Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5208–5212, July 1990
Cell Biology

On the structure of erythrocyte spectrin in partially expanded membrane skeletons

(cytoskeleton/electron microscopy)

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Communicated by Daniel Branton, April 25, 1990 (received for review February 15, 1990)

ABSTRACT **Spectrin is generally believed to play an important role in the erythrocyte membrane's ability to deform elastically. We have studied the structure of negatively stained spectrin in partially expanded membrane skeletons to determine how its molecular structure confers elastic properties on the cell membrane. Fourier analysis of electron micrographs of spectrin reveals that the α and β subunits are twisted about a common axis, forming a two-start helix with twofold rotational symmetry. We propose that elastic deformation of the cell is mediated by transient extension of the helix by mechanical forces.**

In vivo the erythrocyte is elastically deformed during its turbulent passage through the circulation. Although the physicochemical basis of the erythrocyte's elasticity is unknown, it is generally regarded as being a property of the membrane skeleton. The skeleton and its component proteins have been studied extensively (1, 2); however, understanding the elastic properties of the skeleton in terms of the molecular properties of its component proteins is still a major unsolved problem in erythrocyte biology. Our work has focused on spectrin because, of the skeleton proteins, it alone appears to be able to undergo the reversible changes in structure necessary to account for the elastic properties of the erythrocyte.

Erythrocyte spectrin is present in the cell as $(\alpha\beta)_2$ tetramers, which associate with actin dodecamers and band 4.1 to form a planar network. Spectrin tetramers can be artificially extended in low ionic strength media to form a linear molecule that is 2000 Å long (3). However, calculations based on the number of spectrin molecules per unit area of membrane indicate that, in the erythrocyte, the average end-to-end distance of the tetramer is only 700 Å (4–6). This disparity raises questions about spectrin's conformation in the cell and the relationship of the artificially extended spectrin molecule to its native state.

Most electron micrographs of the skeleton have been obtained by using buffers of very low ionic strength in which spectrin is highly extended (7–9). Although low ionic strength represents a nonphysiological state, its use has been a convenient compromise allowing visualization of the distribution of the skeletal proteins. In its highly extended state in the skeleton, spectrin is a straight molecule that does not display a well-defined structure. However, by using conditions closer to the physiological range, it is possible to obtain micrographs of skeletons that are partially expanded. In the work described below we have examined electron micrographs of negatively stained spectrin molecules that are partially rather than fully extended. Fourier analysis of such micrographs reveals molecules in which the α and β subunits twist about one another forming a two-start helix. Both the pitch and the diameter of the helix are variable and are coupled in a manner that conserves a fixed contour length of 2000 Å. These observations led us to propose that the elastic properties of the erythrocyte are the result of variable extension of the spectrin helix in response to mechanical stresses.

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MATERIALS AND METHODS

Preparation of Membrane Skeletons. Ghosts were prepared by the method of Dodge *et al.* (10) from recently outdated blood-bank erythrocytes. Partial expansion of skeletons was achieved by dilution in either 2 mM sodium phosphate/0.05 mM $MgCl_2$/0.5 mM dithiothreitol, pH 8, or 0.2 mM sodium phosphate/0.05 mM $MgCl_2$, pH 8, on ice essentially as described (7, 8). Aliquots were applied to glow-discharge copper grids prepared with fenestrated carbon overlaid with a thin carbon film. Skeletons were rinsed with 0.1 mM sodium phosphate (pH 7) prior to negative staining with 1% uranyl acetate. Electron micrographs were recorded with a Philips EM300 electron microscope at an electron-accelerating voltage of 80 kV.

Computer Image Processing. Electron micrographs were digitized on an Optronics P-1000 rotating drum microdensitometer at a scanning raster of 12.5 μm (≈2.5 Å per pixel). Computations were performed on a DEC Microvax 3200 workstation using software written in this laboratory based upon standard algorithms (11–13). Images were displayed on either a Raster Technologies model one/25 (512 × 512 × 24) display system or a Raster Technologies model one/75 (1280 × 1024 × 8) system. Images were recorded onto photographic film with a Matrix 3000 color graphics recorder. Measurements of particle contour length were performed on enlarged prints of electron micrographs by using a Macintosh SE computer with a Summagraphics bitpad and the computer program MACMEASURE.

RESULTS AND DISCUSSION

Fig. 1 presents electron micrographs of partially expanded skeletons. In these micrographs the spectrin molecules vary substantially in length while maintaining essentially straight contours. Spectrin lengths from such negatively stained preparations range from 1150 Å to 2000 Å (mean = 1664 Å, SD = 204 Å, n = 105). Particle straightness was evaluated by noting the number of inflection points along the length of the molecule. Straight particles would have no inflection points, and convoluted particles would have multiple inflections. We have found an average of 0.45 inflection points per particle; 70% of the particles have no inflection points at all. Thus, spectrin molecules in partially expanded membrane skeletons are essentially straight molecules of variable length. We have examined electron micrographs of negatively stained skeletons for the presence of quaternary structure. Enlargements of some of the spectrin particles examined are shown in Fig. 2.

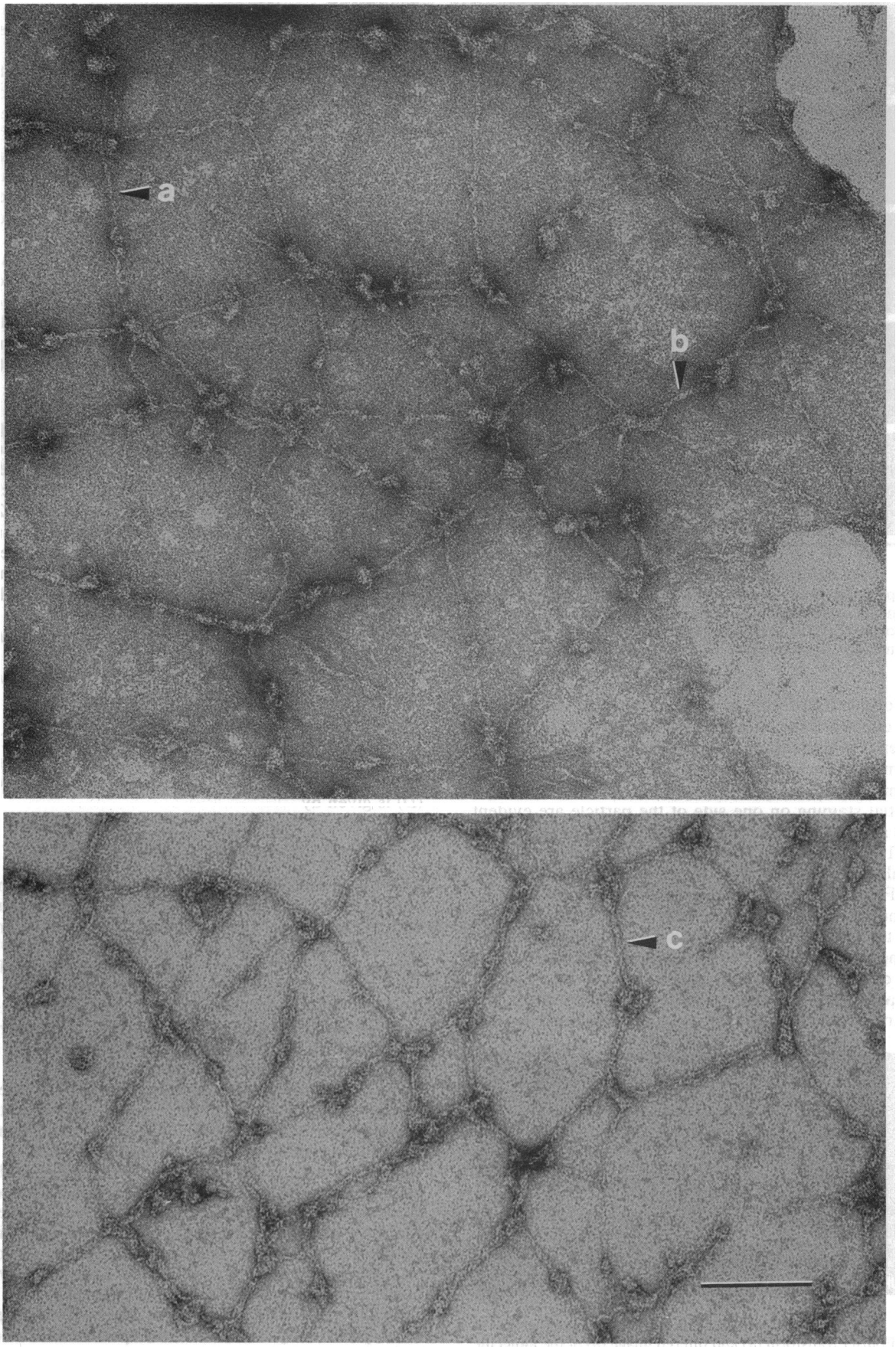

FIG. 1. Electron micrographs of erythrocyte skeletons. Partial expansion of skeletons was achieved by dilution in 2 mM sodium phosphate/0.05 mM $MgCl_2$/0.5 mM dithiothreitol, pH 8 (*a*), or in 0.2 mM sodium phosphate/0.05 mM $MgCl_2$, pH 8 (*b*), on ice. The spectrin molecules are linear structures whose lengths vary from 1150 Å to 2000 Å. (Bar = 1000 Å.)

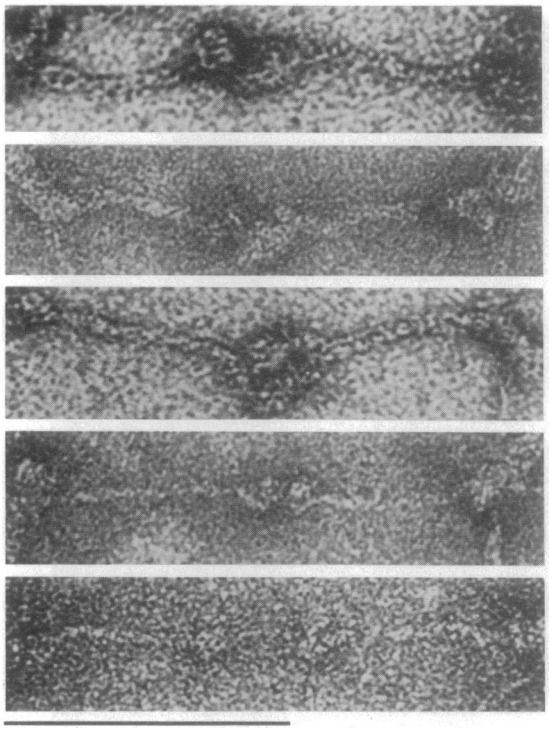

FIG. 2. Electron micrographs of spectrin tetramers. Some of the spectrin particles used in this study are shown enlarged. Actin protofilaments and ankyrin are bound at the ends and near the midpoint of the spectrin tetramers. The regions of spectrin used in the Fourier analysis are on the lefthand sides of these particles. (Bar = 1000 Å.)

The Fourier transforms of selected spectrin tetramers in partially expanded skeletons show distinct maxima reflecting the presence of periodic structure. Fig. 3*a* shows the Fourier transform of particle *b* from Fig. 1. The major peak in the transform has an axial spacing of 52 Å. The effects of preferential staining on one side of the particle are evident from the asymmetric intensity distribution across the meridian (14, 15).

Capitalizing on the periodic nature of these particles, we applied Fourier filtering techniques to remove noise in the image and to reveal their substructure (16). The results of filtering the transform in Fig. 3*a* is shown in Fig. 3*b*. The appearance of the filtered image suggests that this particle consists of two helical strands that are related by a twofold rotation axis. Each strand has a pitch of 104 Å, and the repeat of the particle is 52 Å. This interpretation, which implies that

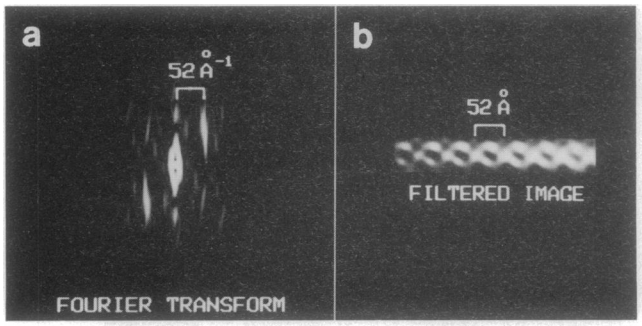

FIG. 3. Results of Fourier filtering an electron micrograph of spectrin. Fourier transform (*a*) and filtered image (*b*) of the molecule indicated in Fig. 1 (molecule *b*). The axial spacings of the strong maxima, 52 Å, correspond to the repeat of the particle. The filtered image was produced by retaining reflections from the equatorial J_0 and the J_2 and J_4.

the reflection on the 52-Å layer line arises from a second-order Bessel function, can be tested by considering the particle diameter.

The particle diameter can be determined from the computed Fourier transform as well as by inspection of the image. If the 52-Å layer line arises from a second-order Bessel function, its radial spacing (38 Å) indicates that the mean staining diameter of the particle is 41 Å. Similarly, the staining diameter calculated from the first maximum of the equatorial J_0 (32 Å) is 39 Å. (Calculations were based on the equation $J_0 = \pi DR = 3.8$, where D is the particle's mean staining diameter and R is the radial distance of the first subsidiary maximum from the meridian. A similar calculation was performed with the first maximum of J_2, which occurs at 3.4.) These determinations of the mean staining diameter from two different layer lines support our proposal that the 52-Å layer line arises from a second-order Bessel function as well as the interpretation that the particle is a two-start helix that has twofold rotational symmetry.

Upon examining the Fourier transforms of other particles (for instance those designated by *a* and *c* in Fig. 1) we observed that both the pitch and diameter of tetramers varied. This variation is illustrated in the filtered images shown in Fig. 4*a*. In this group of images, the pitch ranges from 104 Å to 166 Å and the diameter varies from 52 Å to 36 Å. Measurements of pitch and diameter show that increases in pitch are coupled to decreases in the diameter of the spectrin molecule. Models of these spectrin molecules presented in Fig. 4*b* show these variations in pitch and diameter.

To relate the particle's pitch and diameter, let us consider that the contour length of the tetramer is given by its maximum extension, 2000 Å, and that shorter particles represent axially contracted helical structures in which the contour length of each of the tetramer's helical strands remains 2000 Å. Since the ends of the molecule are attached to actin, a variation in the particle length can be effected by changing the pitch of the helix rather than the number of turns. Thus, the relationship between pitch (P) and diameter (D) is given by

$$C^2 = P^2 + (\pi D)^2, \qquad [1]$$

where C is the contour length of one turn of the particle's helix. Therefore, a plot of P^2 vs. D^2 should be a straight line with a slope of $-\pi^2$ and an intercept of C^2. Fig. 5 presents a plot of P^2 vs. D^2. The slope is -11.52 ($11.52^{1/2} = 3.39$) and the intercept is 3.95×10^4, corresponding to a contour length of 199 Å per turn of the helix. This predicts that a given spectrin tetramer will consist of two helically twisted subunits that make 10 turns over the particle's length. The number of turns is independent of the particle's length because in the skeleton the ends of the tetramer are constrained.

These data support the concept that spectrin extension or condensation occurs by varying the pitch and diameter of the two-stranded helical particle. The pitch and diameter are coupled through the relationship described by Eq. 1. Accordingly, a "native" spectrin tetramer, 700 Å long, would have a pitch of 70 Å and a diameter of 59 Å. These parameters are close to those of the shortest particle we have observed. Thus, while we cannot categorically exclude the possibility that further condensation can occur, forming an even more compact structure, the available data do not require this hypothesis to account for the 700-Å spacing in the membrane.

Finally, we consider how these studies relate to previous work, particularly that on isolated spectrin molecules. Both hydrodynamic and electron microscopic studies have provided considerable support for the view that spectrin is able to assume a convoluted shape in solution (19–21). Our own earlier work (22) along with that of others (7, 23) on isolated

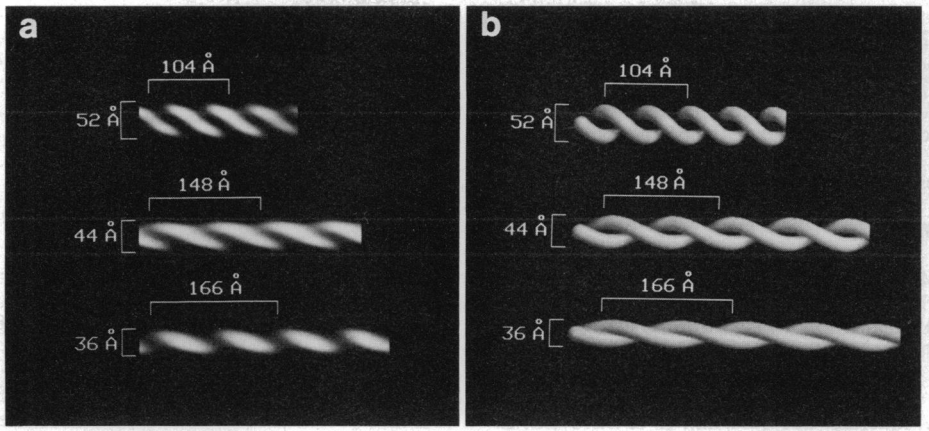

FIG. 4. (*a*) Filtered images of variably extended spectrin showing one surface of the molecule. One-sided images were generated by retaining reflections from the equatorial J_0 and only the strongest pair of conjugates from the J_2 (i.e., reflections from the best-stained side of the particles). Filtered images were derived from molecules *b* (*Top*), *c* (*Middle*), and *a* (*Bottom*) shown in Fig. 1. (*b*) Model of spectrin based on the observed relationship between pitch and diameter in variably extended spectrin. In our proposed structure, each turn of the helix would contain four of the repeating units from the model of spectrin's secondary structure proposed by Speicher and Marchesi (17, 18). Model images were generated by using the program RASTER 3D provided by David Bacon of University of Alberta and modified by Stanley Watowich of University of Chicago.

junctional complexes of spectrin, actin, and band 4.1 has also provided images of spectrin consistent with that of a flexible coiling molecule. How then can these apparently disparate data be reconciled with the findings presented in this work? Waugh (24) has reported that measurements of the shear modulus of erythrocytes show that the energy required to stretch a spectrin tetramer to twice its native length is 1600 (small) calories per mole. This remarkable observation means that at room temperature there are large variations in the equilibrium length of spectrin molecules caused by thermal fluctuations because RT is on the order of 600 calories. Thus, we view spectrin as a weak (or floppy) spring because the energy to double its length is only $2.5RT$. (This corresponds to 2.3 to 3.0 calories per angstrom for linear or harmonic springs, respectively.) It follows from Waugh's work that erythrocytes can be deformed very easily. (This feature is consistent with the physiological requirement that erythrocytes pass through the small capillaries with the smallest possible increase in resistance to blood flow.) When spectrin is free (i.e., unattached to actin), then, because it is such a very weak spring, it appears convoluted and flexible because of thermally induced fluctuations in conformation. On the other hand, when spectrin is attached to the actin protofilaments in the skeleton, it is largely straight, as we and others (7, 9) have observed, because it is constrained at both of its ends. Here the effect of thermal fluctuations is that the length of the spectrin molecules can vary.‡ The demonstration of a helical structure with a variable pitch provides a physical basis to account for the variable length of the straight particle.

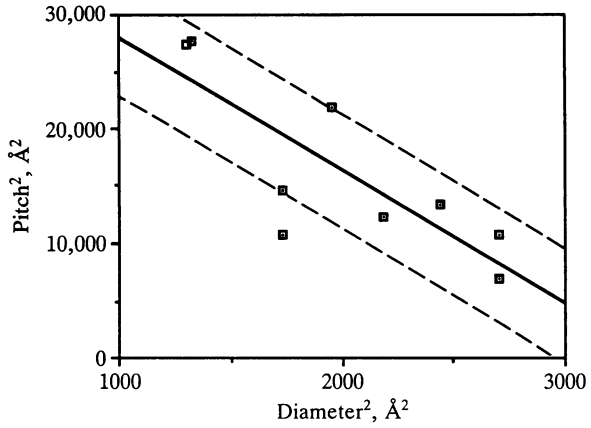

FIG. 5. The relationship between pitch and diameter in negatively stained tetramers. Measurements of particle diameter were made in relation to the outer edges of the filtered images. The intercept corresponds to a contour length of 199 Å for one full turn of a spectrin strand determined from Eq. 1. The standard error of estimate is indicated with the dashed lines.

To account for the elastic properties of the erythrocyte, Elgsaeter and co-workers have proposed that *in situ* the 2000-Å-long spectrin tetramer behaves as a random coil with an end-to-end distance of about 700 Å to 900 Å (25, 26). According to this hypothesis, spectrin's elastic properties result from extending the random coil in response to a mechanical stress. The force restoring the erythrocyte to its biconcave shape arises from the increase in entropy derived from reestablishing the more condensed configuration of the random coil.

Our studies have shown that spectrin tetramers can exist in a continuum of lengths in partially expanded membrane skeletons while maintaining essentially straight contours. This is because spectrin is able to extend axially (as is shown in Fig. 4). Fourier analysis of negatively stained molecules in intact skeletons has revealed that spectrin's α and β subunits align to form an ordered two-start helix with twofold rotational symmetry. Taken together, these results support a model in which spectrin's native length and elastic properties originate from the reversible deformation of a well-defined quaternary structure in the tetramer rather than a random coil. We propose that spectrin is a weak, two-stranded spring whose equilibrium length is determined by a balance of molecular forces that may be transiently disturbed by a mechanical stress. This, in turn, allows transient changes in skeleton shape that immediately recover when the mechanical stress is relaxed.

‡Thermal fluctuations would also result in variations of the helix pitch along the length of individual molecules. When the variation is large, Fourier analysis may not readily reveal periodic features.

We thank Mr. Leon Gross for preparing the images in Fig. 4*b*, Mr. Tom Lee for software development, and Drs. Stanley Watowich and Bridget Carragher for helpful discussions. This work was supported by National Institutes of Health Grants HL22654 to R.J. and GM07183 to A.M.M.

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