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Journal of Cardiovascular Pharmacology

Transition Metal Chelators Reduce Directly Measured Myocardial Free Radical Production During Reperfusion

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Issue: Volume 32(3), September 1998, pp 343-348

Publication Type: [Articles]

Publisher: © 1998 Lippincott Williams & Wilkins, Inc.

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Received April 30, 1997; revision accepted October 31, 1997.

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ISSN: 0160-2446
Accession: 00005344-199809000-00002

Keywords: Free radicals, Transition metals, Reperfusion, Ascorbate, Electron paramagnetic resonance

Summary:

Transition metals such as iron and copper are present in the myocardium and can act as catalysts for the formation of oxygen free radicals during reperfusion after myocardial ischemia. Previous studies suggested that transition metal chelators such as desferrioxamine reduce the production of such radicals and may thereby attenuate postischemic myocardial dysfunction. These studies used spin trapping agents, commonly nitrone compounds, which may themselves influence the severity of the ischemia and reperfusion events being studied. We evaluated two transition metal chelators, desferrioxamine, an iron chelator, and bathocuproine, a copper chelator, by using a new electron paramagnetic resonance technique that does not require the administration of spin traps. We measured ascorbate free radical, an index of free radical production, in the great cardiac vein effluent. Twenty-eight open-chest dogs underwent 20 min of coronary artery occlusion and 30 min of reperfusion. Ten dogs received no drug, 10 dogs received 750 mg bathocuproine, i.v., and eight dogs received 700 mg desferrioxamine, i.v. Both bathocuproine and desferrioxamine blunted the postreperfusion increase in ascorbate free radical generation: no drug, 36 ± 8% increase; desferrioxamine, 13 ± 5% increase; bathocuproine, 21 ± 6% increase ($p < 0.05$ vs. baseline). Thus direct free radical measurements indicate that chelation of the transition metals iron and copper reduces free radical generation during reperfusion.

Myocardial reperfusion after coronary artery occlusion of <20 min, although not resulting in cellular necrosis, results in prolonged abnormalities in contractile function ("stunning") (1). There is extensive evidence that oxygen free radicals play a central role in the pathogenesis of postischemic myocardial reperfusion injury (2,3). Redox-active metals catalyze oxygen radical formation and thus tissue injury during ischemia-reperfusion sequences (3-7). This concept is based on the basic chemistry of oxygen radicals in which trace amounts of a transition metal are required to catalyze the formation of reactive oxygen species such as the hydroxyl radical by the Fenton reaction Equation (1) in which M is a transition metal, and thereby amplify free radical-mediated tissue damage. This chemistry suggests that oxygen radical-mediated damage could be blunted by limiting the availability of transition metal catalysts during reperfusion.

$$H_2O_2 + Mn^{-1} \rightarrow OH^- + HO^\bullet + M^n$$

Equation 1

Studies investigating the role of transition metals as catalysts for formation of oxygen free radicals during reperfusion primarily focused on iron; iron chelators result in improvement in myocardial functional and meta-bolic recovery after ischemia-reperfusion, presumably by preventing oxygen free radical generation (4-7). However, the studies showing a reduction of radical production by chelating agents relied on the administration of spin traps, chemical compounds that combine with the radicals to form stable adducts, detectable by electron paramagnetic resonance (EPR) techniques. The use of such spin traps has been criticized: the agents used have primarily been nitrones that may have toxic effects, or by reacting with radicals, may actually blunt the damage observed or otherwise alter the ischemia-reperfusion sequences under study (8). To overcome these objections, Mergner et al. (8) used an ex vivo trapping technique in which the spin trap was introduced rapidly into the venous effluent of the heart. We took a different approach, developing an on-line, real-time technique that uses EPR spectroscopy directly to measure the ascorbate free radical (9). Ascorbate repairs more oxidizing radicals such as peroxyl radicals; it is itself oxidized by one electron to the ascorbate radical. The amount of ascorbate radical in the

blood is a quantitative parameter of total oxidative stress. Our method does not require administration of spin traps or other chemicals and provides a reliable method for quantitation of free radical generation (9).

Thus one purpose of our study was to use our new ascorbate free radical technique to demonstrate that a transition metal chelator, such as desferrioxamine, reduces free radical flux after myocardial ischemia-reperfusion sequences. A second purpose was to evaluate the possible role of copper chelators in ischemia-reperfusion sequences. Less is known regarding the role of copper in reperfusion injury. The heart contains a significant amount of copper (10,11). Further, copper can be 10-60 times more potent than iron in catalysis of the Fenton reaction (12). Thus we hypothesized that use of a chelator that targets copper, rendering it less catalytically active, would reduce overall free radical generation in the myocardium during a brief coronary occlusion/reperfusion sequence. We report our results from using the copper chelator bathocuproine in an open-chest canine model.

METHODS

EPR technique

The EPR technique used was described in detail elsewhere (9). In brief, ascorbate is considered to be the terminal small-molecule donor antioxidant in physiologic systems. Ascorbate free radical (AFR) is generated when ascorbate donates an electron to more oxidizing radicals such as peroxyl, alkoxyl, and hydroxyl radicals. Thus AFR generation is proportional to the total oxidative load of the system (9). The AFR is resonance stabilized, allowing its detection by EPR spectroscopy. We used a Varian E-4 spectrometer (Varian Associates, Inc., Palo Alto, CA, U.S.A.) with a TM_{110} cavity and an aqueous flat cell to monitor AFR concentration in the coronary venous effluent as a real-time marker of free radical generation within the ischemic reperfused hearts (9).

Spectra were obtained on blood being continuously with-drawn from either the great cardiac vein or the femoral artery within seconds of leaving the animal. The following instrument settings were used for all studies, as they provide the largest ascorbate radical signal within the constraints of the needed [AFR] versus time: nominal power, 40 mW; modulation amplitude, 0.63 gauss; time constant, 1 s; and scan rate, 1 gauss/24 s (13). The concentration of AFR was determined from its EPR signal height after calibration by using double integration techniques and 3-carboxy proxyl as the standard. The AFR EPR signal is partially saturated at 40 mW power. Appropriate corrections were made to determine steady-state AFR concentrations (13). We found that for our experimental conditions, 1 mm of signal height corresponded to 0.0734 n*M* ascorbate free radical, after saturation effects were accounted for. All signal heights were normalized to full gain of the instrument, 10^5.

Animal preparation

Twenty-eight dogs (20-25 kg) were anesthetized (fentanyl-droperidol, 0.13 ml/kg, and pentobarbital, 20 mg/kg) and mechanically ventilated. Arterial blood gases were maintained in physiologic range by adjusting respiratory rate, inspired O_2, and serum bicarbonate. The electrocardiogram and central aortic pressure were continuously monitored. Through a left lateral thoracotomy, the heart was suspended in a pericardial cradle. The left anterior descending coronary artery was isolated proximally and a snare placed around the vessel for subsequent coronary occlusion. A catheter was passed from the external jugular vein into the coronary sinus and secured with its tip in the great cardiac vein for withdrawal of blood for EPR analysis. A venous-to-venous shunt was fashioned so that after EPR scanning, the blood from the cardiac vein was returned to the femoral vein. The amplitude of the AFR signal is proportional to the AFR concentration in blood, as previously described (9). Ascorbic acid was infused intravenously as a 1-g bolus then at a maintenance rate (8-30 mg/min) to obtain a constant endogenous AFR signal. The systemic arterial blood was scanned by EPR intermittently to assure steady-state arterial AFR concentration. If needed, the maintenance ascorbate infusion was adjusted.

Echocardiography

Two-dimensional echocardiography was performed by using a 5-mHz transducer (ATL Ultramark-4; Advanced Technology Laboratories, Bothell, WA, U.S.A.). Transthoracic images of the parasternal short-axis at the level of the midpapillary level were obtained in all conditions. Recordings were taken at baseline, at 10 min of occlusion, and after 30 min of reperfusion. The occlusion echo was analyzed off-line for the extent of abnormal wall contraction by digitizing end-diastolic frames and by using these frames as the reference for systolic contraction abnormalities. The cross-sectional myocardial area was computed by digitizing epicardial and endocardial borders (including papillary muscles); the area enclosed by the endocardial border [i.e., left ventricular (LV) cavity] was then subtracted from the area enclosed by the epicardial border. The area of abnormal wall contraction (hypokinesis, akinesis, or dyskinesis) was then digitized on the same end-diastolic frame. The percentage of abnormal wall contraction during occlusion, or "% occlusion area," was computed as Equation (2)

$$\frac{(\text{Area of abnormal occlusion contraction})}{(\text{Total myocardial area})} \times 100$$

Equation 2

The 30-min reperfusion echocardiograms were similarly analyzed: the percentage abnormal wall contraction during reperfusion Equation (3)

$$\%\ \text{reperfusion or ``stunned'' area} = \frac{(\text{Area of abnormal reperfusion wall contraction})}{(\text{Total myocardial area})} \times 100$$

Equation 3

The percentage of myocardium that was salvaged was then estimated as the difference between the occlusion area and reperfusion area. Equation (4)

$$\% \text{ Salvage area} = \frac{(\text{Occlusion area} - \text{Reperfusion area})}{(\text{Total myocardial area})} \times 100$$

Equation 4

As the size of the ischemic occlusion area varied among the animals, the salvaged area was normalized to the size of the occlusion area, by using the formula: Equation (5)

$$\text{Salvage ratio} = \frac{(\% \text{ Salvage area})}{(\% \text{ Occlusion area})}$$

Equation 5

Experimental protocol

After a steady-state concentration of AFR was established, 20 min of coronary occlusion was performed by using the left anterior descending snare. The snare was then released and the animals monitored for 30 min of reperfusion. Great cardiac vein AFR concentration was continuously monitored. As the absolute AFR value for each animal is different, the AFR radical concentration was normalized to the baseline value (preocclusion). Thus AFR data are expressed as percentage change from preocclusion AFR level and reported at the following nine time points after reperfusion: 1, 3, 5, 7, 10, 15, 20, 25, and 30 min. Three groups of animals were studied. Group 1 (n = 10) was given no drug. Group 2 (n = 8) was treated with desferrioxamine (700 mg) as a continuous infusion starting 15 min before occlusion and continued through reperfusion. Group 3 (n = 10) was treated with bathocuproine (750 mg) as a continuous infusion started 5 min before occlusion and continued through reperfusion. The dose of desferrioxamine was selected as a representative dose from prior studies of its use in coronary is-chemia-reperfusion experiments (4-7,14-18). Higher doses of desferrioxamine were associated with hypotension if infused over a 20-min period. The bathocuproine dose was empiric and was estimated to provide a relatively equimolar dose compared with the desferrioxamine dose. Data from seven of the control animals were previously reported (19).

Statistical analysis

The percentage change in AFR from baseline in the time interval of 1-30 min in bathocuporine and desferrioxamine treat-ment groups was compared with that of the no-drug group by using repeated measures analysis of variance. The analysis was performed by using SAS procedure MIXED (20) with an unstructured within-subject covariance matrix. Mean contrasts were estimated and tested by using the Bonferroni *t* test to compare mean percentage change in AFR in bathocuporine and desferrioxamine with the control group at each of the nine postreperfusion time points reported.

The same analysis was used to test whether there was a significant change from baseline at each time point. A value of $p < 0.05$ was considered significant.

RESULTS

The hemodynamic data of Table 1 show that there were no differences in heart rate, systolic blood pressure, diastolic blood pressure, or rate-pressure product at baseline, during occlusion, or during reperfusion between the three conditions.

	No-drug dogs			Desferrioxamine dogs			Bathocuproine dogs		
	Base	Occ	Rep	Base	Occ	Rep	Base	Occ	Rep
HR	138 ± 15	132 ± 17	133 ± 23	130 ± 17	137 ± 25	139 ± 23	136 ± 20	136 ± 21	138 ± 24
SBP	90 ± 18	86 ± 18	81 ± 20	94 ± 24	96 ± 27	98 ± 31	104 ± 15	92 ± 21	96 ± 19
DBP	59 ± 16	56 ± 14	56 ± 17	57 ± 21	55 ± 20	61 ± 24	66 ± 16	57 ± 17	60 ± 14
RPP	12,500 ± 3,100	—	—	12,400 ± 4,100	—	—	14,000 ± 2,900	—	—

Base, baseline; OCC, occlusion; Rep, reperfusion; SBP, systolic blood pressure; DBP, diastolic blood pressure; RPP, rate–pressure product.

TABLE 1. Hemodynamic data

The no-drug animals showed an increase in AFR con-centration that began immediately after initiation of reperfusion and was statistically greater than baseline value at 1-25 min of reperfusion ($p < 0.01$; Fig. 1). The peak increase was 36 ± 8%, and the time to peak increase was 5 min. After reperfusion, the animals treated with the iron chelator desferrioxamine showed no significant increase in AFR compared with the baseline level. The animals treated with the copper chelator bathocuproine had an increase in AFR concentration that was statistically greater than baseline at 3, 5, and 7 min after reperfusion ($p < 0.05$). The peak increase was 21 ± 6% at 7 min.

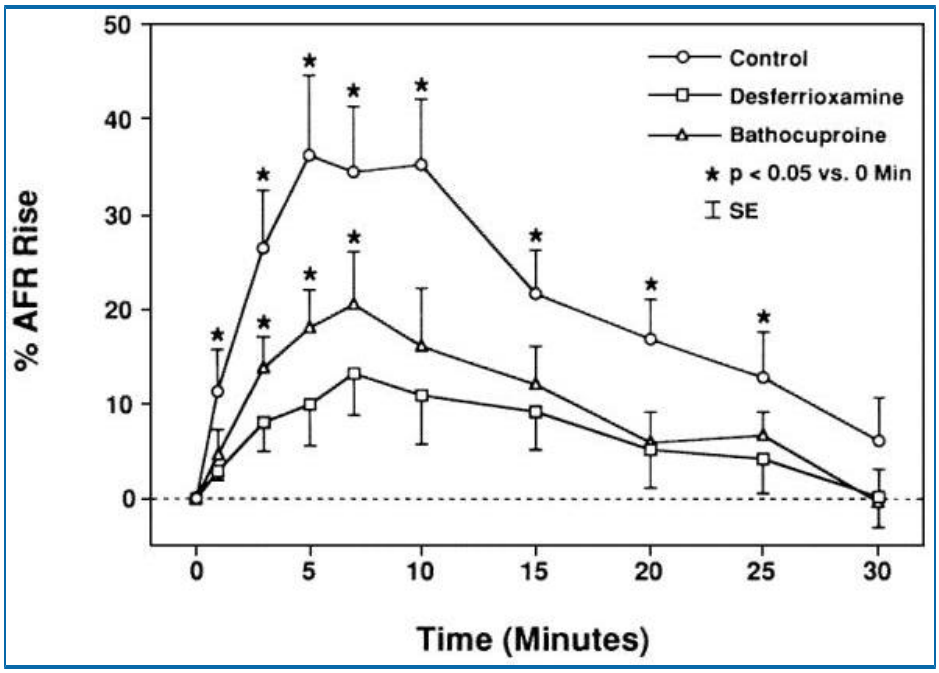

FIG. 1. Percentage increase in ascorbate free radical (AFR) during reperfusion after 20 min of coronary occlusion. When no drug was given (control dogs), all time points from 1 to 25 min show a significant increase in AFR compared with 0 min (baseline). After desferrioxamine treatment, there was no significant increase in AFR. After bathocuproine treatment, increases in AFR occurred only from 3 to 7 min.

Comparing the treated animals with no-drug animals, both treated groups had a lower peak increase in AFR than did the no-drug group (no drug, 36%; desferrioxamine, 13%; bathocuproine, 21%). The peak AFR increase with desferrioxamine was significantly less than the increase in the no-drug animals ($p = 0.01$); the peak AFR increase with bathocuproine versus no-drug animals showed a trend toward statistical significance ($p = 0.07$). Comparing the integrated areas under the percentage increase in AFR versus time curves, both treatment groups had smaller areas under the curve compared with the no-drug animals, but again, the desferrioxamine comparison achieved significance ($p = 0.02$; Fig. 2), whereas the bathocuproine versus no-drug comparison nearly achieved significance ($p = 0.07$). The desferrioxamine-and bathocuproine-treated groups were not different from each other.

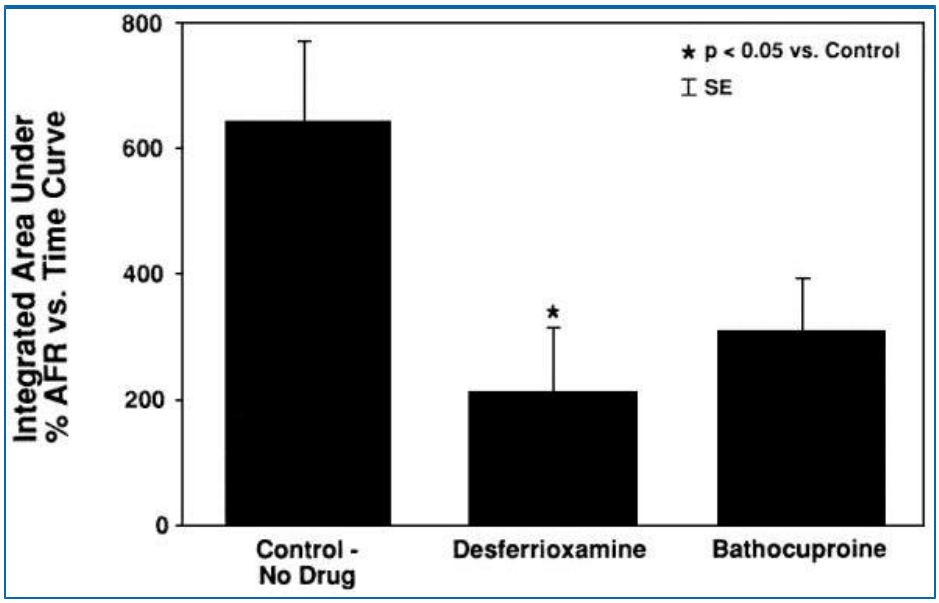

FIG. 2. Integrated area under the ascorbate free radical (AFR) time-activity curve. Desferrioxamine significantly reduced AFR generation ($p = 0.02$). The reduction with bathocuproine showed a trend toward significance ($p = 0.07$). The units are n*M* × min.

All animals had observable wall-motion abnormalities by echocardiography during occlusion (systolic expansion or bulging) and after 30 min of reperfusion. Table 2 shows the echocardiographic wall-motion analysis. The abnormal wall-motion area during occlusion was 37% in the no-drug and 47% in the desferrioxamine group, which is not significantly different. The bathocuproine-treated dogs had larger abnormal wall-motion areas than did the no-drug dogs at 54% ($p < 0.05$). The myocardium that was contracting abnormally after 30 min of reperfusion ("stunned") is shown in Table 2. There was

no difference between the no-drug and desferrioxamine groups (21 and 27%), but the stunned myocardial area was larger in the bathocuproine group (33%) versus the control dogs ($p < 0.05$). The percentage salvage was not statistically different among the three groups (Table 2). When nor-malized for the different occlusion abnormal wall-motion areas (salvage ratio), the difference between groups was also not significant.

	No-drug dogs	Desferrioxamine dogs	Bathocuproine dogs
Abnormal contraction (%)			
% Occlusion area	37 ± 6	47 ± 16	54 ± 10[a]
% Reperfusion area	21 ± 10	27 ± 9	33 ± 15[a]
Percentage salvage area	16 ± 13	20 ± 23	19 ± 17
Salvage ratio	0.24 ± 0.3	0.24 ± 0.3	0.32 ± 0.3

[a] $p < 0.05$ vs. no-drug dogs.

TABLE 2. Contraction abnormalities by echocardiography

DISCUSSION

The major finding of this study is that both the iron chelator desferrioxamine and the copper chelator bathocuproine reduced free radical generation after a brief coronary occlusion-reperfusion sequence. This was shown by using a technique that directly measures the ascorbate free radical without the potentially confounding effects of chemical spin traps.

As we previously showed (9), AFR concentration in the coronary venous effluent increases rapidly during reperfusion after brief coronary occlusion. AFR concen-tration is a measure of the total oxidative stress in the myocardium during reperfusion. The increase in AFR after an occlusion-reperfusion sequence can be attenuated by the free radical scavengers superoxide dismutase and catalase (9). In this study, the AFR increase was attenuated by giving two different transition metal chelators. These chelators presumably prevent copper and iron from acting as catalysts for the amplification of oxygen free radical formation during reperfusion.

Multiple studies focused on iron and showed improved functional and metabolic recovery of isolated hearts sub-ject to global hypoperfusion when treated with desfer-rioxamine (14-18). Others showed improved myocardial contractile recovery in intact animals treated with desferrioxamine after an ischemia-reperfusion sequence (4-7). Presumably this benefit is from the desferrioxamine inhibition of free radical generation during reperfusion. Two prior ischemia-reperfusion studies with spin traps showed that myocardial production of radical adducts is decreased with desferrioxamine treatment (4,8). Our study supports those; it is the first study to use a tech-nique that does not require chemical spin traps and to show by using real-time direct radical measurement that desferrioxamine given before reperfusion decreased the production of AFR from the coronary effluent.

Although our AFR method requires supplementation by exogenous ascorbate given intravenously, we previously showed that ascorbate alone, at the levels used, did not modify postischemic dyskinesis after an is-chemia-reperfusion sequence and does not artifactually alter the phenomenon we are studying (9).

We also showed the free radical-attenuating effect of a copper chelator, bathocuproine. Copper is a potent catalyst of the Fenton reaction, which generates hydroxyl radical during reperfusion (21). When isolated rat hearts are subjected to a global ischemia-reperfusion sequence, hearts from rats fed a diet low in copper have improved functional recovery compared with that of rats with adequate copper diet (22). Others showed that isolated hearts that are loaded with copper before ischemia-reperfusion have markedly reduced functional recovery (10). Powell et al. (11) showed that, when given with an oxygen free radical-generating system, copper causes a decrease in myocardial function (no ischemia-reperfusion involved). Applebaum et al. (23) showed that a copper chelator, neocuproine, protected isolated rat hearts during an is-chemia-reperfusion sequence. Our data support the con-cept that the cardioprotection in copper chelator-treated hearts in the work of Applebaum et al. may be secondary to decreased free radical production.

Although the increase in AFR after reperfusion was reduced by bathocuproine, it was not abolished; in this respect, bathocuproine was inferior to desferrioxamine. This may in part be dose related; the dose of bathocuproine we chose was empiric and may have been too low. A dose-response study would be necessary to demonstrate this point. It is also worth noting that the percentage of abnormal contraction during coronary occlusion in the bathocuproine-treated dogs was significantly higher than that of the no-drug dogs but not so in the desferrioxamine dogs, implying a larger risk area in the bathocuproine-treated dogs; this would tend to increase AFR generation in the bathocuproine groups compared with the desferrioxamine-treated dogs.

Despite reducing the amount of AFR generated during reperfusion, we were not able, by using echocardiography, to demonstrate improvement in myocardial function with iron and copper chelation. There are several possible explanations for this. First, we evaluated the effect only after 30 min of reperfusion. It is possible that the reperfusion sequence was not followed long enough to demonstrate eventual recovery of function in the metal chelator-treated groups. Other studies that have shown benefit from chelator intervention monitored the animals for 3-4 h of reperfusion (4-7). Second, the dose of these agents may not have been sufficient, as there still was AFR production in the treated groups, and this may have allowed myocardial damage. The dose of desferrioxamine in some previous animal studies was higher (6) and was been administered as an intracoronary dose in others (4). As noted earlier, the dose of bathocuproine was empiric. A dose-response study would be useful here also.

The copper chelator we used, bathocuproine, is used as a reagent for the colorimetric determination of copper and is considered to be copper specific (24,25). Superoxide dismutase is a copper/zinc-containing enzyme; potent copper chelators can reduce its activity, some so well that they are used as superoxide dismutase inhibitors (26). However, bathocuproine does not inhibit superoxide dismutase, which is important in the context of

ischemia-reperfusion.

This work supports the concept that transition metals such as iron and copper play an important role in the generation of free radicals during reperfusion after brief coronary occlusion. Chelation of these metals produces smaller increases in the ascorbate free radical in myocardial effluent during reperfusion. Whether copper chelation could have a clinical role as a cardioprotective strategy in patients undergoing ischemia-reperfusion sequences remains to be determined.

Acknowledgment: This study was supported in part by NHLBI grants HL07121 (Dr. Spencer) and RO1-HL53284 (Dr. Kerber). We gratefully acknowledge the technical assistance of Robin Smith and the secretarial assistance of Diane Phillips.

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