Ferro cyanide Protection against Uncoupling of Excitation and Contraction by Manganese in Rabbit Ventricular Myocardium

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SUMMARY Recent studies of cardiac and skeletal muscle suggest that the sites at which Mn\(^{2+}\) uncouples excitation and contraction are restricted to a space functionally equivalent to the interstitial. We sought to corroborate this hypothesis by using the ferrocyanide anion, Fe(CN)\(_6^{3-}\). This anion is relatively innocuous to myocardial tissue, is compatible with solutions containing alkaline and alkaline earth cations, but immediately precipitates when combined with cations of the transition-element series, particularly Mn\(^{2+}\). The finding of interest is that heart tissue pretreated with 20 mM Na\(_4\)Fe(CN)\(_6\) is "protected" from the normal action of Mn\(^{2+}\), the uncoupling of excitation and contraction. This finding would be expected if Fe(CN)\(_6^{3-}\) could precipitate Mn\(^{2+}\) influx in one or several compartments. Protection was not a function of the duration of the pretreatment as long as an interval of several minutes was allowed. However, protection markedly declined as a function of the interval following the cessation of Fe(CN)\(_6^{3-}\) treatment and preceding the addition of Mn\(^{2+}\). Quantified in this way using thin-diameter papillary and trabecular muscles from rabbits, protection was found to be approximately monoexponential and most likely confined to a single, rapidly effluxing compartment, the interstitium. Studies were conducted using the perfused rabbit septum to divulge the washout kinetics of Mn\(^{2+}\) and [\(^{14}\)C]Na\(_4\)Fe(CN)\(_6\). Analyses of these washouts suggest that Mn\(_4\)Fe(CN)\(_6\) precipitated in the interstitium not because this was the only compartment reached by both agents but because this was the space in which Mn\(^{2+}\) exerted its uncoupling action.

NO MODEL of excitation-contraction coupling in adult mammalian myocardium is unanimously supported by all investigators. This can be traced to the inferential nature of the data thus far produced.\(^{1,7}\) A major and certainly fundamental point in any model is the morphological location of the primary sites from which Ca\(^{2+}\) is released to the contractile proteins during an action potential. On the one hand these sites are thought to reside inside the confines of the sarcolemmal membrane and its invaginations, most likely in the terminal cisternae.\(^{1,3,4}\) On the other hand, the sites are thought to reside at the cell surface in association with the unit-membrane, surface-coat complex.\(^{5,6}\) Notwithstanding these differences of opinion, there is one point on which there is consistent agreement: tissue calcium in general, and specifically contraction-related Ca\(^{2+}\), appear to be highly compartmentalized.

Efforts to identify the Ca\(^{2+}\)-releasing sites have been hampered both by the ubiquity of calcium distribution in cardiac tissue and by the small fraction of tissue calcium residing at these sites.\(^{7}\) Estimates based on recent data\(^{8}\) suggest that during normal rhythmical contraction the quantity of Ca\(^{2+}\) released per beat would not exceed 1% of the total tissue calcium. To circumvent some of these problems, investigators have searched for a suitable Ca\(^{2+}\)-release inhibitor, i.e., an agent that would block contraction by acting specifically on the releasing sites. Studies aimed at understanding the blocking agent might then reveal additional information about the nature and the location of these sites.\(^{9,10}\) Three agents have surfaced as, perhaps, the most viable candidates: La\(^{3+}\) for its potency and its high electron density, verapamil and D-600 for their apparent specificity, and Mn\(^{2+}\) for its reversibility. For the study reported here, the primary focus is on the action of Mn\(^{2+}\), although Ni\(^{2+}\), Cd\(^{2+}\), and Pb\(^{2+}\) also were examined briefly. Although one of the major sites of action of Mn\(^{2+}\) (with respect to E-C coupling) would appear to be the sarcolemmal and/or T-tubular membrane,\(^{11}\) recent reports by Ochi\(^{12,13}\) and by Delahayes\(^{10}\) indicate that Mn\(^{2+}\) can and does enter the cardiac cell. Once inside, Mn\(^{2+}\) could act at other sites to interfere with E-C coupling. The purpose of this project was to examine the tissue distribution of Mn\(^{2+}\) through the use of a second agent, Na\(_4\)Fe(CN)\(_6\), and thereby infer a more exact definition of the sites at which Mn\(^{2+}\) acts to uncouple excitation and contraction.

The experimental design developed for this study makes use of "Mn\(^{2+}\) and ferrocyanide, Fe(CN)\(_6^{3-}\), a substance which when accumulated by cardiac tissue prior to an exposure to Mn\(^{2+}\) "protects" the tissue against the usual action of Mn\(^{2+}\), i.e., the uncoupling of excitation and contraction. The ferrocyanide anion is relatively innocuous to cardiac tissue and does not precipitate biologically important cations such as Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\), although the dissociation of these ferrocyanide salts is

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Supported by Grant HL-11351-9-10 from the United States Public Health Service, a Grant-in-Aid from the American Heart Association, Greater Los Angeles Affiliate, and funds from Veterans Administration Medical Research.

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Received June 9, 1977; accepted for publication February 23, 1978.
FERROCYANIDE PROTECTION AGAINST E-C UNCOUPLING/Sanborn and Stremel 179

incomplete. A vigorous and marked precipitation reaction occurs, however, when most of the divalent transition-element cations are added to Fe(CN)$_6^{3-}$-containing medium. This is especially true for Mn$^{2+}$.

This paper describes the results from experiments designed to reveal the interactions among tissue, Fe(CN)$_6^{3-}$, and Mn$^{2+}$. The findings corroborate the hypothesis that Mn$^{2+}$ uncouples excitation and contraction in a space functionally equivalent to the interstitium.

Methods

Preparation and Perfusion

Rabbit heart tissue was selected for this study to permit cross-correlation between mechanical data, recorded from bath-perfused papillary and trabecular muscles, and radiioisotopic data, obtained from arterially perfused interventricular septa. Young adult, male New Zealand white rabbits were killed by an overdose of pentobarbital injected iv. When the septal preparation was used, heparin was injected first. Details of the septal-perfusion technique have been published elsewhere.14 Hearts from rabbits were killed by an overdose of pentobarbital in- 

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The effect on force development was identical in each the usually quiescent papillary or trabecular muscles.

The spontaneous rhythmicity usually encountered with the septum appeared enhanced, whereas and then partially recovers. The following statistics were exhibited little or no such tendency.

Both the superfused rabbit papillary and trabecular preparation. The effect of Na₄Fe(CN)₆ on steady state force development. Solution containing 1.0 mM Ca²⁺ + 20 mM Na₄Fe(CN)₆ introduced at a. This muscle remained in 20 mM Na₄Fe(CN)₆ containing medium for 75 minutes. A new steady state was attained at approximately the 10th minute and maintained at that level to 75 minutes. Traces a' and b' are typical steady state contractions in 1.0 mM Ca²⁺ medium without and with 20 mM Na₄Fe(CN)₆, respectively. Both peak isometric tension and dP/dt behaved identically. An apparent new steady state (see trace b') is reached 22.8 minutes after the change (range, 9–33 minutes) and the developed force measures 87.6 ± 16.4% (dP/dt) or 81.6 ± 16.9% for dP/dt and 80.7 ± 12.1% for tension. The magnitudes and time courses of these changes are similar quantitatively to those reported for the case of y-OH-butyrate in frog ventricle. After the removal of Na₄Fe(CN)₆ from the perfusion medium, force development transiently overshoots and then recovers to the control steady state.

Definition of the Term "Protection." The measurement of Fe(CN)₆ protection as applied to this study is illustrated in Figure 2. Only the general features of such an experiment will be treated at this time. Later, in connection with the presentation of Figure 3, a more rigorous description will be undertaken. In trial M, the muscle is equilibrated with the 1.0 mM Ca²⁺ medium and, at some arbitrary time (defined as "0" time), the value of [Ca²⁺], is reduced to 100 μM. Because of the number of trials required to complete one experiment and because of the possible deleterious effects of O Ca²⁺ perfusion, the concentration of the low Ca²⁺ medium was set at 100 μM. Separate trials established that the results were not biased by this selection.) After several minutes of perfusion with the low Ca²⁺ medium (a maneuver designed to deplete all contraction-related stores, irrespective of location), the [Ca²⁺], is returned to 1.0 mM concurrently with the onset of quiescence. Stimulation is resumed 30 seconds later. Trace m is the record of the first contraction on resimulation. For the case of a quiescent interval of 0.5 minute, during which replenishment of the previously depleted stores of contraction-related Ca²⁺ is presumably occurring, and a thin diameter muscle (d < 0.5 mm), trace m will equal or nearly equal steady state force.
FERROCYANIDE PROTECTION AGAINST E-C UNCOUPLING/Sanborn

I mM Ca 100 µM Co 1 mM Ca

1 mM Ca 25 mM Mn

20 mM Fe(CN)6 20 mM Fe(CN)6

O

I mM Ca 100 µM Co and

20 mM Fe(CN)6 20 mM Fe(CN)6

Stimulation rate: 25/min

Temperature: 29° C

Area; 0.16 mm2

FIGURE 2

Trial sequence used to demonstrate protection by Fe(CN)63-. Trial M: solution containing 1.0 mM Ca2+ changed to one containing 100 µM Ca2+ at 0 time and continued for 3.0 minutes, at which time the stimulus was turned off. Concurrently the [Ca2+]o was returned to 1.0 mM and stimulation was resumed 0.5 minute later. Trial N: same sequence followed as for trial M except 2.5 mM Mn2+ was also introduced at the onset of quiescence. Trial O: prior to t = 0, the perfusion medium contained 1.0 mM Ca2+ and 20 mM Na4Fe(CN)6. At 0 time, medium was changed to one containing 100 µM Ca2+ and 20 mM Na4Fe(CN)6 and continued for 3.0 minutes. The stimulus was turned off at t = 3.0 minutes, and concurrently the Na4Fe(CN)6 was removed and the [Ca2+]o was returned to 1.0 mM. Stimulation resumed 0.5 minute later. Trial P: same sequence followed as for trial O except 2.5 mM Mn2+ was also introduced at the onset of quiescence. Inset shows superposition of the four contractions manifest upon restimulation. Equivalent results were measured for both peak isometric tension and for dP/dt.

The concept of protection can be formulated in the following way:

\[
\text{protection} = \frac{p - n}{o - n} \times 100
\]

where the parameters n, o, and p are measured in units of dP/dt or peak tension. Thus, in this formulation, protection will vary between 0 and 100, depending on the amplitude of p.

Effect of Filled vs. Unfilled Stores of Contraction-Related Ca2+ on Protection. This experimental scheme may raise a few questions because Mn2+ is added at a time when the contraction-related stores should be nearly empty. Thus Mn2+ may be acting to block Ca2+ at some site(s) other than the Ca-releasing site(s). This proposition was examined by repeating the scheme illustrated in Figure 2 with one exception: perfusion with 100 µM Ca2+ was eliminated. With this modification, the amount of Ca2+ in the contraction-related stores at the time of the addition of Mn2+ would be little different from the steady state levels. For the case of relatively thin-diameter muscles (d = 0.3 mm, area = 0.07 mm2), the relationships among the amplitudes of contractions m, n, o, and p were found not to change (Sanborn, unpublished data, and Fig. 3, this paper).

The experimental design chosen for this study was dictated by the limitations of time. As muscle diameter increases, the length of the interval required for Mn2+ to exert its maximal uncoupling effect also increases (Sanborn, unpublished results). During this same interval, however, Na4Fe(CN)6 efflux from the muscle is occurring, and, as a consequence, protection diminishes. As will become clear later in connection with Figures 7 and 8, the shorter the interval between the cessation of treatment with Na4Fe(CN)6 and the resumption of stimulation, the greater the potential for protection. Since protection per se can be demonstrated only after Mn2+ has had sufficient time to exert its maximal effect, the minimal interval will be limited by muscle diameter (i.e., the Mn2+). Upon restimulation, the uncoupling action is evident in trace n. The two remaining trials leading to traces o and p demonstrate the protective effect of treatment with Na4Fe(CN)6. Note that, in this particular example, perfusion with Na4Fe(CN)6-containing medium was begun prior to the change from 1.0 mM to 100 µM Ca2+ and terminated at the conclusion of the perfusion with the 100 µM Ca2+ medium. Concurrently with the onset of quiescence, the [Ca2+]o is changed back to 1.0 mM without 2.5 mM Mn2+ (trial O) and with 2.5 mM Mn2+ (trial P).

Note that the duration of the quiescent interval and, equivalently, the duration of exposure to Mn2+ lasts only 0.5 minute. The protection against the action of Mn2+ conferred upon the muscle by prior treatment with Na4Fe(CN)6 is evident in trial P. All four contractions are overlaid for comparison in the inset. Note (1) that contraction p, recorded in the presence of 2.5 mM Mn2+, is very nearly equal to contraction o, recorded in the absence of Mn2+ and (2) that the relationship between contractions m and o is little different from the pattern exhibited in Figure 1.

development (Sanborn, unpublished results). This sequence completes one trial. This procedure is repeated a second time, except concurrently with the onset of quiescence the medium is changed to 1.0 mM Ca2+ + 2.5 mM Mn2+. Upon restimulation, the uncoupling action is evident in trace n. The two remaining trials leading to traces o and p demonstrate the protective effect of treatment with Na4Fe(CN)6. Note that, in this particular example, perfusion with Na4Fe(CN)6-containing medium was begun prior to the change from 1.0 mM to 100 µM Ca2+ and terminated at the conclusion of the perfusion with the 100 µM Ca2+ medium. Concurrently with the onset of quiescence, the [Ca2+]o is changed back to 1.0 mM without 2.5 mM Mn2+ (trial O) and with 2.5 mM Mn2+ (trial P).
time for Mn

 indicates its uncoupling action. Had we adopted an experimental design without the Ca

 depletion step, only the thinnest muscles (d < 0.3 mm) would suffice. By including the Ca

 depletion step, replenishment and Mn

 blockade are given more or less equal weight and muscle diameter is much less critical. As muscle diameter exceeds 0.5–0.6 mm, the quiescent interval required for full replenishment takes longer and the force developed on the first beat becomes diminished due to the interval-strength relationship

 or, alternatively, the rest-decay phenomenon.

 We chose to test for protection after a period of quiescence because, in preliminary studies, this experimental scheme yielded the clearest measure of protection.

 **Effect of the Duration of Perfusion with Na

 \text{Fe(CN)}_6^- \text{Containing Medium on Protection.}** The total time during which the muscle was exposed to Na

 \text{Fe(CN)}_6^- \text{-containing medium was varied between 3.5 and 75 minutes. Within this range, protection was not a function of the exposure time.**

 **Protection and Other Mn-like Cations.** Additionally, three other transition-element cations were tested within the experimental scheme illustrated in Figure 2, Ni

 \text{Fe(CN)}_6^- \text{, Cd

 \text{Fe(CN)}_6^- \text{, and Pb

 \text{Fe(CN)}_6^- \text{. For Ni

 \text{Fe(CN)}_6^- \text{and Cd

 \text{Fe(CN)}_6^- \text{(HEPES buffer substituted for HCO}_3^- \text{), the protection fell within the range found for Mn

 \text{Fe(CN)}_6^- \text{. For the case of Pb

 \text{Fe(CN)}_6^- \text{(HEPES substituted for HCO}_3^- \text{) the protection was substantially less, measuring approximately 20%. (This marked difference in protection was judged from test tube experiments to be in agreement with the rate of metal cation-Fe(CN)_6^- precipitation, as discussed later in the "Results." Thus the combination of Na

 \text{Fe(CN)}_6^- \text{, and Mn

 \text{Fe(CN)}_6^- \text{is not unique; other cationic uncouplers of excitation and contraction can be protected against as well.**

 **Major Findings**

 **Studies with Superfused Papillary and Trabecular Muscles**

 Our working hypothesis is that after cessation of perfusion with Na

 \text{Fe(CN)}_6^- \text{-containing medium, the residual Na

 \text{Fe(CN)}_6^- \text{in the tissue will precipitate influxing Mn

 \text{Fe(CN)}_6^- \text{, thereby blocking the expected effect of Mn

 \text{Fe(CN)}_6^- \text{on E-C coupling. The longer this interval, the less residual Na

 \text{Fe(CN)}_6^- \text{and consequently the less the expected protection (a finding substantiated in preliminary trials). An experimental scheme, based upon that illustrated in Figure 2, was devised to quantify the rate of decline of protection as a function of the elapsed time between the cessation of Fe(CN)_6^- treatment and the resumption of stimulation. The duration of exposure to Fe(CN)_6^- was set at 10 minutes, the duration of low Ca

 \text{Fe(CN)}_6^- \text{ perfusion at 5 minutes, and the duration of the quiescent interval (and equivalently the period of exposure to Mn

 \text{Fe(CN)}_6^- \text{) at 0.5 minute. Ten muscles, ranging in diameter from 0.30 to 0.44 mm (area range, 0.071 to 0.15 mm

 \text{), were tested. Thus the dependent variable was protection and the independent variable the length of time between the cessation of treatment with Na

 \text{Fe(CN)}_6^- \text{and the onset of restimulation. Note that, with this experimental design, the earliest time at which protection could be measured was at 0.5 minute, exactly as illustrated in Figure 2. To measure protection at 5 minutes, perfusion with control Ca

 \text{Fe(CN)}_6^- \text{ medium containing Na

 \text{Fe(CN)}_6^- \text{ began at —9.5 minutes and ended at 0.5 minute. The medium then was changed to 100 \text{\mu M} \text{Ca}^{2+} \text{ without Na

 \text{Fe(CN)}_6^- \text{ and continued for 4.5 more minutes, at which time stimulation was stopped concurrently with a change back to the control medium without (trial F) Mn

 \text{Fe(CN)}_6^- \text{. After 0.5 minute, stimulation was resumed. Additional trials were conducted on three of the thinnest muscles to test the effect of omitting the Ca

 \text{Fe(CN)}_6^- \text{ deple tion step. The results are plotted in Figure 3. Except for the one set of points at 4.5 minutes, the data show that protection is effectively 0 for elapsed times exceeding 3.5 minutes. The apparent first-order decline of protection is seen to be independent of perfusion with low Ca

 \text{Fe(CN)}_6^- \text{ medium prior to the exposure to Mn

 \text{Fe(CN)}_6^- \text{. The rate constant, fitted by eye, measures 1.66 \text{min}^{-1} \text{ (equivalent to a t

 \text{1/2 of 0.445 minute or 26.7 seconds). Thus protection appears to occur in a single kinetic compartment.**

 In Figure 4 are plotted the amplitudes of dP/dt for the 5-minute interval during which the thinnest and thickest muscles were perfused with low Ca

 \text{Fe(CN)}_6^- \text{ medium (dP/dt was judged preferable because time-to-peak tension lengths in low Ca

 \text{Fe(CN)}_6^- \text{ medium). The points were calculated according to the equation F = A_o e^{-kt} + B, where F = the amplitude of the dP/dt signal, A_o = the steady state amplitude of that fraction of the dP/dt signal attributable to the difference between the two Ca

 \text{Fe(CN)}_6^- \text{ concentrations,
FERROCYANIDE PROTECTION AGAINST E-C UNCOUPLING/Sanborn and Stremel

![Figure 4 Decline of dP/dt during constant stimulation in low Ca\textsuperscript{2+} medium compared for two muscles of differing diameters.](image)

...and B = the steady state amplitude of the dP/dt signal in 100 \textmu M Ca\textsuperscript{2+}. Thus, after the change from 1.0 mM Ca\textsuperscript{2+} to 100 \textmu M Ca\textsuperscript{2+} medium, dP/dt decays from F to B. For the case of the thinnest muscle, the rate constant measures (by eye) 1.44 min\textsuperscript{-1} and for the thickest, 0.85 min\textsuperscript{-1}. Thus the rate constant for the decline of protection is at least as fast as the rate constant descriptive of the decline of dP/dt following a change from 1.0 mM to 100 \textmu M Ca\textsuperscript{2+} medium.

Studies with the Perfused Septum

\textsuperscript{55}Mn Washout. Ten \textsuperscript{55}Mn\textsuperscript{2+} washout curves were obtained from five septa (two washouts per muscle). The [Ca\textsuperscript{2+}], was either 1.0 or 1.5 mM and the [Mn\textsuperscript{2+}], ranged from 0.1 to 2.5 mM. Exposures to Mn\textsuperscript{2+} and to the label, which varied between 4 and 30 minutes, were always concurrent. Hence the washouts reflect only the efflux of the Mn\textsuperscript{2+} accumulated during the label. These experiments were not designed to measure the steady state exchange of Mn\textsuperscript{2+}. Muscle temperature was nominally 28°C and the stimulation rate, 24/min.

The following general comments are offered (Fig. 5A): The washout curves are multiphasic in the sense that a minimum of four exponential functions is required to describe their time course. Extended labeling compared to brief labeling, or labeling in high and then in low Mn\textsuperscript{2+} medium, yield pairs of curves that appear simply as scaled versions of one another (i.e., only the intercept values are changed, the rate constants can be considered constant). Except after the briefest label (few minutes) with the lowest [Mn\textsuperscript{2+}], the tissue always contains substantially more isotope than could be predicted from the time course of the washout. This indicates either of two pos-
min. Integration of the upper curve between 90 minutes and ∞ will yield the amount of $^{54}$Mn expected in the muscle ash, unless there were much slower undetected phases. The predicted value was $1.86 \times 10^7$ counts/min but the actual value in the muscle ash was $4.78 \times 10^6$

counts/min.

Phasic resolution of each curve was accomplished by reverse, serial hand subtraction. Each phase was integrated with respect to time between 0 and ∞ to yield a hypothetical pool size. The following expressions were derived for the upper and lower curves, respectively:

$$\text{counts/min} = (1.06e^{-0.44t} + 3.25e^{-0.54t} + 1.97e^{-0.129t} + 0.614e^{-0.0272t}) \times 10^6$$

$$\text{counts/min} = (1.11e^{-0.55t} + 2.24e^{-0.26t} + 1.06e^{-0.393t} + 0.285e^{-0.0325t}) \times 10^6$$

The bulk (88%) of the extra Mn$^{2+}$ accumulated in response to the additional 25.5 minutes of labeling time can be assigned to the two slowest phases. For example, the contents of the slowest phase have increased by approximately 300% and the next slowest, by approximately 200%. The gain found in the second fastest phase was only 22%. Both the rate constants for the second phases (0.441 min$^{-1}$, upper, and 0.441 min$^{-1}$, lower) and their water spaces (639 ml/liter tissue H$_2$O, upper, and 524 ml/liter tissue H$_2$O, lower) are comparable to phase 1 values obtained from $^{45}$Ca washouts of perfused rabbit septa.

An analysis of force development for this muscle during and after treatment with Mn$^{2+}$ also is of interest. In response to the 4.5-minute exposure to Mn$^{2+}$, dP/dt declined to 42% of steady state. Between 10 and 11 minutes after the removal of Mn$^{2+}$, recovery was 98% complete despite the finding that the muscle still contained considerable Mn$^{2+}$. In terms of a recovery function of the form $1 - e^{-\lambda t}$, the return of force development to the steady state is described by a rate constant lying between 0.36 and 0.40 min$^{-1}$. If there is parallelism between the recovery of force development and the efflux of Mn$^{2+}$, then the uncoupling of excitation and contraction cannot be ascribed to Mn$^{2+}$ residing in the two slowest phases.

$[^{14}C]Na_4Fe(CN)_6$ Washout. Consideration also was given to the reaction between cardiac tissue and Na$_4$Fe(CN)$_6$. Thirteen muscles were labeled with 20 mM Na$_4$Fe(CN)$_6$ for periods lasting between 4 and 40 minutes. All washouts were followed for 90 minutes and all exhibited a similar time course to that illustrated in Figure 5B. These washouts, like those of $^{54}$Mn, bear a qualitative resemblance to the washout of $^{45}$Ca (see ref. 7). They are multiphasic, requiring a minimum of four phases for their description, and, as was the case for Mn$^{2+}$, the washout curves do not account for all of the Fe(CN)$_6^{3-}$ accumulated during the labeling period.

A visual examination of the washout curves in Figure 5B reveals that significantly more Na$_4$Fe(CN)$_6$ is gained in response to the extra 35 minutes of labeling time, that the two curves exhibit a similar time course, and that their separation remains nearly constant between the 20th and 90th minute. These particular two curves were subjected to curve fitting with a Fourier-based computer program developed by Provencher. Both 4- and 5-phase resolutions were obtained, but the statistical evidence was inadequate to support the more complex solution. The following expressions, converted to pool size, were returned for the upper and lower curves, respectively:

$$\text{counts/min} = (1.06e^{-0.44t} + 3.25e^{-0.54t} + 1.97e^{-0.129t} + 0.614e^{-0.0272t}) \times 10^6$$

The amount of isotope increased by approximately 100% in the slowest phase, by 100% in the next slowest phase, by 45% in the second fastest phase, and did not change in the fastest phase. As in the case for $^{45}$Ca$^{2+}$ and $^{54}$Mn$^{2+}$ washouts, there is a phase with a rate constant of approximately 0.5 min$^{-1}$. Conversion of the counts/min contained in this phase to a water space yields values of 522 (upper curve) and 366 (lower curve) ml/liter tissue H$_2$O. As was the case with $^{54}$Mn$^{2+}$, these analyses indicate that the bulk of the extra Na$_4$Fe(CN)$_6$ accumulated in response to additional labeling time, is attributable not to the faster but to the slower phases. On the assumption that the fastest phase represents Na$_4$Fe(CN)$_6$ in the stopcocks, cannula, and vascular space (see Langer), the content of Na$_4$Fe(CN)$_6$ in the slowest two phases (upper curve) equals 44% of the total in the three phases ascribable just to the tissue.

The presumption underlying this study is that Fe(CN)$_6^{3-}$ can be used to precipitate Mn$^{2+}$ in the tissue and that the kinetic identification of the site or sites of this action will provide inferential information about their morphological location. Knowledge about the reaction between Mn$^{2+}$ and Fe(CN)$_6^{3-}$ will, therefore, be of value in the design of an isotopic methodology and in the interpretation of the results. Accordingly, test tube experiments were conducted to establish the limitations on the reaction between Na$_4$Fe(CN)$_6$ and other divalent, Mn$^{2+}$-like cations.

Data were acquired both on the visual identification of metallic cation-Fe(CN)$_6^{3-}$ precipitation and on $^{54}$Mn$_2$Fe(CN)$_6$ precipitation. Reaction mixtures were prepared (using the control Ca$^{2+}$ medium) in which the products, [Mn$^{2+}$]$^2$ $\times$ [Na$_4$Fe(CN)$_6$], were approximately equal to and greater than the reported solubility product of Mn$_2$Fe(CN)$_6$ between $10^{-12}$ and $10^{-13}$ mol$^3$ liter$^{-3}$ (solid Mn$_2$Fe(CN)$_6$)$_{21}$. There was clear agreement between both kinds of studies. As long as the product [Mn$^{2+}$]$^2$ $\times$ [Na$_4$Fe(CN)$_6$] > $10^{-9}$ to $10^{-10}$ mol$^3$ liter$^{-3}$ the reaction was essentially instantaneous and stoichiometrically complete (i.e., Mn$^{2+}$ will strip all of the Fe(CN)$_6^{3-}$ from the Na$_4$Fe(CN)$_6$ complex). If the product < $10^{-9}$ to $10^{-10}$, the reaction required several hours for completion. The rates of formation of Na$_2$Fe(CN)$_6$, Cd$_2$Fe(CN)$_6$, and Pb$_2$Fe(CN)$_6$ were also visibly checked. Rates of precipitation of the Ni$^{2+}$ and Cd$^{2+}$ complexes were judged equivalent to that for Mn$^{2+}$. The rate of Pb$^{2+}$ precipitation, however, appeared much slower.

Additional tests were conducted to determine whether $[^{14}C]Mn_2$Fe(CN)$_6$ precipitate would count with the same efficiency as $[^{14}C]$Na$_4$Fe(CN)$_6$. Two scintillation vials
were prepared. Each received 0.6 ml of control Ca2+ medium containing 1.0 mM [14C]Na4Fe(CN)6. Mn2+ was added to one vial to yield a concentration of 2.5 mM. Both vials were treated for liquid scintillation counting according to the steps outlined in Methods. Statistically identical count levels were registered in each vial.

Based on these data, there is reason to believe that a Na4Fe(CN)6 washout curve from a muscle previously treated with this salt will be perturbed by an exposure to 2.5 mM Mn2+ as long as the [Na4Fe(CN)6] exceeds approximately 100 μM in the compartment(s) where precipitation is possible. This expectation was tested in a series of five experiments (two exploratory and a set of three, two of which are illustrated in Figures 6 and 7).

Each muscle in this series received duplicate 6- to 7-minute labels with medium containing 200 mM [14C]Na4Fe(CN)6. The [Ca2+]o of the medium was either 1.0 mM or 100 μM. After each label, the accumulated Na4Fe(CN)6 was washed out and the effluent sampled for 90 minutes. In one case, the washout was uninterrupted except for a possible change of the [Ca2+]o, which yielded approximately 100 μM in the compartment(s) where precipitation is possible. This expectation was tested in a series of five experiments (two exploratory and a set of three, two of which are illustrated in Figures 6 and 7).

Each muscle in this series received duplicate 6- to 7-minute labels with medium containing 200 mM [14C]Na4Fe(CN)6. The [Ca2+]o of the medium was either 1.0 mM or 100 μM. After each label, the accumulated Na4Fe(CN)6 was washed out and the effluent sampled for 90 minutes. In one case, the washout was uninterrupted except for a possible change of the [Ca2+]o, which yielded the control curve. In the other case, the washout sequence was identical, except the muscle was exposed to 2.5 mM Mn2+ at some specified time for a period of 3 minutes.

**Figure 6** [14C]Na4Fe(CN)6 washout with ( Philosophical Society of Washington, 1854 ) and without ( lower curve ) 2.5 mM Mn2+ treatment. Arrows (†) indicate placement and duration of Mn2+ treatment. Inset identifies envelope of active tension during experimental routine. Traces corresponding to radioactive washout curves are labeled "a" (control) and "b"(2.5 mM Mn2+). Perfusion sequence yielding tension record (a) and control washout ( , lower curve ) was: 1.0 mM Ca2+ changed to 100 μM Ca2+ + 20 mM [14C]-Na4Fe(CN)6 7 minutes prior to washout ( ). At t = 0 ( ), washout initiated in 100 μM Ca2+ continued for 20 minutes, then changed to 1.0 mM Ca2+ medium for duration of washout. The same perfusion sequence was used to obtain tension record (b) and perturbed washout ( upper curve ) except 2.5 mM Mn2+ solution was added at t = 20.0 minutes ( ) concurrently with return to 1.0 mM Ca2+ medium. After a 30-minute exposure, the Mn2+ solution was removed ( ) and washout continued in 1.0 mM Ca2+ medium. The perfusion sequence followed to obtain tension record (c) was the same as that used to obtain (b) but the [14C]Na4Fe(CN)6 label period was omitted. Temperature = 27°C, stimulation rate = 24/min, tissue wet weight = 1.37 g, and percent tissue H2O = 86.4.

**Figure 7** [14C]Na4Fe(CN)6 washout with ( Philosophical Society of Washington, 1854 ) and without ( lower curve ) 2.5 mM Mn2+ treatment. Arrows (†) indicate placement and duration of Mn2+ treatment. Inset identifies envelope of active tension during experimental routine. Traces corresponding to radioactive washout curves are labeled "a" (control) and "b"(2.5 mM Mn2+). Perfusion sequence yielding tension record (a) and control washout ( , lower curve ) was: 1.0 mM Ca2+ changed to 100 μM Ca2+ + 20 mM [14C]-Na4Fe(CN)6 6 minutes prior to washout ( ). Washout initiated at t = 0 ( ) in 1.0 mM Ca2+ for 12.0 minutes, then changed to 100 μM Ca2+ for 8.0 minutes, and finally returned to 1.0 mM Ca2+ ( ) for the duration of the washout. The same perfusion sequence was followed to obtain tension record (b) and perturbed washout ( upper curve ) except 2.5 mM Mn2+ solution was added at t = 20.0 minutes ( ) concurrently with return to 1.0 mM Ca2+ medium. After a 30-minute exposure, the Mn2+ solution was removed ( ) and washout continued in 1.0 mM Ca2+ medium. The perfusion sequence followed to obtain tension record (c) was the same as that used to obtain (b) but the [14C]Na4Fe(CN)6 label period was omitted. Temperature = 27°C, stimulation rate = 24/min, tissue wet weight = 1.37 g, and percent tissue H2O = 86.4.

Toward the end of each experiment, the effect of 2.5 mM Mn2+ on force development was measured in the absence of Na4Fe(CN)6 treatment. The inset of each figure diagrams these three sequences. As was the case for the papillary and trabecular muscles, protection was calculated for each septum. These and other relevant data pertaining to the set of three experiments, two of which are illustrated in Figures 6 and 7, are listed in Table 1.

The curves in Figure 6 show the reaction between residual Fe(CN)63- and 2.5 mM Mn2+ two minutes into the washout. Within seconds after the change to the perfusate containing 2.5 mM Mn2+ ([Ca2+]o = 1.0 mM), there was a marked decline in the appearance of Na4Fe(CN)6 in the effluent. During the 3-minute period of exposure to Mn2+, the curve declined and approached a course parallel to the control curve. Immediately after the removal of Mn2+ there was a marked reappearance of Na4Fe(CN)6 in the effluent; the curve overshoot the control and remained significantly elevated until the 90th minute.

The physical interpretation of this event is the following: As Mn2+ enters the interstitial space, precipitation of
have ensued, a finding not evidenced by the washout of Na$_4$Fe(CN)$_6$ available for reaction was markedly less than the washout curve was markedly reduced. (3) Protection was observed when Mn$^{2+}$ was added 20 minutes after the beginning of the washout: (1) The onset of the decline of Fe(CN)$_6^{2-}$ counts probably means that the free Na$_4$Fe(CN)$_6$ remaining in the tissue was 1.13 fmol, equivalent to 0.827 mmol/kg wet tissue, 0.971 mmol/liter tissue H$_2$O, or 1.86 mmol/liter interstitial H$_2$O (assuming a space of 520 ml/liter tissue H$_2$O). Had any of these values been representative of the free Na$_4$Fe(CN)$_6$ in any compartment open to Mn$^{2+}$, a prompt and vigorous precipitation should have ensued, a finding not evidenced by the washout curve. The delayed and minimal decline in [C]Fe(CN)$_6^{2-}$ counts probably means that the free Na$_4$Fe(CN)$_6$ available for reaction was markedly less than these values. The data presented in Table 1 permit a more quantitative understanding of the three experiments. The lack of agreement among columns 1, 2, and 6 (protection) implies that protection is not directly related to the total tissue content of Na$_4$Fe(CN)$_6$. Among columns 4, 5, and 6, agreement is much better. Protection declines approximately in parallel with the percent Na$_4$Fe(CN)$_6$ remaining in a phase 1-like pool. Lack of agreement between columns 5 and 6 is not surprising because the influx of Mn$^{2+}$ and the efflux of Na$_4$Fe(CN)$_6$ are both functions of time. For example, if the amount of isotope remaining in the phase 1-like pool (8- to 11-minute exposure) is calculated at the 9.8-minute point instead of the 8-minute point, the percent [$^{14}$C]Fe(CN)$_6^{2-}$ not seen increases from 58.9 to 100.

Discussion

These results demonstrate that the normal effect of Mn$^{2+}$ on cardiac tissue (uncoupling of excitation and contraction) can be blocked by pretreatment with 20 mM Na$_4$Fe(CN)$_6$. This protection, however, dissipates rapidly after the removal of this salt from the perfusion medium. The data shown in Figure 3 indicate that the decline of protection follows apparent first order kinetics, suggesting that protection is confined to a single kinetically defined compartment. Since the rate constant for the decline of protection is at least as fast as that for the decline of force (contraction) can be blocked by pretreatment with 20 mM Na$_4$Fe(CN)$_6$, this compartment is most likely equivalent to the one necessary for the maintenance of normal force development. By comparison with the data of Shine et al. 7 (see also ref. 19), we would conclude that this space has the characteristics of the interstitium.
Insights into these issues were provided by the isotopic experiments conducted with $^{54}$Mn$^{2+}$ and $[^{13}C]$-Na$_4$Fe(CN)$_6$. Based on recent data presented by Ochi and Delahayes, we must presume that some of the Mn$^{2+}$ seen in the washout curves had a cellular origin. Calculation of the water spaces (Fig. 5A) occupied by Mn$^{2+}$ after the 4.5-minute label (71% of the wet tissue weight or 81% of the total tissue H$_2$O) and after the 30-minute label (148% of the wet tissue weight or 171% of the total tissue H$_2$O exclusive of the first phase) certainly indicates that more Mn$^{2+}$ was accumulated than could have been accounted for by the interstitial space alone. When the washout of Mn$^{2+}$ was correlated with the recovery of force development to the steady state (98% complete within 10–11 minutes), we found that all Mn$^{2+}$ effluxing with a rate constant slower than 0.36 to 0.40 min$^{-1}$ was unrelated to the uncoupling of excitation and contraction. By comparing the washout of Mn$^{2+}$ with the washout of Ca$^{2+}$ (ref. 7) from rabbit septa, we conclude that Mn$^{2+}$ acts to uncouple excitation and contraction in a compartment functionally equivalent to the interstitial space. These isotopic data thus appear to agree with recent mechanical data obtained from thin-diameter papillary and trabecular muscles (Sanborn, unpublished observation). (Note that the influx and efflux or accumulation and washout of Mn$^{2+}$, or any other substance yet tested, is markedly slower for the case of septa compared to thin-diameter papillary and trabecular muscles. This must be kept in mind when juxtaposing data from these two preparations.)

In the recent paper by Delahayes, an intracellular role for the action of Mn$^{2+}$ was linked to the development of contracture that was manifest when guinea pig ventricular strips were stimulated in medium containing 2 mM Ca$^{2+}$ and 10 mM Mn$^{2+}$. Contracture did not develop quiescence. The question of interest is whether Mn$^{2+}$ acts at intracellular sites during "normal" uncoupling of excitation and contraction. In most instances, contracture does not accompany the diminution of force development as long as the exposure is brief—minutes rather than tens of minutes—and the Mn$^{2+}$ is below approximately 2.5 mmol/kg cell H$_2$O. This would mean that the [Na$_4$Fe(CN)$_6$], and the Na$_4$Fe(CN)$_6$ space (Fig. 5A) measured 71% of the wet tissue weight. The simplest interpretation of these data is that the interstitial space contains a paucity of anion-binding sites. Thus, the origin of Mn$^{2+}$ in the two slowest phases may be cellular. A similar conclusion was reached concerning the extra slow, time-dependent uptake of SO$_4^{2-}$.

Since the 38.5-minute Na$_4$Fe(CN)$_6$ space equals 93.6% of the total tissue H$_2$O, at least some cellular spaces could have accumulated this substance. If cellular H$_2$O amounts to approximately 2.5 kg/kg dry tissue, the apparent Na$_4$Fe(CN)$_6$, due to the two slowest phases (after 38.5 minutes of labeling) equals 21.7 mmol/kg cell H$_2$O. This would mean that the Na$_4$Fe(CN)$_6$, and the Na$_4$Fe(CN)$_6$ space (Fig. 5A) measured 71% of the wet tissue weight. The simplest interpretation of these data is that the interstitial space contains a paucity of anion-binding sites. Thus, the origin of Mn$^{2+}$ in the two slowest phases may be cellular. A similar conclusion was reached concerning the extra slow, time-dependent uptake of SO$_4^{2-}$.

Of interest, then, is an analysis of the possible location of the pools of Na$_4$Fe(CN)$_6$, contributing to these slow phases. The raw data themselves, in conjunction with data in the literature, can provide some insight into this issue. By comparing the washout of Mn$^{2+}$ with the washout of Ca$^{2+}$ (ref. 7) from rabbit septa, we conclude that Mn$^{2+}$ acts to uncouple excitation and contraction in a compartment functionally equivalent to the interstitial space. These isotopic data thus appear to agree with recent mechanical data obtained from thin-diameter papillary and trabecular muscles (Sanborn, unpublished observation). (Note that the influx and efflux or accumulation and washout of Mn$^{2+}$, or any other substance yet tested, is markedly slower for the case of septa compared to thin-diameter papillary and trabecular muscles. This must be kept in mind when juxtaposing data from these two preparations.)

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minute treatment, because Mn\(^{2+}\) only reacts with the Fe(CN)\(_6^{3-}\) in the phase 1-like pool. Thus the apparent first order decline of protection, rather than delimiting the restricted accumulation of Na\(_4\)Fe(CN)\(_6\) to (the phase 1-like pool), most likely delimits the sites at which Mn\(^{2+}\) (and Ni\(^{2+}\), Cd\(^{2+}\), and Pb\(^{2+}\) also) acts to uncouple excitation and contraction. Based on the comparison of \(^{55}\)Mn\(^{2+}\) washouts with \(^{40}\)Ca washouts, the phase 1-like pool probably represents Na\(_4\)Fe(CN)\(_6\) localized to the interstitial spaces.

The reader should be mindful that the results and analyses presented in this paper do not prove that the reaction sites, and, hence, the sites of action of Mn\(^{2+}\), are in the interstitial space. They only set a kinetic limitation on the physical description of this space. However, the actual reaction sites could be anywhere as long as their exchange with the interstitial space were at least as rapid as the exchange of material with the interstitial space itself. In this sense, the location of these sites would be functionally equivalent to the interstitial space. This is in agreement with the conclusions reached in other studies (Sanborn, unpublished observations).

Acknowledgments

We express our appreciation to Lacy Goode for her contribution to the preparation of this manuscript.

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Ferrocyanide protection against uncoupling of excitation and contraction by manganese in rabbit ventricular myocardium.

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*Circ Res.* 1978;43:178-188
doi: 10.1161/01.RES.43.2.178

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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