High-Density Lipoproteins Estimated by an Enzymatic Cholesterol Procedure, with a Centrifugual Analyzer

K. Owen Ash and Wayne M. Hentschel

We describe an assay for high-density lipoprotein cholesterol, adapted to a centrifugual analyzer, the GEMSAEC System 3, which includes use of an increased Mn^{2+} concentration (91 mmol/liter) [J. Lipid Res. 19, 65 (1978)] and ethylenediaminetetraacetate [Clin. Chem. 22, 98 (1976)]. Modifications to the GEMSAEC system include reducing the mixing burst and preconditioning the sample tip. Accuracy of this procedure, as assessed by analysis of a control pool from the Center for Disease Control, was 99.2%. Day-to-day precision for two control pools was 320 ± 13 and 506 ± 17 mg/liter. Serum sample volume was decreased to 0.5 ml. In blanks with heparin/Mn^{2+} present, the pseudocholesterol concentrations resulting from a reaction of the enzymatic cholesterol reagent and the heparin/Mn^{2+} precipitating reagent depend on the source of the enzymatic reagent and appear to be enhanced slightly by the use of ethylenediaminetetraacetate. Pseudocholesterol concentrations reach a maximum at heparin/Mn^{2+} concentrations well below those needed to completely precipitate the low-density and very-low-density lipoprotein fractions. Population reference values were obtained from analyses done on 224 local physicians (mean: male, 500 mg/liter; female, 620 mg/liter) and 156 ambulatory patients (mean: male, 463 mg/liter; female, 553 mg/liter).

Additional Keyphrases: coronary heart disease - hyperlipoproteinemia - normal values - GEMSAEC - variation, sources of

Interest in high-density lipoproteins (HDL)\(^1\) is increasing because of their demonstrated inverse relationship to cardiovascular disease, with low HDL concentrations being recognized as a major risk factor for coronary heart disease (1-8). This has resulted in an increased demand for HDL quantitation in the clinical laboratory. Until recently, HDL studies have been performed at a few specialized lipid-research laboratories by ultracentrifugation techniques, which are impractical for most clinical service laboratories (9, 10). Consequently, investigators have sought more convenient methods for the quantification of HDL. An approach that has received considerable attention involves addition of polyaniions and divalent cations to precipitate the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) fractions, which can then be removed by centrifugation (9, 11-15). In the most widely studied precipitation method, heparin and MnCl\(_2\) are used. The HDL fraction remains in the supernate and is conveniently estimated by measuring the cholesterol content. Enzymatic cholesterol methods can provide excellent precision and improved specificity (16, 17); however, difficulties have been encountered in adapting the enzymatic methods to the determination of HDL cholesterol when heparin and MnCl\(_2\) are used as the precipitating agents (11, 18, 19).

We report here a method adapted to a centrifugual analyzer, which incorporates the recent advances reported by Warnick and Albers relating to the MnCl\(_2\) concentration (20) and the use of EDTA as recommended by Steele et al. (18). Our HDL values correlate well with those obtained by the Lipid Research Clinics on specimens supplied by the Center for Disease Control. We also report clinical studies of HDL cholesterol determinations on 224 local physicians and 156 ambulatory patients.

Materials and Methods

Reagents

Heparin: "Panheparin" (10 000 USP units/ml; Abbott Laboratories, North Chicago, Ill. 60064) was used in all experiments; it was stored at 4 °C.

Manganese chloride solution: A 1.29 mol/liter manganous chloride solution was prepared with 25.64 g MnCl\(_2\)-4H\(_2\)O (J. T. Baker Chemical Co., Phillipsburg, N.J. 08865) diluted to 100 ml with de-ionized water.

Combined heparin/manganese chloride reagent (heparin/Mn\(_{2+}\)): To 2.4 ml of the heparin stock is added 8.2 ml of the 1.29 mol/liter MnCl\(_2\) stock solution. This reagent is prepared fresh weekly and stored at 4 °C. This reagent contains 2264 USP units of heparin per milliliter and 1.0 mol of MnCl\(_2\) per liter as suggested by Warnick and Albers (20, 21).

Cholesterol reagent diluted: A 0.2 mol/liter EDTA stock solution contained 7.45 g Na\(_2\)EDTA-2H\(_2\)O (Sigma Chemical Co., St. Louis, Mo. 63178) in 100 ml of de-ionized water, the pH adjusted to 6.7 with NaOH. A 4 mmol/liter EDTA working solution was prepared by 50-fold dilution (18).

Enzymatic cholesterol reagent: Unless specified otherwise we used the "Calbiochem Enzymatic Cholesterol S.V.R." (Calbiochem, La Jolla, Calif. 92037) reconstituted with 10.0 ml of the 4 mmol/liter EDTA.\(^2\) When specifically noted, the "Spinchem" enzymatic reagent for cholesterol (SmithKline Instruments, Inc., Sunnyvale, Calif. 94086) was used; this

\(1\) Nonstandard abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; EDTA, ethylenediaminetetraacetate.

\(2\) We use these reagents for both HDL cholesterol and total cholesterol assays. If only HDL cholesterol assays are to be run with the Calbiochem reagent, then the vial can be reconstituted with 15.5 ml of the 4 mmol/liter EDTA.
reagent was found to be satisfactory when reconstituted with 20.0 ml of the 4 mmol/liter EDTA.²

Calibration: Vial B from the “Calbiochem Lipid Mix-Pack” (Calbiochem) was used as the secondary standard and was reconstituted at least every five days and a minimum of 2 h before its first use.

Procedure

LDL and VLDL precipitation: The procedure given by Warnick and Albers (20, 22) was used, with the volume of added heparin/Mn²⁺ precipitating reagent proportionately reduced for the 1.0- and 0.5-ml serum samples. To correct for the dilution produced during the precipitation step, the determined HDL cholesterol concentrations are multiplied by 1.1.

HDL cholesterol analysis: The GEMSAEC System 3 (Electro-Nucleonics, Inc., Fairfield, N.J. 07006) is set up as follows:

<table>
<thead>
<tr>
<th>Vol. Pump</th>
<th>% Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample: 10 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>Flush: 100 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>Reagent: 500 µl</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Sample tip: stainless steel
Blank switch: reagent
Reaction mode: Auto/End-Point
Reaction temp.: 37 °C
Wave length: 500 nm
Filter position: 430–560 nm
Blank: H₂O in position #1 of sample wheel

IR Initial Reading 720
RI Reading Interval 5
NR Number of Readings 1
SC Standard Concentration *
KT Computational Factor for International Units
TF Temperature Factor 1.0
TC Type of Computation 1
AD Absorbance Decimal Locator 3
CD Concentration Decimal 0
HI Upper Limit of Normal 640*
LO Lower Limit of Normal 320*
SA Starting Absorbance 0.500
RM Print-Out Format 2
XX Linear Region Indicator 0
DI Direction Indicator 1
DA Early Reading Delta 0.000
ER Early Reading Parameter 0²

* Enter the concentration of the calibration specimen or standard.
† The values for HI and LO are set to flag HDL cholesterol values that would be greater than 700 mg/liter or less than 350 mg/liter after correcting for the 1.1 dilution factor. These samples are repeated before results are reported.
² The Early Reading switch on the control module, if present, must be in the normal, “NOR,” position.

The air pressure has been lowered to 69 kPa (10 psi) instead of the recommended 103 kPa (15 psi). The assay has a 1/61 ratio of specimen volume to total reaction volume.

Results and Discussion

Modifications to the GEMSAEC analyzer: Implementation of cholesterol assays with this analyzer presented several problems, which were eliminated by minor modifications. These modifications are discussed here for readers who plan to use centrifugal analyzers. Initially, we observed carryover that was independent of the pipetting; results were affected by the samples in neighboring cuvettes. By monitoring the reaction at 60-s intervals and observing the oscilloscope, we determined that the contamination coincided with the mixing burst in the run. This “splash” phenomenon was overcome by lowering the air pressure from 103 to 69 kPa and by eliminating the short mixing burst in the “early reading” system. These modifications have proven to be compatible with all other chemistries presently performed on our analyzer. Due to the decreased air pressure, a second wash cycle (manual mode) has been beneficial after each cholesterol run, to remove residual material. This extra wash has not been needed with the other assays. Incubation time has been maintained at 12 min so that both the HDL cholesterol and total cholesterol assays will go to completion if included in the same run. For the HDL cholesterol assays alone, the running time might be shortened, but we have not confirmed this experimentally.

Another problem involved calibration of the cholesterol run. When the disc was loaded with the Rotoloder, samples of known cholesterol concentrations were underestimated. This problem has also been noted by others (23), but we observed that this sampling error seemed to decrease with prolonged use. We found that preconditioning the stainless-steel sample tip essentially eliminated the problem. Our preconditioning procedure includes a 10-min soak in a serum containing a high cholesterol concentration, a priming step of 20 cycles, and a wash step wherein the tip is flushed 12 times with water. Using an assayed serum, 4530 mg/liter cholesterol, we obtained, before preconditioning the sample tip, a mean value of 4392 mg/liter; the 32 determinations ranged from 4270 to 4480 mg/liter with a standard deviation of 42.8 mg/liter. After the tip was preconditioned the mean was 4523 mg/liter; the 21 determinations ranged from 4470 to 4560 mg/liter with a standard deviation of 26.5 mg/liter. At this point, the rationale for the preconditioning is pragmatic; the information is provided for the benefit of those using GEMSAEC analyzers.

Pseudocholesterol concentrations: A fine precipitate develops in the reaction mixture when enzymatic cholesterol reagents are used to analyze samples precipitated with the heparin/Mn²⁺ reagent. Observable turbidity disappears when the enzymatic cholesterol reagent contains EDTA as suggested by Steele et al. (18), although pseudocholesterol concentrations due to an interaction of the enzymatic reagent with Mn²⁺ are not entirely eliminated (18, 19). The pseudocholesterol concentrations detected in HDL reagent blanks (water plus heparin/Mn²⁺ reagent) also depend somewhat on the source of the enzymatic reagent and the use of EDTA. When reconstituted with the 4 mmol/liter EDTA, the Calbiochem reagent gave a mean pseudocholesterol concentration of 33 mg/liter (70 determinations) whereas the Spinchem reagent gave a mean of 9 mg/liter (48 determinations). When reconstituted with water, the Calbiochem reagent gave a mean pseudocholesterol concentration of 18 mg/liter (21 determinations) whereas the Spinchem reagent did not demonstrate a cholesterol concentration in the assayed HDL reagent blanks (a mean of 0 mg/liter in 22 determinations). Thus, at least when assayed with the GEMSAEC which gives a mean determination from eight readings, EDTA seems to slightly enhance the pseudocholesterol concentrations, even though the precipitate has been eliminated. The component concentrations of the enzymatic reagents, when reconstituted as described in the Materials and Methods section, are quite similar except for the phosphate buffer, 4-aminoantipyrine, and peroxidase which are somewhat more concentrated in the Calbiochem reagent. The differences due to the reagent source are not reflected in the patients' samples that were simultaneously assayed. However, the differences due to the enhancement by EDTA are reflected to some degree (6 to 12 mg/liter) in the patients' samples.
Fig. 1. Pseudocholesterol absorbance as a function of heparin/Mn²⁺ reagent dilution

The HDL reagent blanks were assayed against a water-reagent blank. Enzymatic cholesterol reagents were reconstituted with 4 mmol/liter EDTA. For the undiluted HDL reagent blank 100 µl of the combined heparin/Mn²⁺ reagent was added to 1.0 ml of water simulating the precipitation step. Dilutions of the heparin/Mn²⁺ reagent with water are specified on the abscissas. The undiluted HDL reagent blank contained, per liter, 20 X 10⁶ USP units of heparin and 91 mmol of MnCl₂. Each point is the mean of five determinations; standard deviations are shown by the vertical bars.

Fig. 2. Linearity of the enzymatic cholesterol assay

Each point represents the mean of four determinations. The stock serum contained 4530 mg of cholesterol per liter, from which serial dilutions of 1 to 2 were made with isotonic saline. Slope, 1.00; y-intercept, −18.79; correlation coefficient, 0.99.

The HDL reagent blank effect reached a maximum at relatively low concentrations of the precipitating agents (Figure 1); the pseudocholesterol concentrations were not significantly decreased even by a 10-fold dilution of the heparin/Mn²⁺. However, such low concentrations of heparin/Mn²⁺ are insufficient to completely precipitate the LDL and VLDL fractions (20).

Linearity: Both the HDL cholesterol and total cholesterol assays were implemented on our GEMSAEC analyzer at the same time. Consequently, linearity studies included a range appropriate for both assays. The procedure was linear from 70 mg/liter up to at least 4530 mg/liter (Figure 2). Our experience in diluting and repeating specimens for which the total cholesterol concentration exceeds 4530 mg/liter has demonstrated that the linear range extends well above 4530 mg/liter, to approximately 6000 mg/liter.

Accuracy, precision, and recovery: We assayed four vials of a HDL cholesterol control pool, alpha Q 2, obtained from the Center for Disease Control (CDC), Atlanta, Ga. These samples were stored at −70 °C until used. The mean concentration for alpha Q 2 was reported by CDC as "about 5 mg/dl" [noted in Liedtke et al. (19) as 511 mg/liter] as determined by the Lipid Research Clinics. In 45 determinations, after the selective precipitation of LDL and VLDL, we obtained a mean concentration of 507 mg/liter, with a standard deviation of 13 mg/liter (Table 1), representing 99.2% accuracy (24). The alpha Q 2 samples were assayed randomly during a 43-day period as a continual monitoring of our procedure, and to establish HDL cholesterol concentrations for two control sera pools used throughout our laboratory to monitor precision (Table 1).

Analytical recovery was assessed by mixing equal portions of the two control pools. In two runs the individual control pools were assayed at 502.5 and 330 mg/liter; the calculated mean value for the two is 416.25 mg/liter. Included in the same analysis runs were four replicate samples of the combined serum. All four determinations gave a concentration of 420 mg/liter, a 99.1% recovery (24).

Warnick and Albers (20), using plasma samples, have recommended an increased Mn²⁺ concentration (92 mmol/liter) to ensure complete precipitation of the LDL and VLDL fractions. When we assayed serum specimens, using the lower Mn²⁺ concentration (46 mmol/liter) used by several earlier investigators (e.g., 1, 15, 18, 25), the measured HDL cholesterol concentrations were in fact slightly higher (20 to 30 mg/liter); values for the alpha Q 2 and several other samples

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**Table 1. Establishing Assay Controls for HDL Cholesterol Determinations**

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Mean (mg/liter)</th>
<th>SD (mg/liter)</th>
<th>CV, %</th>
<th>2 SD limits, mg/liter</th>
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<tbody>
<tr>
<td>Alpha Q 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vial &quot;A&quot;</td>
<td>27</td>
<td>507.8</td>
<td>13.3</td>
<td></td>
<td></td>
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<tr>
<td>Vial &quot;B&quot;</td>
<td>4</td>
<td>512.5</td>
<td>15.0</td>
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<tr>
<td>Vial &quot;C&quot;</td>
<td>4</td>
<td>500.0</td>
<td>9.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vial &quot;D&quot;</td>
<td>10</td>
<td>506.0</td>
<td>15.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>45</td>
<td>507.0</td>
<td>13.0</td>
<td>2.5</td>
<td></td>
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**Control sera:**

<p>| | | | | | |</p>
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<th></th>
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<tbody>
<tr>
<td>H 1 low</td>
<td>118</td>
<td>320</td>
<td>13</td>
<td>4.1</td>
<td>290–350</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H 2 &quot;normal&quot; control</td>
<td>153</td>
<td>506</td>
<td>17</td>
<td>3.4</td>
<td>470–540</td>
</tr>
</tbody>
</table>

**Table 2. Effect of Decreasing the Volume of the Initial Serum Sample Used in the Precipitation Step**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial serum volume</th>
<th>Difference</th>
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<tr>
<td></td>
<td>1.0 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>HDL cholesterol, mg/liter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H 1 low control</td>
<td>327 (23)</td>
<td>329 (17)</td>
</tr>
<tr>
<td>H 2 &quot;normal&quot; control</td>
<td>501 (24)</td>
<td>503 (17)</td>
</tr>
</tbody>
</table>

Patients:

<table>
<thead>
<tr>
<th></th>
<th>OP/0069</th>
<th>OP/0407</th>
<th>OP/2507</th>
<th>OP/3463</th>
<th>OP/3535</th>
<th>OP/3764</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>170 (3)</td>
<td>560 (3)</td>
<td>535 (2)</td>
<td>258 (4)</td>
<td>485 (4)</td>
<td>435 (4)</td>
</tr>
</tbody>
</table>

Number of replicate assays performed on each specimen is in parentheses. Patients' values were determined over a five-day period. The 1.0-ml and 0.5-ml samples were precipitated by adding, respectively, 100 and 50 µl of the combined heparin/Mn²⁺ reagent. Further details are provided in the Materials and Methods section.
were significantly higher (50 to 100 mg/liter),\(^3\) perhaps suggesting incomplete precipitation of the LDL and VLDL fractions (20). Even though these experiments suggested that HDL cholesterol concentrations may have been slightly overestimated by some of the earlier methods, we decided to calibrate our assay to the alpha Q 2 HDL cholesterol concentration obtained by the Lipid Research Clinics and reported by CDC. For this reason the pseudocholesterol values are not subtracted out in our procedure because, when the 45 replicate assays of the alpha Q 2 specimen were corrected for the HDL reagent blank concentrations, the mean was 475 mg/liter, which is 35 mg/liter lower than the 510 mg/liter concentration reported by CDC. The mean concentration for these same 45 determinations without the blank correction was 507 mg/liter, in agreement with the concentration reported by CDC. This facilitated comparison of our results with earlier studies.

Sample size: Initially, serum aliquots of 1.0 to 3.0 ml were used for the precipitation step, with the volume of heparin/Mn\(^{2+}\) adjusted proportionately; no significant difference was observed in the determined HDL cholesterol concentrations. To conserve serum, we investigated a 0.5-ml serum aliquot. The volume of the combined heparin/Mn\(^{2+}\) reagent to be added was reduced proportionately to maintain the same final concentrations. Precision of the HDL cholesterol concentrations was not compromised by this change in sample volume (Table 2). We are now using 0.5-ml serum samples routinely for our HDL assay, except for lipemic sera, where a 1.0-ml serum sample (with 100 \(\mu\)l of heparin/Mn\(^{2+}\)) is used in case further centrifugation is needed (20–22).

Reference population: Before offering the HDL cholesterol assay for patient work we screened 224 “healthy” physicians. The results are summarized in Table 3 and the distribution for the male participants is shown in Figure 3a. The mean values for both the male and female groupings were slightly higher than those reported from population studies such as the Framingham Study (8, 21, 25); however, this difference could be due, at least in part, to the younger age of the participants in our population study; 175 of the 202 males were under 40 years of age.

Table 3 also gives the mean HDL cholesterol value for male and female ambulatory patients whose sera samples have been evaluated in our laboratory since the introduction of this assay. The distribution, Figure 3b, is comparable to other published population studies (25).

We thank Dr. Gerald R. Cooper and Barbara Batero of the Center for Disease Control for providing us with the alpha Q 2 samples and the information regarding their HDL cholesterol concentration. These investigations were supported by the Development Fund of the Department of Pathology, College of Medicine, University of Utah.

References


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\(^3\) HDL cholesterol = 1.09 (uncorrected HDL cholesterol – HDL reagent blank).

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### Table 3. HDL Cholesterol Reference Population Values

<table>
<thead>
<tr>
<th></th>
<th>Mean Deviation</th>
<th>Observed Range</th>
<th>2 SD Limits</th>
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</thead>
<tbody>
<tr>
<td>Physicians</td>
<td>n</td>
<td>mg/liter</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>202</td>
<td>500</td>
<td>90</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>620</td>
<td>130</td>
</tr>
<tr>
<td>Ambulatory patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>107</td>
<td>463</td>
<td>113</td>
</tr>
<tr>
<td>Female</td>
<td>49</td>
<td>553</td>
<td>172</td>
</tr>
</tbody>
</table>

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**Fig. 3. Distribution of values for reference populations**

(a) 202 male physicians, symbol O; (b) 107 male ambulatory patients, symbol O; and 71 females, including ambulatory patients and female physicians, symbol O.
Analytical Precautions in Measurement of Blood Cyanide

F. Lee Rodkey and Robert F. Robertson

Solutions of KCN in 0.1 mol/liter NaOH, stored for six months at 4 °C, were unchanged as determined by silver titration and color development with pyridine–pyrazaline reagent. At room temperature the cyanide concentration of such solutions decreased by 0.079% per day (half life = 880 days) and the extent of color development changed in direct proportion to the change in silver titer. Rubber stoppers absorb (or dissolve) HCN when in contact with this gas. The cyanide tends to come out of the stopper long after the source of the gas is removed. Rubber stoppers and expired air contaminated by HCN must be carefully avoided during cyanide analysis. Expired air contains HCN, generally in larger amounts for persons who smoke.

We recently published a procedure for measuring blood cyanide concentration (1). During the development of this procedure we occasionally observed unexplained, aberrant results. Further experience with the method has allowed us to define several additional factors that must be controlled. These include the stability of stock KCN standard solution and two sources of cyanide contamination: expired air and contaminated rubber stoppers.

Materials and Methods

The reagents, apparatus, and procedures used were exactly as previously reported (1). Stock KCN solutions were made in 0.1 mol/liter NaOH, prepared with de-ionized water. They were then stored at different temperatures and in different containers as noted. Techniques of silver titration of the stock KCN solutions and color development of the working standards were unchanged from those described (1).

Results and Discussion

Stability of KCN: A long-term instability of stock KCN solutions in 0.1 mol/liter NaOH was suspected when we analyzed several KCN stock solutions that had been prepared several months previously and stored at room temperature without contact with rubber. These solutions gave lower silver-titration values, and the diluted working standards developed less color with the pyridine–pyrazaline reagent. We then prepared three stock KCN solutions and analyzed them periodically by both the silver titration and the colorimetric pyridine–pyrazaline procedures (Table 1). Stock KCN solutions stored at room temperature in glass or polyethylene containers decreased in titer at about the same rate. Only 86.5% of the initial cyanide remained after six months. The decrease in cyanide at room temperature follows first-order kinetics, with a loss of about 0.079% per day—or a half life of 880 days. The sample stored in plastic at refrigerator temperature, 4 °C, did not change its silver titer by more than about 2 parts per thousand throughout this entire period.

Each of these solutions was diluted 1000-fold with 50 mmol/liter NaOH for colorimetric assay with the pyridine–pyrazaline reaction by the routine procedure (1). On the day the stock solutions were prepared, the absorbance was 0.0225 (range, 0.0227–0.0229) for each nanomole of cyanide for all solutions. This value remained constant for the stock solution.