Problems and practical considerations in assessing accuracy with NIST SRM 909a: report of defective vials

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During an experimental period of 12 months in 1992–1993, while we were comparing the effectiveness of monthly vs quarterly use of the National Institute for Standards and Technology Standard Reference Material (NIST SRM) 909a as an accuracy material for the projected 30-year Fernald Medical Monitoring Program, we encountered three random defective vials with a glucose recovery of less than 30% of the NIST-assigned value. Analysis with five different multichannel instruments confirmed the original finding. Concomitant glucose recovery from adjacent vials was 97%-104%, as determined by using the same instruments, reagents, calibrators, and quality-control criteria on the same days. Recoveries of uric acid and cholesterol were also low (53–75% and 75–80%, respectively) in the three defective vials. Other analytes were unaffected. Studies to identify the cause of the defective vials were carried out with microbiological, electron microscopic, and biochemical techniques. When used for accuracy studies, each vial of NIST SRM 909a should have a concomitant check for glucose recovery to detect whether the vial is defective.

INDEXING TERMS: standardization • clinical chemistry • glucose • Reference Material

In 1990 our laboratories assumed the responsibility for maintaining the comparability of 23 chemical analytes over a 30-year period in support of the funded Fernald Medical Monitoring Program developed for a cohort of 15,000 individuals who resided near a uranium processing plant between 1954 and 1982 and who were exposed to uranium metal dust (personal communication, R.B. Wones, Department of Internal Medicine, College of Medicine, University of Cincinnati). This study is now in its sixth year.

We selected Standard Reference Material (SRM) 909a from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) as an accuracy material for nine of the analytes to be studied because the values assigned by NIST are based on analyses by individual NIST-developed Definitive Methods [1–9]. The unique feature of SRM 909a for long-term comparability purposes is that values for successive lots are assigned on the basis of actual Definitive Method measurement data rather than on the long-term stability of a single material.

We used SRM 909a to assess the ongoing accuracy of results obtained with six multianalyte instruments during the 3 years 1991–1993. During this period, we identified as defective three vials of SRM 909a, in which the glucose recovered was less than 30% of the assigned NIST value. Other vials from the same shipments yielded 95–104% recovery for glucose measured on the same day with the same instruments, reagents, calibrators, and daily quality-control materials and procedures [10].

Here, we report the analyte recovery studies for the three defective vials (two 909a-1, and one 909a-2) and for three concurrent acceptable vials and summarize the results of eight studies to determine the cause of the problem.

Materials and Methods

Multianalyte instruments, calibrators, controls, and reagents

We used two Hitachi 737, one Hitachi 717, and one Hitachi 747 analyzer [Boehringer Mannheim Corp. (BMC), Indianapolis, IN] and two Synchron CX3 analyzers (Beckman Instruments, Brea, CA).

Beckman calibrators (Standard 1 and Standard 2) at two concentrations were used to calibrate the Synchron CX3 analyzer.
lyzers. BMC calibrator (Precial) was used to calibrate the Hitachi instruments.

Ciba Corning controls (lot nos. 083001, 084001; Ciba Corning Diagnostics, Irvine, CA) were used to monitor the instrument day-to-day performance for all analytes except cholesterol. For cholesterol quality control on the Hitachi analyzers, we used Serachem cholesterol control (lot no. 400128; Fisher Scientific, Cincinnati, OH).

All instruments operated within acceptable daily control limits during the collection of all data reported in this paper.

BMC reagents were used in the Hitachi 737 A and 737 B instruments for all analytes, except that glucose and urea nitrogen reagents were from Sigma Diagnostics (St. Louis, MO) and creatinine reagent was from Sclavo (Wayne, NJ). BMC reagents were used for all analytes on the Hitachi 747 and 717 instruments. Beckman reagents were used for all CX3 determinations.

STANDARD REFERENCE MATERIALS
Two separate shipments of SRM 909a were received in dry ice (1 box/shipment) from NIST: the first in March 1992 and the second in October 1992. The boxes were stored at 4 °C. The vials for which results are reported here were used within 1 year from the date of shipment, as specified in the package insert.

A box of SRM 909a consists of six vials, three bottles each of two concentrations of analyte (909a-1 and 909a-2) in freeze-dried serum. All vials were reconstituted according to direction method B in the product insert descriptive data for SRM 909a. After reconstitution, the analytes were assayed within 1-2 h. The NIST-recommended period is to assay within 8 h after reconstitution.

ANALYSIS PROTOCOL
All vials were analyzed in the same manner as patients' samples and in triplicate for all analytes. When a defective vial was identified, we reconstituted a second vial of the same 909a lot and concentration and analyzed it that same day within 2 h, using the same instruments, the same reagents, the same calibrators, and the same daily quality-control acceptance criteria. We also analyzed one vial of the other concentration from that lot.

Analysis by Hitachi analyzers 717, 737, and 747. Calcium was measured by a 3-cyclohexylaminopropanesulfonic acid colorimetric method (BMC); cholesterol was measured spectrophotometrically by a cholesterol esterase and cholesterol oxidase reaction method [11, 12]; creatinine was measured by a modified Jaffé reaction involving kinetic measurements to reduce interference by noncreatinine substances [13, 14]; electrolytes were measured by indirect potentiometry [15, 16]; glucose was measured by the reduction of NAD⁺ in a coupled reaction between hexokinase and glyceraldehyde-3-phosphate dehydrogenase [17]; urea nitrogen was measured by a complex enzyme assay in which the rate of oxidation of NADH to NAD⁺ is monitored spectrophotometrically [18]; and uric acid was measured by uricase and catalase enzymes with production of a quinone-oxidime dye complex, the color of which was measured bichromatically [19].

Analysis by Beckman CX3 analyzers. Glucose was measured by oxygen consumption (amperometry) with use of glucose oxidase [20]; monovalent ions were measured by indirect potentiometry [21-23]; creatinine was measured by a modified Jaffé reaction [13, 14]; and urea nitrogen was measured by the increase in conductivity after addition of urease [24].

INVESTIGATIVE METHODOLOGY
The eight methods used to investigate the causes of the defective vials were: pH (Ciba-Corning Diagnostics method), lactic acid [25, 26], bacterial Gram stain [27], bacterial culture [28], sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [29], ultracentrifugation [30], total protein [31, 32], and electron microscopy [33]. Analytically accepted procedures were used throughout. A detailed description of the investigative methodology is provided in an Appendix, which is available from the corresponding author upon request.

Results
NIST accuracy studies as part of a 30-year program for monitoring chemical analytes in a local healthcare project led us to test 16 vials of NIST SRM 909a between March 1992 and February 1993 to determine whether monthly or quarterly accuracy studies were preferable. In the course of these studies, between August 1992 and February 1993, we encountered three defective vials of SRM 909a. Table 1 shows the glucose recovery as a percent of the labeled NIST SRM 909a value for each defective vial and for its concomitant acceptable vial.

Table 2 shows the complete analytical recovery data for

<table>
<thead>
<tr>
<th>Defective vial</th>
<th>Date found</th>
<th>909a level</th>
<th>Glucose recovery, % (mean and range, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Defective vial</td>
</tr>
<tr>
<td>1</td>
<td>08/22/92</td>
<td>a-1</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.1-12.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26-28</td>
</tr>
<tr>
<td>2</td>
<td>11/04/92</td>
<td>a-2</td>
<td>10.2</td>
</tr>
<tr>
<td>3</td>
<td>02/10/93</td>
<td>a-1</td>
<td>9-16</td>
</tr>
</tbody>
</table>

* a = Synchron CX3 A, b = Synchron CX3 B, c = Hitachi 737 A, d = Hitachi 737 B, e = Hitachi 717, f = Hitachi 747.
defective vial no. 1 (909a-1; 8/22/92) and the concomitant acceptable vial 909a-1 for nine analytes determined with four multichannel analyzers. As Table 3 shows, the sentinel analyte value for a defective vial was a glucose recovery of <30%.

However, two other analytes also showed diminished recovery. Recovery of uric acid in the defective vials was 53–71% vs 92–104% in the acceptable vials (Table 3). Cholesterol recovery in the defective vials was 75–86%, whereas 96–101% of cholesterol was recovered from the acceptable vials (Table 3). The recovery ranges for all other analytes in the three acceptable vials, used for verification on 8/22/92, 11/4/92, and 2/10/93, were between 89% and 113%, except for high creatinine recovery in Hitachi instruments—which represents a special problem of creatinine methodology not related to this study. The Synchron CX3 did not show any problem with creatinine recovery.

Table 3 also indicates that urea nitrogen and calcium, and monovalent ions Na, K, and Cl, were recovered by 84–110% in the defective vials—similar to the 89–107% range of recovery in the acceptable vials.

The detailed data for defective vials 2 (11/4/92) and 3 (2/10/93) and their acceptable adjacent vials are similar to those listed in Table 2 for defective vial 1 (8/22/92) and its adjacent acceptable vial. (Complete data are provided in an Appendix, available from the corresponding author upon request.)

Precision of analysis is indicated by the fact that triplicate assays of analytes in the defective and acceptable vials were within 2% of the mean values. (Details are available from the corresponding author as Appendix Tables 5 and 6.)

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Table 2. Recovery analysis of SRM 909a-1 defective and acceptable vials (8/22/92) for nine analytes by four multichannel analyzers.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>NIST-assigned average and target range, mmol/L</th>
<th>Defective vial</th>
<th>Acceptable vial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hitachi analyzers</td>
<td>Beakman analyzers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>737A</td>
<td>737B</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.37</td>
<td>0.27</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>5.25–5.50</td>
<td>(5.1)</td>
<td>(8.2)</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.234</td>
<td>0.166</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>0.231–0.237</td>
<td>(71.2)</td>
<td>(68.7)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4.89</td>
<td>ND</td>
<td>4.03</td>
</tr>
<tr>
<td></td>
<td>4.83–4.95</td>
<td>(82.5)</td>
<td>ND</td>
</tr>
<tr>
<td>Sodium</td>
<td>148.5</td>
<td>144.0</td>
<td>146.0</td>
</tr>
<tr>
<td></td>
<td>146.1–150.9</td>
<td>(99)</td>
<td>(98.3)</td>
</tr>
<tr>
<td>Potassium</td>
<td>3.66</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>3.58–3.74</td>
<td>(95.7)</td>
<td>(109)</td>
</tr>
<tr>
<td>Chloride</td>
<td>92.4</td>
<td>86.0</td>
<td>89.0</td>
</tr>
<tr>
<td></td>
<td>90.7–94.1</td>
<td>(93.1)</td>
<td>(96.3)</td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>5.54</td>
<td>5.36</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>5.46–5.61</td>
<td>(96.8)</td>
<td>(90.4)</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.32</td>
<td>2.12</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>2.28–2.36</td>
<td>(91.3)</td>
<td>(90.2)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.084</td>
<td>0.176</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>0.083–0.085</td>
<td>(210)</td>
<td>(221)</td>
</tr>
</tbody>
</table>

* The ranges assigned by the NIST and the stated uncertainties, defined as 95%/95% statistical tolerance intervals, which reflect the combined effects of measurement imprecision and the variability of the mass of dry serum among vials. Thus, 95% of the reconstituted vials would have analyte values falling within these specified ranges.

* Average of three measurements each.

* Percent of recovery is listed in parentheses.

* Aberrant methodology.

* ND, not done due to lack of method channel.

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Table 3. Range of recoveries of SRM 909a analytes from three defective and three acceptable vials by all analyzers.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Percent recovery, range</th>
<th>Defective vials</th>
<th>Acceptable vials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>8.8–27</td>
<td>95–105</td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>53–71</td>
<td>92–104</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>75–86</td>
<td>96–101</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>96–100</td>
<td>98–103</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>96–110</td>
<td>97–103</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>84–101</td>
<td>89–103</td>
<td></td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>90–110</td>
<td>97–107</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>90–100</td>
<td>96–101</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>130–232</td>
<td>119–137</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>84–105</td>
<td>99–113</td>
<td></td>
</tr>
</tbody>
</table>

* Three acceptable vials were measured on the same days, with the same instruments and reagents that were used when the three defective vials were discovered.

* Hitachi measurements.

* Synchron CX3 measurements.
INVESTIGATION OF CAUSES FOR DEFECTIVE RECOVERY OF GLUCOSE

Eight aspects of the defective vial material were examined to identify an assignable cause for the defective glucose recovery. Acceptable vials were subjected to the same studies. Table 4 compares the results of seven of the eight investigational procedures for two pairs of defective and acceptable vials (11/4/92 and 2/10/93); the eighth comparison, protein electrophoresis, is shown in Fig. 1.

The pH of the defective material was 0.6 pH unit lower than in the acceptable vials. The lactic acid content in defective vials was 86% greater than the baseline values of the acceptable vials. There was no difference in the quantitative total protein concentration in the defective and the acceptable vials.

After ultracentrifugation, we measured the total protein in the pellet and the supernatant independently. The insoluble pellet protein concentration had increased by 2.4 times in the defective vial 909a-1 (2/10/93) and by 3.1 times in the defective vial 909a-2 (11/4/93) compared with the similarly prepared insoluble pellet protein from the corresponding acceptable vials. The visual turbidity of the reconstituted material correlated with the increase in the insoluble pellet protein.

Total protein recovery was between 99% and 102% of the preultracentrifuged value. This indicates that the difference in insoluble protein between the defective and the acceptable vials is real and not a recovery artifact from the separation of the soluble and insoluble fractions.

The precision (SD) of total protein measurement at 50 g/L is 1.2 g/L by the Hitachi biuret method [31]. Therefore, we conclude that there was no significant difference in total protein between acceptable and defective vials. Because the pellet protein concentrations were very low, we confirmed these protein values by the more precise method of Lowry et al. [32].

To account for the increase in insoluble protein in the defective vial, we changed the pH of an acceptable vial (pH 7.44) to pH 6.85 (that of a defective vial) by adding dilute hydrochloric acid. Because this did not change the amount of pelletable protein, we concluded pH was not the primary cause of this difference.

The last aspect of protein comparison between defective and acceptable vials was SDS-PAGE. We used reagents and methodology from Bio-Rad Labs. (South Richmond, CA) to analyze the whole reconstituted SRM and the pelleted and supernatant fractions obtained by ultracentrifugation of defective 909a-2, acceptable 909a-2, and acceptable 909a-1 vials. Use of a series of seven molecular-mass standards (Gibco Research Products, Life Technologies, Gaithersburg, MD) covering 10–200 kDa allowed clear separation of 14 protein bands (Fig. 1). All bands were consistently present in all fractions in both defective and acceptable vials, and no new bands were visible in the lanes of sample from defective vial 909a-2.

In addition, Gram stains of the reconstituted defective and acceptable vial materials were negative for stainable bacteria [27] (Table 4). Use of a sensitive bacterial growth method, i.e., broth culture at 37 °C, revealed no identifiable microorganisms in either defective or acceptable vials [28].

Finally, electron microscopic sections of the insoluble pellet materials obtained from ultracentrifugation of samples from defective and acceptable vials of 909a at both concentrations, showed no evidence of intact bacterial organisms, bacterial membranes, or bacterial fragments or cellular components (Fig. 2).

**Discussion**

Comparison of the results for three defective vials gives convincing evidence of discrepancies between the glucose, cholesterol, and uric acid concentrations in SRM 909a-1/a-2 and their listed target values. The most affected analyte was glucose, followed by uric acid and cholesterol, in all three defective vials; other analytes in the defective vials were unaffected.

When evidence is presented indicating a flaw in an important

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**Table 4. Other properties of defective and acceptable vials.**

<table>
<thead>
<tr>
<th>SRM and date of analysis</th>
<th>Vial</th>
<th>pH</th>
<th>Lactic acid, mmol/L</th>
<th>Protein, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total 🅾</td>
</tr>
<tr>
<td>909a-2</td>
<td>Defective</td>
<td>6.85</td>
<td>2.34</td>
<td>65.0</td>
</tr>
<tr>
<td>11/4/92</td>
<td>Acceptable</td>
<td>7.44</td>
<td>1.24</td>
<td>66.0</td>
</tr>
<tr>
<td>909a-1</td>
<td>Defective</td>
<td>6.76</td>
<td>2.49</td>
<td>44.9</td>
</tr>
<tr>
<td>2/10/93</td>
<td>Acceptable</td>
<td>7.10</td>
<td>1.35</td>
<td>44.0</td>
</tr>
</tbody>
</table>

* Reconstituted SRM before ultracentrifugation.

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**Fig. 1. SDS-PAGE of NIST SRM 909a-1 and 909a-2.**

W, whole, reconstituted, nonultracentrifuged SRM; P, pellet suspensions and, S, supernatant fractions after ultracentrifugation. The reconstituted SRM 909a-1/909a-2 proteins were run in 15% polyacrylamide gel and afterwards stained with Coomassie Blue. The total amount of protein applied in each lane was: lanes 1 and 10, molecular mass marker, 20 μg; lane 2, 7.1 μg; lane 3, 36 μg; lane 4, 18 μg; lane 5, 9 μg; lane 6, 58 μg; lane 7, 9 μg; lane 8, 4 μg; lane 9, 10 μg; lane 11, 36 μg; lane 12, 25 μg. Marker proteins (from top): myosin-H chain, phosphorylase-B, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme. a₂², material from the defective vial.
national standard, specific precautions must be taken to rule out the possibility that the observation could be a defect in the measurement system. Hence, we report the specific precautions we took:

1. *Multiple instruments* were used in the same way, which ruled out the possibility of idiosyncratic performance by a single instrument.

2. Both defective and acceptable vials were *analyzed on the same day*, within the 8-h NIST-recommended postreconstitution time limit. This ruled out the possibility of a time-related sporadic change in instrument, reagent, calibrator, or NIST sample.

3. *Analysis of acceptable vials from the same box* that contained a defective vial ruled out a localized phenomenon that would affect all vials in a specific box, e.g., unfavorable shipping or storage conditions.

4. The occurrence of defective vials in different shipments over a 6-month period pointed toward a *prestorage event* rather than a localized storage depot problem.

5. In addition, because the triplicate analyses used throughout the study checked within a range of 2% or less, the consistent level of precision ruled out the possibility that the observed changes in the defective vials were attributable to poor *instrument precision*.

6. Between the two CX3 instruments, the widest analyte recovery range was 6% (94–100% for urea nitrogen). Thus, *variability between similar analyzers* is not an assignable cause for the large discrepancies observed in glucose, uric acid, and cholesterol analyte recovery in the three defective vials of SRM 909a-1 and 909a-2.

**POSSIBLE CAUSES OF LOW GLUCOSE RECOVERY**

The most probable cause of a low recovery of glucose from a sample known to contain glucose in a protein and salt matrix would be bacterial consumption of the glucose. The pH change and the increase in lactic acid would support this bacterial hypothesis. However, the negative results for bacterial culture and Gram stain, the failure to find any bacteria-associated structures (by electron microscopy), and a lack of any extraneous protein band(s) in SDS-PAGE do not support this hypothesis.

Possibly, however, the lyophilization step in the manufacturing process would destroy any contaminating bacteria or other cellular material so that organisms or cells would not be visible in electron micrographs. The absence of new extraneous protein band(s) in protein electrophoresis gives further support against any bacterial or cellular contamination.

**COMMUNICATION OF RESULTS TO NIST SRM PROGRAM**

The low glucose recovery from the first defective vial 909a-1, which was reconstituted and analyzed on 8/22/92, was communicated to NIST on 9/28/92 (personal communication: letter from S. Ghosh to J. Colbert, NIST SRM Program). This report was acknowledged to be the first documented report of a defective 909a vial to NIST (personal communication: telephone call from J. Colbert to B.K. De, 10/19/92).

In addition, NIST responded that the expected recovery of glucose would decrease because of normal degradation at a rate of 0.94 mg/dL/gm/year (0.052 mmol/L/gm/year) [sic] as stated in the NIST SRM 909a product insert. Thus, in the 5-year dating period the maximum change would be −0.26 mmol/L/gm/5 years, or −4.7%. However, this does not account for the −70% discrepancy in glucose recovery values that we observed in the three defective vials.

On March 16, 1993, NIST issued a revised certificate for SRM 909a: J. Colbert, Letter to Users of SRM 909a Standard Reference Materials Program, and W.P. Reed, Revision of certificate dated 7/24/91: Standard Reference Material 909a SRM Program NIST. This revision lowered the initial assigned glucose value from 967 to 892 mg/L, a 7.7% change. However, this modification also did not account for the −70% discrepancy.

**PRECAUTIONARY ADVICE**

Because NIST SRMs are routinely used to examine the accuracy and recovery of analytical methods, we urge users to check each vial of SRM 909a for glucose recovery concomitant with use. From June 1992 to February 1994 (20 months), we have analyzed a total of 36 vials: 19 vials of 909a-1 and 17 vials of 909a-2 (including the 16 vials analyzed from March 92 to February 93). Four vials of 909a-1 were defective (21%), as were two vials of 909a-2 (12%).

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**Fig. 2.** Electron microscopy of insoluble protein pellets from SRM 909a-1 and 909a-2. (A) 909a-1 acceptable; (B) 909a-1 defective; (C) 909a-2 acceptable; (D), 909a-2 defective. A–C, ×11 500; D, ×6480.
We acknowledge the cooperation of Michael J. Welch, A. Cohen, and Jennifer Colbert at NIST. Gram stain and culture are due to the courtesy of Joseph Staneck, Director, Laboratory Medicine, and Steve Glenn in the Microbiology Division, Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati. Credit for electron microscopic preparation and observation is extended to Richard Montione, Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati. We acknowledge Nelson Horsemann, Department of Physiology, University of Cincinnati Medical Center, for use of his laboratory facilities in biochemical analysis. B.A.K. discovered the first defective vial.

References