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# Change in State of Spin Labels Bound to Sarcoplasmic Reticulum with Change in Enzymic State, as Deduced from Ascorbate-Quenching Studies

**(transport/adenosinetriphosphatase/rotation)**

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**ABSTRACT** **The ATPase (EC 3.6.1.3) of sarcoplasmic reticulum vesicles was reacted to various extents with thiol-directed spin labels. By suspension of the preparation in appropriate solutions, the enzyme could be placed and held in certain intermediate states of the ATPase cycle, or it could be set into steady-state catalysis. Ascorbate added to the system destroyed the spin-label signals with undetectable distortion of the electron paramagnetic resonance spectrum. In general, in the presence of ascorbate, undestroyed signal as a function of time could be described as the sum of two first-order reductions going on in separate compartments with different ascorbate concentrations. In different enzymatic states the proportion of total signal in the two compartments was different, but the first-order velocity constants remained the same. If the labeled membrane was first attacked with Triton, then exposed to ascorbate, signal was destroyed according to a single first-order constant, equal to the faster of the two constants observed with intact membrane, and equal to the constant whereby ascorbate attacks free label in solution. The data were reconciled by a simple rotary model, envisioning that an enzymatic state corresponds to an average angular position of the ATPase and thereby determines the proportion of labeled thiols exposed to external and internal ascorbate concentrations.**

As the chemistry of calcium transport ATPase (EC 3.6.1.3) of sarcoplasmic reticulum (SR) has become clearer, it has become logical to associate some chemical states of the enzyme (E) with an "outward" or "external" (in reference to the vesicles of SR) location of the $Ca^{2+}$-binding site of enzyme, and some states with an inward or internal location, the presumption being that the site is carried from one location to another by a rotation, or some inversion, of enzyme (1). For example, it has been demonstrated (2) that $Ca^{2+}$ ions are translocated from the outside to the inside of SR during the formation of a phosphorylated intermediate, $E_{\sim P}$ from an enzyme–ATP complex, $E_{ATP}$, and that the $Ca^{2+}$ ions are released again on addition of EGTA + ADP to reverse the step, but the $Ca^{2+}$ ions remain inside the membrane when $E_{\sim P}$ is decomposed into E + $P_i$ even if EGTA + ADP are added. Except for such correlations, however, there does not appear to be evidence for external-internal transitions. In this work we sought such evidence by observing how the accessibility of enzyme-bound spin labels to externally presented ascorbate changed with changing chemical state†. Although our data are not uniquely explained in this way, our results are suggestively consistent with a rotary hypothesis of $Ca^{2+}$ translocation.

Abbreviations: SR, sarcoplasmic reticulum; E, enzyme; NEM spin label, 4-maleimido-2,2,6,6-tetramethyl piperidinooxyl spin label; EPR, electron paramagnetic resonance.

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## MATERIALS AND METHODS

At 0°C for 21 hr, 4 mg/ml of purified SR protein (4) were incubated with either 1 ("light" labeling) or 5 ("heavy" labeling) moles of "NEM spin label"‡ per $10^5$ g of SR protein, in the absence of ATP§. After exposure to the label but before the unbound label was dialyzed away, the electron paramagnetic resonance (EPR) spectrum of the system was compared with a standard curve of signal height against concentration of free label. Such comparisons showed 0.9 and 4.0 moles of label/$10^5$ g of SR protein had bound in light and heavy labeling, respectively. For stoichiometry, the amount of enzyme was estimated from the area of the main peak in the electrophoretogram (7–9).

Spectra were obtained at room temperature with a Varian E-3 EPR spectrometer, with the following settings: modulation amplitude, 1 G¶; modulation frequency, 100 kHz; microwave power, 20 mW; microwave frequency, 9.536 GHz.

By solvent choice (2, 10, 11) we could impose any one of four states on the ATPase enzyme:

80 mM KCl, 50 mM Tris-maleate, pH 7.0, plus:
2 mM EGTA, 15 mM $MgCl_2$, to obtain "E",
2 mM EGTA, 15 mM $MgCl_2$, 4 mM ATP, to obtain "$E_{ATP}$",

† The ascorbate trick of deducing the facing direction of spin-labeled molecules in membranes originated in the laboratory of H. M. McConnell (3), to whom we are also indebted for some coaching in its use.

‡ 4-Maleimido-2,2,6,6-tetramethyl piperidinooxyl spin label; from Synvar Associates, Palo Alto, Calif. Some work was also performed with "IAA spin label" [4-(2-iodoacetamido)-2,2,6,6-tetramethyl piperidinooxyl spin label]; provided through the courtesy of Dr. Richard Haugland.

§ No ATP was used because Nakamura *et al.* (5) showed that the extent of $E_{\sim P}$ formation is unaffected by these degrees of labeling. The ATPase activity, however, is reduced to about 43% of control. Even by the "heavy-labeling," no indication of aggregation was observed (see ref. 6), while when 10 moles of NEM spin label were incubated with $10^5$ g of SR, aggregates of vesicles were formed.

¶ For measurement of ascorbate-quenching of lightly labeled SR, we used a modulation amplitude of 4-G to increase the signal-to-noise ratio, since the rate of signal disappearance was the same at various field strengths.

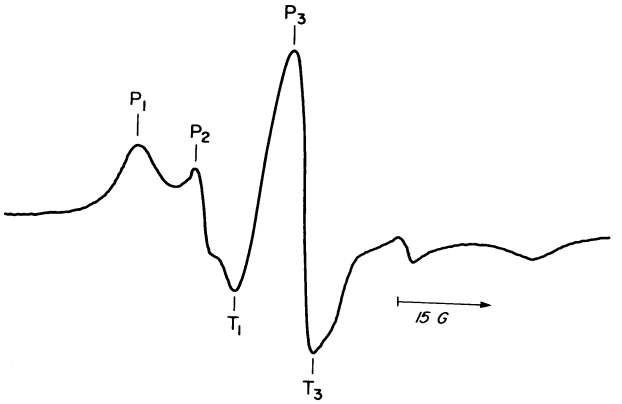



FIG. 1. EPR spectrum of SR labeled with 4 moles of "NEM spin label"/$10^5$ g. SR is 6 mg/ml in 0.1 M KCl, 5 mM Tris-maleate (pH 7.0), room temperature. Field set, 3385 G; scan range, 100 G. P, peak; T, trough.

5 mM $CaCl_2$, 30 $\mu$M $MgCl_2$, regenerator‖, to obtain "$E^{Ca}$",

5 mM $CaCl_2$, 30 $\mu$M $MgCl_2$, 4 mM ATP, regenerator, to obtain "$E_{\sim P}{}^{Ca}$" and very slow continous translocation.

In the foregoing solvents, SR protein was 1.8 mg/ml if ascorbate quenching was to be studied, and 6.0 mg/ml if EPR spectra were to be taken.

## RESULTS

Marked on a spectrum of heavily labeled fresh SR (Fig. 1) are certain peaks ($P_i$) and troughs ($T_j$). Of particular interest are $H_1$ and $H_2$, the heights from baseline of $P_1$ and $P_2$, respectively; as is well known, $P_1$ (3357 G) and $P_2$ (3366 G) arise from strongly and weakly immobilized labels. The ratio, $H_2/H_1$, of such a system is unchanged by formation of $E_{ATP}$ or $E_{\sim P}{}^{Ca}$; on the other hand, in the spectrum of aged SR, $H_2/H_1$ is much larger and is slightly decreased by $E_{\sim P}{}^{Ca}$ formation (Table 1); therefore, we used only fresh SR for ascorbate-quenching studies. Qualitatively the same behavior is observed with lightly labeled SR.

At 0.25–0.50 mM, ascorbate reduces nitroxyl (and destroys the EPR signal) at a convenient rate. Ascorbate creates a new trough ($T_2$) in the spectrum at 3384.75 G, but the rate of signal disappearance is the same at various field strengths tested. Therefore, $H_1$ will be used to describe the time course of quenching by ascorbate, but the subscript will henceforth be omitted when no confusion can result. Over 1–2 hr, H(t) appears to decay to a nonzero value; however, if the SR is first treated with Triton X-100 (a detergent thought to disassemble the membrane structure**), H(t) decays to zero, i.e., what initially appears as an ascorbate-resistant portion of the signal disappears after Triton treatment. We analyzed this phenomenon using conditions wherein ascorbate decrease in the course of label reduction is slight, and can be corrected for. For a single kind of label:

$$-dH/dt = k_0[\text{ascorbate}]H \equiv k_0[A]H \equiv k_0'H$$

‖ For ATP regeneration, 10 mM creatine phosphate and 0.5 mg/ml of creatine kinase were used. Unlike other transphosphorylases, creatine kinase operates without $Mg^{2+}$ if $Ca^{2+}$ is present.

** Triton decreases $H_2/H_1$ from 0.93 to 0.71; but in Triton, $E_{\sim P}{}^{Ca}$ formation still has no effect on $H_2/H_1$.

TABLE 1. *The ratio, $H_2/H_1$, of signal height from weakly immobilized labels ($H_2$) to signal height from strongly immobilized labels ($H_1$), for two different preparations (I, II) of SR*, as a function of additions to standard medium†*

| Addition (state) | $H_2/H_1$ | |
|---|---|---|
| | *I-fresh* | *II-fresh* |
| None (E) | 0.75 | 0.67 |
| 2 mM EGTA, 15 mM $MgCl_2$, 4 mM ATP ($E_{ATP}$) | 0.77 | 0.67 |
| 5 mM $CaCl_2$, 30 $\mu$M $MgCl_2$, 4 mM ATP, 10 mM creatine-P, 0.5 mg/ml of creatine kinase ($E_{\sim P}{}^{Ca}$) | 0.76 | 0.70 |
| | | *II-aged* |
| 4 mM ATP, 10 mM creatine-P, 0.5 mg/ml of creatine kinase | | |
| + 2 mM EGTA, 30 mM $MgCl_2$ ($E_{ATP}$) | | 1.03 |
| + 2 mM EGTA, 1.2 mM $MgCl_2$, 0.1 mM $CaCl_2$ ($E_{ATP}$) | | 1.01 |
| + 0.1 mM $CaCl_2$, 30 mM $MgCl_2$ ($E_{\sim P}{}^{Ca}$) | | 0.95 |
| + 0.1 mM $CaCl_2$, 30 $\mu$M $MgCl_2$ ($E_{\sim P}{}^{Ca}$) | | 0.93 |
| + 5 mM $CaCl_2$, 30 $\mu$M $MgCl_2$ ($E_{\sim P}{}^{Ca}$) | | 0.94 |

* The SR was at 1.8 mg/ml, labeled with "NEM spin label," at the higher degree of labeling.

† Standard medium was 80 mM KCl, 50 mM Tris-maleate, at pH 7.0 and room temperature.

Therefore, if the ascorbate concentration is quasi-constant, H(t) should be a first-order decay, the decay constant being proportional to ascorbate concentration. Such behavior is observed with *free* NEM spin label, and also with free IAA spin label; using either substance and various ascorbate concentrations, we found $k_0 \sim 0.5$ $mM^{-1}$ $min^{-1}$ in solvents like those used with SR.

Labels bound to Triton-treated SR are destroyed at very nearly the same rate as free label in solution. Signals from labels attached to untreated SR are attacked by ascorbate in more complicated fashion; a minimal but quite satisfactory description follows. Suppose that in the reduction kinetics sense there are two classes of labels: those attacked slowly (s), and those attacked rapidly (f). Then, if the spectrometer detects only the total unreduced signal,

$$H_{obs}(t) = H_s(0)\exp(-k_s't) + H_f(0)\exp(-k_f't).$$

If $k_f'$ is sufficiently larger than $k_s'$, analysis of semilogarithm plots of $H_{obs}(t)$ yields $H_s(0)$, $H_f(0)$, $k_s'$, and $k_f'$. We analyzed in this way the decay curves of SR in various enzymatic states, and at each of two degrees of labeling with NEM spin label. Since the only known ascorbate concentration is that bathing the external surfaces of the SR vesicles, $[A]_f$, we used that concentration to obtain the reported $k_s = k_s'/[A]_f$, and $k_f = k_f'/[A]_f$. The model to be developed below, however, would have made it equally natural to say that $k_s = k_f$, but that $[A]_f/[A]_s = k_f'/k_s'$, i.e., to say that the ascorbate concentrations are different between the classes s and f. Fig. 2*A* and *B* illustrates the procedure with heavily labeled SR, and Table 2 gives, for each enzymatic state and degree of labeling, the values of $k_f$ and $k_s$ and the percentages of $H_{obs}(0)$ that are in the s-class. Similar experiments and analyses were attempted with IAA-spin label (Fig. 2*D*), but

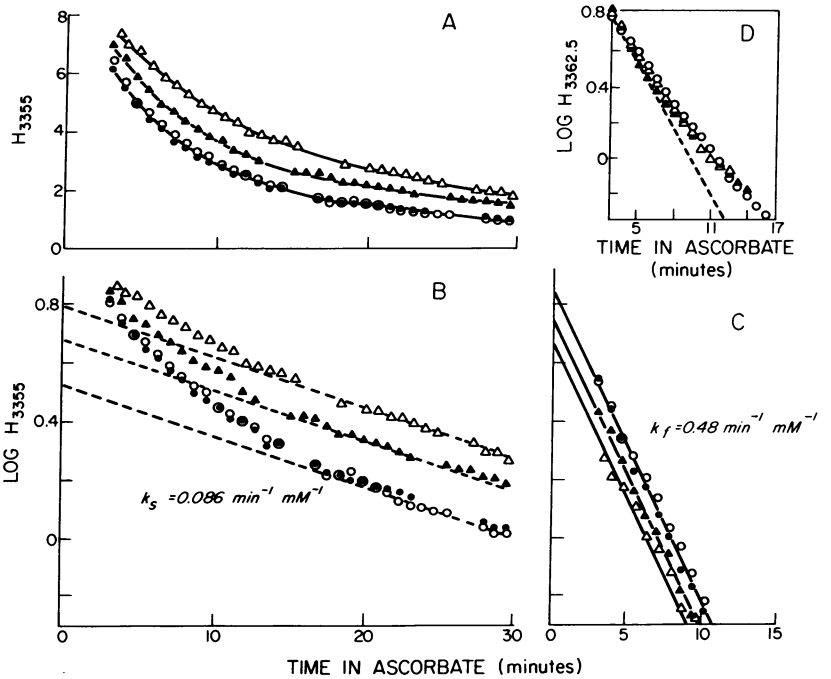



FIG. 2. Kinetics of destruction by ascorbate of EPR signal from spin-labeled SR vesicles in various enzymatic states. SR was labeled at 4 moles of "NEM spin label"/$10^5$ g, and used at 1.8 mg/ml, in 80 mM KCl, 50 mM Tris-maleate, pH 7.0, room temperature. Various additions to the medium (see *text*) impose various enzymatic states, namely, "E", ○; "$E^{Ca}$", ●; "$E_{ATP}$", △; "$E_{\sim P}{}^{Ca}$", ▲. At zero time, the medium is made 0.5 mM in ascorbate, and H, the peak height at 3355 G, is measured as a function of time. *Part A* shows data as measured. From a semilogarithmic analysis (*parts B* and *C*) there is calculated what portion of $H_{obs}(0)$ is attacked slowly ($k_s$ = 0.086), i.e., $H_s(0)$, and what portion is attacked rapidly ($k_f$ = 0.48), i.e., $H_f(0)$. *Part D* shows an experiment with SR labeled at 0.425 mole of "IAA spin label"/$10^5$ g, used at 3.17 mg/ml; experiments and symbols are otherwise as in *parts A–C*. The *dashed line* in *part D* indicates the rate at which signal from free "IAA spin label" is destroyed; evidently, $H_s(0)$ is very small no matter what enzymatic state is imposed.

we found the percentage of $H_{obs}(0)$ in the s-class to be always too small to be a useful parameter††.

The results with NEM spin label are gratifyingly simple (Table 2). The rate constants, $k_s$ and $k_f$, are quite independent of enzymatic state or degree of labeling; moreover, signal from the f-class of labels decays with the same rate constant as signals from free labels in solution, i.e., $k_f = k_0$. On the other hand, the distribution of label between s-class and f-class depends sensitively on both enzymatic state and degree of labeling.

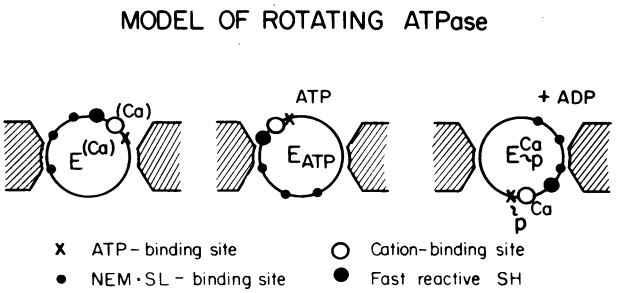
†† In the case of IAA-spin label, $H_{3365}/H_{3361}$ changed from 0.88 to 0.98 on addition of MgATP, with pK of 4.5. As noted by Inesi and Landgraf (12), this pK value was almost equal to that of the binding of MgATP required for modulation of ATPase, i.e., acceleration of the step $E_{ATP}{}^{Ca} \rightarrow E_{\sim P}{}^{Ca}$ (4).

## DISCUSSION

To be explained as a result of the foregoing results is that: (*i*) The EPR spectrum of NEM spin label bound to SR does not indicate a change in conformation around the label with change in enzymatic state. (*ii*) The reduction by ascorbate of SR-bound spin labels is adequately described by assuming two kinetic classes of spin label, the faster of these behaving like label in free solution or like label bound to Triton-treated SR. (*iii*) Upon ascorbate addition, signal decays at almost the same rate at various field strengths, so the EPR spectrum of labels in one class must be the same as that in the other class, i.e., there seems to be no difference in the environments of the two classes other than in ascorbate concentration. (*iv*) Enzymatic state does not alter the rate constants of reduction by ascorbate, but markedly affects the proportion of label in one class or another.

TABLE 2. *Reduction of SR-bound "NEM spin label" by ascorbate: rate constants ($k_f$, $k_s$) and percentage, P, of slowly attacked label, at two degrees of labeling and in various media*

| Medium | Intended state | 4 moles of label/$10^5$ g | | | | | | | | 0.9 mole of label/$10^5$ g | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $k_f$ | $k_s$ | $P_{obs}$ | $P_{corr}$ | $k_f$ | $k_s$ | $P_{obs}$ | $P_{corr}$ | $k_f$ | $k_s$ | $P_{obs}$ | $P_{corr}$ |
| | | *Preparation I* | | | | *Preparation II* | | | | | | | |
| 2 mM EGTA, 15 mM $MgCl_2$ | E | 0.48 | 0.086 | 32 | 32 | 0.46 | 0.11 | 34 | 34 | 0.46 | 0.085 | 18 | 18 |
| 5 mM $CaCl_2$, 30 μM $MgCl_2$, creatine kinase | $E^{Ca}$ | 0.48 | 0.086 | 32 | 32 | 0.46 | 0.11 | | | | | | |
| 2 mM EGTA, 15 mM $MgCl_2$, 4 mM ATP | $E_{ATP}$ | 0.48 | 0.086 | 57 | 77 | 0.46 | 0.11 | 52 | 72 | 0.46 | 0.085 | 25 | 33 |
| 5 mM $CaCl_2$, 30 μM $MgCl_2$, 4 mM ATP, creatine kinase | $E_{\sim P}{}^{Ca}$ | 0.48 | 0.086 | 46 | 57 | 0.46 | 0.11 | 45 | 57 | 0.46 | 0.085 | 42 | 69 |




| ENZYME STATE | CATION SITE | | ATP SITE | | EPR SIGNAL | % OF NEM·SL INSIDE | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ENZYME KINETICS | MODEL | ENZYME KINETICS | MODEL | OBS. MODEL | 4 moles / $10^5$ g CORR., (I) | OBS. (II) | MODEL | 0.9 mole / $10^5$ g CORR., OBS. | MODEL |
| E & $E^{Ca}$ | out | out | out | out | no change | 32 | 34 | 25 | 18 | 14 |
| $E_{ATP}$ | out | out | out | out | | 77 | 72 | 75 | 33 | 43 |
| $E^{Ca}_{\sim P}$ | in | in | ? | in | | 57 | 57 | 50 | 69 | 71 |

FIG. 3. Summary of how a "rotary ATPase" model accounts for the results of this paper. Upper and lower parts of the model correspond to outside and inside of the membrane, respectively. The model assumes that one thiol (●) reacts with "NEM spin label" four times as fast as the other three thiols (•). Biochemical experiments reported elsewhere (1,2,4) show that $E_{ATP}{}^{Ca}$ is formed by the bindings of enzyme with $Ca^{2+}$ ions and ATP on the outside of the membrane in a random sequence. $E_{\sim P}{}^{Ca}$ formation induces the translocation of $Ca^{2+}$ ions from the outside into the inside. ADP appears outside the membrane. $E_{\sim P}$ formation induces a large increase in affinity of $Mg^{2+}$ ions to the enzyme. $Ca^{2+}$ ions are replaced by $Mg^{2+}$ ions, and $E_{\sim P}$ decomposes, $P_i$ appears outside the membrane, and the ATPase resumes the original state.

Also to be explained is much biochemical information suggesting (1, 2, 4) that in $E^{Ca}$ or $E_{ATP}$ the active site of the ATPase is external, but that once $E_{\sim P}{}^{Ca}$ is formed the active site quickly moves to an internal orientation.

Before we attempt to construct a model it is necessary to "correct" the findings of item *iv*. The electrophoretogram of SR shows that about 40% of the protein is not ATPase, but of course such protein could have thiol groups that are labeled. We found that the binding extent of NEM spin label is almost equal to that of NEM (*N*-ethylmaleimide) itself, and that the number of thiol groups per $10^5$ g of ATPase modified by *N*-ethylmaleimide is slightly lower than that for the other proteins found in the electrophoretogram. A "correction" for this effect can be made by assuming that the proportion of s- and f-class labels on this "inert" protein is the same as on "E", but that on inert protein this proportion is unchanged by addition of $Ca^{2+}$ or ATP (or both). In the *table* of Fig. 3, the corrected percentages of $H_s(0)$ in different enzymatic states are tabulated (along with those directly observed).

A simple model that would account for many facts is depicted in Fig. 3, along with some of its quantitative predictions: The ATPase is spheroidal, capable of rotating about an axis in the plane of the membrane. Corresponding to each static chemical state of the active site is a particular angular position in the rotation; corresponding to steady-state translocation of $Ca^{2+}$ is a steady-state turning, but a definite time-average angle. On the surface of the sphere (for simplicity we assume that rotation is uniaxial, and that all chemical groups of interest—active site and thiols—are on the equator) are the active site ($Ca^{2+}$, ATP) as well as four thiol groups that may or may not be spin labeled, depending on the degree of labeling. Choice of angular position then determines, for the active site and for every thiol or spin label, whether it is external or internal. To account for reduction of labels by ascorbate we suppose that the effective internal concentration of ascorbate is only $(k_s/k_f)$-times the external, and that a bound label is attacked as readily as a free label. A maintained transmembrane gradient in ascorbate could arise from very rapid penetrability and a transmembrane electrical potential difference. To account for observed differences in labeling, and in percentage of labels in the s-class, $P_{obs} = 100[H_s(0)/H_{obs}(0)]$, we assume one thiol near (n), and three thiols remote (r) from, the active site, and that the former has a reactivity toward spin label that is $\kappa$ ($\kappa = 4$ fits our data best) times the reactivity of the latter. Then, if an enzymatic state calls for $N_n$ "near" thiols, and $N_r$ "remote" thiols to be internal, we can also *calculate* the percentage. It is $P_{calc} = 100(N_n\kappa + N_r)/(\kappa + 3)$ for "light" labeling, and $P_{calc} = 100(N_n + N_r)/4$ for "heavy" labeling.

The foregoing model accommodates previous "biochemical" facts, and also those reported in this work: At no point are conformational (spectrum-changing) transitions invoked. A "natural" explanation for the observed two compartments is given, yet the environment "seen" by protein surface in the two compartments is the same (aqueous); only the ascorbate concentration is different, and therefore the rate constant of label reduction is different. Finally, the assumption of a "rotary" ATPase provides a very simple explanation for changes in label reduction with enzymatic state, degree of labeling, etc. Naturally, the model is much oversimplified, and of course it was *designed* to explain the facts that it explains, so at this stage it can hardly be taken literally. Yet it seems to us important that rather simple physical features can account for complex observational phenomena. Also of some significance may be the striking similarity between the rotary model invoked here and rotary models of the force-generating process in muscular contraction (1, 13–15).

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