no trapping of K^+ occurs when the amount of bound Mg^{2+} (in nmoles/mg non-haemoglobin ghost dry wt) is

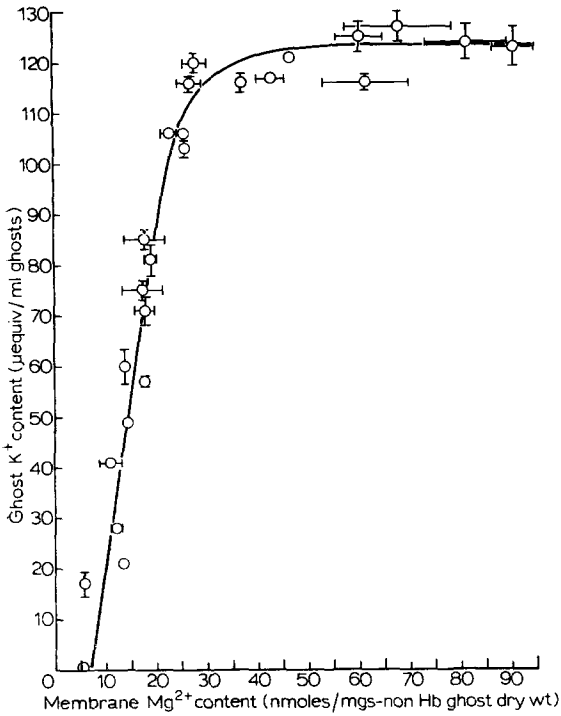

Fig. 4. The dependence of K^+ retention on bound Mg^{2+}. Membrane Mg^{2+} levels were varied by lysing and washing with hypotonic media at different pH values between 5 and 7.5, in the presence of 2 mM Mg^{2+}. The amount of K^+ retained by the ghosts was related to the corresponding membrane-bound Mg^{2+}. Collected results from at least twelve experiments are shown as mean values ±S.D. of three determinations on samples from replicate conditions. When bars are not shown, the S.D. is enclosed by the circle.

about 5. Raising this content up to about 25 causes a linear increase in K^+ retention. By further changing membrane Mg^{2+} to nearly 40, the K^+ content reaches its maximum value of about 125 μequiv/ml ghosts and remains constant at higher Mg^{2+} levels.

A large scattering of values was found when K^+ retention was related to membrane haemoglobin. Thus, the amount of K^+ retained by ghosts having the same haemoglobin content varied by a factor of about 4. Conversely, ghosts having haemoglobin concentrations differing from each other by a factor of 3 retained K^+ to the same extent. These results indicate that there is not a clear relationship between membrane haemoglobin and ghost K^+ content, but a general trend towards a relation was found, which may arise casually from a dependence of membrane haemoglobin on adsorbed Mg^{2+}.

In order to demonstrate such a correspondence a number of plots were made, including linear, reciprocal, log–linear, log–log and Hill plots. The latter fitted the experimental data best and is presented in Fig. 5. A linear correlation coefficient of 0.80 was obtained, indicating a good fit of the data. It appears, therefore, that the amount of haemoglobin in the ghost membrane is related sigmoidally to their Mg^{2+} content as the pH varies from 5 to 7.5.

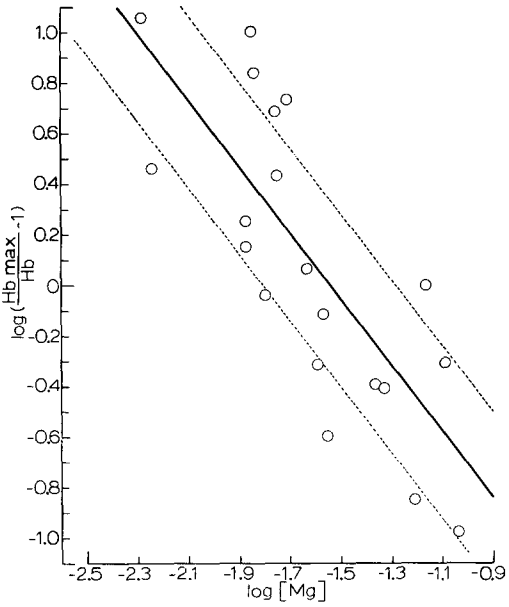

Fig. 5. The relationship between haemoglobin and Mg^{2+} binding to ghosts. A Hill plot of membrane-bound haemoglobin against the corresponding amount of bound Mg^{2+}. Each point is the mean value of duplicate determinations from a single experiment. The continuous line corresponds to the regression equation $Y = -2.03 - 1.31X$. The dashed lines show $\pm$S.E. of this equation.

The reversibility of the action of Mg^{2+}

To test if the amount of membrane-bound Mg^{2+} determines the extent of K^+ retention, ghosts were prepared by hypotonic washing at pH 5.5 in order to lower the bound Mg^{2+}. They were then divided in two portions. One lot was resuspended

TABLE I

THE REVERSIBILITY OF THE ACTION OF Mg^{2+} ON GHOST PERMEABILITY

Red cells were both lysed and washed with a hypotonic medium at pH 5.5. The ghosts were then resealed at pH 5.5 and 6.5 in a medium of lower osmolarity and in the presence of 2 mM Mg^{2+}. Ghost K^+ and Mg^{2+} contents and the amount of Mg^{2+} and haemoglobin bound to the membranes were determined after washing with an isotonic Na^+ medium. The results from 5 experiments are given as mean values $\pm$S.D. *P* denotes the probability obtained from Student's *t* tests.

	pH of resuspension medium		*P*
	5.5	6.5	
K^+ content (μequiv/ml ghosts)	63 $\pm$3.5	72 $\pm$4.5	< 0.05
Mg^{2+} content (μmoles/ml ghosts)	1.7$\pm$0.12	2.0$\pm$0.21	> 0.05
Membrane-bound haemoglobin (μg/mg non-haemoglobin ghost dry wt)	50 $\pm$8.6	34 $\pm$14.1	> 0.05
Membrane-bound Mg^{2+} (nmoles/mg non-haemoglobin ghost dry wt)	16 $\pm$1.2	20 $\pm$1.4	< 0.05

in a medium of lower osmolarity at pH 5.5 and the other in the same medium but at pH 6.5 to favour Mg^{2+} binding.

As was expected, the K^+ retention was significantly greater ($P < 0.05$) at pH 6.5 than at 5.5 (Table I). Similarly, Mg^{2+} binding was also raised in a statistically significant way ($P < 0.05$) whilst haemoglobin showed little changes when the pH was increased from 5.5 to 6.5.

These findings clearly demonstrate that the K^+ retention is determined by the level of membrane Mg^{2+} and not by that of haemoglobin. The results also show that within certain limits the action of Mg^{2+} is reversible.

DISCUSSION

It is well known that the binding of haemoglobin to erythrocyte membranes is markedly altered by pH at haemolysis, and is maximal at about 6.5 [10, 11]. A similar effect of pH on the K^+ retention was recently described in human erythrocyte ghosts [4]. These findings have been confirmed and extended in the present work. Thus, the retentions of K^+, Mg^{2+} and haemoglobin by ghosts resealed immediately after lysis, are optimal at pH 6.5 and decrease above or below it. However, if the ghosts are washed several times with hypotonic media and resuspended in a medium of lower osmolarity before reversal, haemoglobin retention does not show the same pH dependence as K^+ and Mg^{2+}.

These results demonstrate that the trapping of haemoglobin does not correspond to that of small ions. Moreover, haemoglobin is not directly related to the lowering of membrane permeability, as shown by the following findings. First, no clear relationship between haemoglobin binding and K^+ content was obtained. Secondly, ghosts washed with hypotonic media at pH 7.5 had more haemoglobin bound to their membranes but retained lesser amounts of K^+.

On the other hand, K^+ retention is paralleled by corresponding changes in the level of membrane Mg^{2+} and a strict relationship between bound Mg^{2+} and K^+ content was found at all pH values studied. Furthermore, if ghosts washed with hypotonic media at pH 5.5 are resealed at pH 6.5, both Mg^{2+} binding and K^+ retention are significantly increased whereas membrane haemoglobin remains practically unaffected. These observations clearly show that the amount of bound Mg^{2+} determines the extent of K^+ retention, thus indicating that the interaction of Mg^{2+} with the membrane accounts for the lowering of permeability. This is compatible with a requirement for ionic Mg^{2+}, as indicated by the fact that ghosts cannot reseal to K^+ if prepared in media containing Mg^{2+} and unbalanced amounts of chelating agents such as ATP or EDTA [2, 5].

The above point of view is different from that of Lepke and Passow [4], who interpreted the effect of pH on K^+ retention as due to the exposure of the ionizable groups which were formerly buried in the membrane, and which are capable of exerting control on the cation permeability.

The highest level of bound Mg^{2+} was obtained in ghosts resealed immediately after lysis at pH 6.5 and was about 80 nmoles Mg^{2+}/mg non-haemoglobin ghost dry wt or roughly equivalent to 0.4 μmole Mg^{2+}/ml ghosts. This value is slightly higher than that of 0.12 found after lysing in distilled water [14].

The Mg^{2+} content of membranes is decreased by hypotonic washing in the

presence of 2 mM Mg^{2+}, removal of Mg^{2+} being more marked at pH 6.5 and 7, where the haemoglobin retention by unwashed ghosts is maximal. As Mg^{2+} facilitates haemoglobin binding, it is expected that the removal of the latter by washing will cause a diminution of bound Mg^{2+}.

Membrane Mg^{2+} is also decreased by pH values below or above 6.5. Since H^+ compete very effectively with Mg^{2+} for binding sites in the membrane [15], the diminution of bound Mg^{2+} by decreasing the pH is to be expected from a displacement by H^+. The effect above pH 6.5 cannot be explained on similar grounds. Mg^{2+} adsorption to the ghost membrane at constant temperature and ionic strength, is fundamentally determined by the free ion concentration in the lysing and washing media. The amount of free Mg^{2+} may be appreciably reduced due to both $Mg(OH)_2$ formation and binding to haemoglobin, but at pH 7.5 this effect will be almost negligible in washed ghosts. Since negatively charged binding sites are presumably involved in the interaction of Mg^{2+} with the membrane and these cannot reduce their number by increasing pH, it is possible that the decrease in bound Mg^{2+} above pH 6.5 may be due to a release of membrane ligands. In this context, it is of interest to point out that some non-haemoglobin proteins are released from erythrocyte membranes after washing with hypotonic media at alkaline but not acid pH [7, 10].

These observations indicate that although Mg^{2+} alone can account for a reduction of the K^+ permeability, there are some unknown factors which affect its binding and are thus involved in the control of cation permeability.

In this line of thought it is tempting to speculate further on the role of Mg^{2+} and the action of Ca^{2+} previously described [8]. Mg^{2+} may act from the inside of ghosts by cross-linking some membrane molecules which are essential for maintaining the membrane integrity and hence a low cation permeability. The action of internal Ca^{2+} may be explained, perhaps, on the basis of a competition for Mg^{2+}, resulting in a rearrangement or conformational change of such membrane molecules so that cation permeability is increased. Experiments along this line are already in progress in our laboratory.

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