Spectrophotometric Measurement of Plasma 2-Thiobarbituric Acid-Reactive Substances in the Presence of Hemoglobin and Bilirubin Interference (43552)

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Abstract. **The 2-thiobarbituric acid reaction with malondialdehyde has been used to assess lipid peroxidation in a variety of biologic systems. However, in an attempt to measure plasma thiobarbituric acid-reactive substances (TBARS) during extracorporeal membrane oxygenation, a form of sustained cardiopulmonary bypass, it became apparent that the absorbance signal at the 532-nm wavelength was composed not only of the peak absorbance of TBARS, but also of interfering substances from heme pigments and bilirubin. A method of subtracting interfering substances was developed and applied to normal human plasma. The method was tested by adding varying amounts of red blood cell hemolysate, bilirubin, and 1,1,3,3-tetramethoxypropane (TMP) standard to plasma and determining TBARS in the resulting mixture. In addition, varying the amount of added desferoxamine was investigated to determine the effects of iron chelation on the assay. This was important because the different samples would have varying amounts of free iron from hemoglobin to catalyze the reaction. It was found that the following equation could be used in this system to determine that amount of 532-nm absorption due to TBARS:**

$$MDA_{532} = 1.22[(A_{532}) - (0.56)(A_{510}) + (0.44)(A_{560})].$$

Regression analysis revealed an 86.6% recovery of the TMP spike. Analysis of variance showed that the variability in the model could be explained mainly by the additive increments of TMP spike (94.6%). [P.S.E.B.M. 1993, Vol 202]

The 2-thiobarbituric acid (TBA) reaction with malondialdehyde (MDA) has been used to assess lipid peroxidation in a variety of biologic systems (1–8). However, in an attempt to measure thiobarbituric acid-reactive substances (TBARS) during extracorporeal membrane oxygenation (ECMO), a form of cardiopulmonary bypass, it became apparent that the absorbance signal at the 532-nm wavelength was composed not only of the peak absorbance of the TBA-MDA chromagen, but also of interfering substances from heme pigments and bilirubin. Since the test was being employed in neonates with hyperbilirubinemia from physiologic jaundice who then experienced red blood cell hemolysis as a side effect of exposure to nonbiologic surfaces in the ECMO circuit, these interfering substances represented a significant problem. A method of arithmetically subtracting the effect of interfering substances was tested by applying it to normal human plasma with varying amounts of red blood cell hemolysate, bilirubin, and 1,1,3,3-tetramethoxypropane (TMP) standard added to the plasma. In addition, varying amounts of added desferoxamine were investigated to determine the effects of iron chelation on the assay.

The subtraction method is based on that of Allen (9) after Morton and Stubbs (10) and depends upon proportionality relationships in right triangles. It defines the portion of 532-nm absorbance (A_{532}) due to the TBA-MDA chromagen (MDA_{532}) in terms of absorbances at 532 nm and 560 nm and 510 nm. Figure 1

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presents a diagram of a representative spectrum from an incubated plasma sample with the absorbance at a given wavelength (A_i) shown to be composed of interfering substances (I_i) and absorbance due to the MDA-TBA chromagen (MDA_i).

$$A_i = MDA_i + I_i \quad [1]$$

specifically

$$A_{532} = MDA_{532} + I_{532} \quad [2]$$

The fundamental relationship of proportional triangles that validates the arithmetic subtraction method is demonstrated by variables x, y, and z introduced in Figure 1 and defined below.

$$x = I_{510} - I_{560};\ y = I_{532} - I_{560};\ z = I_{560}$$

$$y/a = x/(a + b) \text{ or, } y = [a/(a + b)]\ x. \quad [3]$$

The supplementary wavelengths were chosen after inspecting spectra of samples incubated in the presence of hemolysate and bilirubin. The TBA-MDA peak of 532 nm was chosen for point y. Points x and z were chosen to be colinear with y but at a distance from y, maximizing MDA_i at y and minimizing MDA_i at x and z. The relationships defined in Equation 3 will be used to solve the following equation:

$$MDA_{532} = A_{532} - I_{532} \quad [4]$$

in terms of A_{532}, A_{510}, and A_{560}. Referring again to Figure 1, interfering substances at 532-nm wavelength can be defined as:

$$I_{532} = y + z. \quad [5]$$

Substitution of Equation 3 into Equation 5 yields

$$I_{532} = [a/(a + b)]x + z. \quad [6]$$

Substitution of specific I_i for x and z defines interfering substances at 532 nm in terms of interfering substances at 510 nm and 560 nm and yields

$$I_{532} = [a/(a + b)][I_{510} - I_{560}] + I_{560} \quad [7]$$

or

$$I_{532} = [a/(a + b)][I_{510}] + [b/(a + b)]I_{560}. \quad [8]$$

Substitution into Equation 4 yields

$$MDA_{532} = A_{532} - [a/(a + b)][I_{510}] + [b/(a + b)][I_{560}]. \quad [9]$$

In samples containing interfering substances, only A_i can be measured, not I_i. Therefore, the term $\{[a/(a + b)]\ [MDA_{510}] + [b/(a + b)]\ MDA_{560}\}$ is subtracted from both sides, producing

$$MDA_{532} - \{[a/(a + b)][MDA_{510}] + [b/(a + b)][MDA_{560}]\}$$
$$= A_{532} - \{[a/(a + b)][I_{510}] + [b/(a + b)][I_{560}]\} - \{[a/(a + b)][MDA_{510}] + [b/(a + b)][MDA_{560}]\}. \quad [10]$$

Remembering that $A_i = MDA_i + I_i$, Equation 10 becomes:

$$MDA_{532} - \{[a/(a + b)][MDA_{510}] + [b/(a + b)]MDA_{560}\}$$
$$= A_{532} - \{[a/(a + b)][A_{510}] + [b/(a + b)]A_{560}\}. \quad [11]$$

Multiplication of the equation by the term $1/MDA_{532}$ yields

$$1 - \frac{\{[a/(a + b)][MDA_{510}] + [(b/(a + b)]MDA_{560}\}}{MDA_{532}} = \frac{A_{532} - \{[a(a + b)][A_{510}] + [b/(a + b)]A_{560}\}}{MDA_{532}} \quad [12]$$

Rearrangement of terms yields

$$\frac{MDA_{532}}{} = \frac{A_{532} - \{[a/(a + b)][A_{510}] + [b/(a + b)][A_{560}]\}}{1 - \{[a/(a + b)][MDA_{510}] + [b/(a + b)][MDA_{560}]\}/MDA_{532}} \quad [13]$$

Recalling that a and b are constants that represent differences in wavelength, substitution of 28 nm and 22 nm, respectively, results in the following equation:

$$\frac{MDA_{532}}{} = \frac{A_{532} - [(0.56)(A_{510}) + (0.44)(A_{560})]}{1 - [(0.56)(MDA_{510}) + (0.44)(MDAA_{560})]/MDA_{532}} \quad [14]$$

The denominator of the right side of Equation 14 is composed of terms related only to the MDA-TBA spectrum. The relative MDA absorbances at the various wavelengths can be determined experimentally by measuring absorbance in the pure preparation obtained by incubating 1,1,3,3-tetramethoxypropane with 2-thiobarbituric acid. The term $1/\{1 - [(0.56)\ (MDA_{510}) + (0.44)\ (MDA_{560})]/MDA_{532}\}$ is, therefore, an experimentally determined constant. These data will be presented. The final equation, to be used to correct for absorbance due to interfering substances in measuring the MDA-TBA chromagen, is as follows:

$$MDA_{532} = 1.22[(A_{532}) - (0.56)(A_{510}) + (0.44)(A_{560})]. \quad [15]$$

Methods

TBARS Incubation Method. The TBARS incubation method was essentially that of Ohkawa *et al.* (4). and reported previously (11, 12). Plasma (0.3 ml) and various additives were diluted in 0.9% NaCl to a vol-

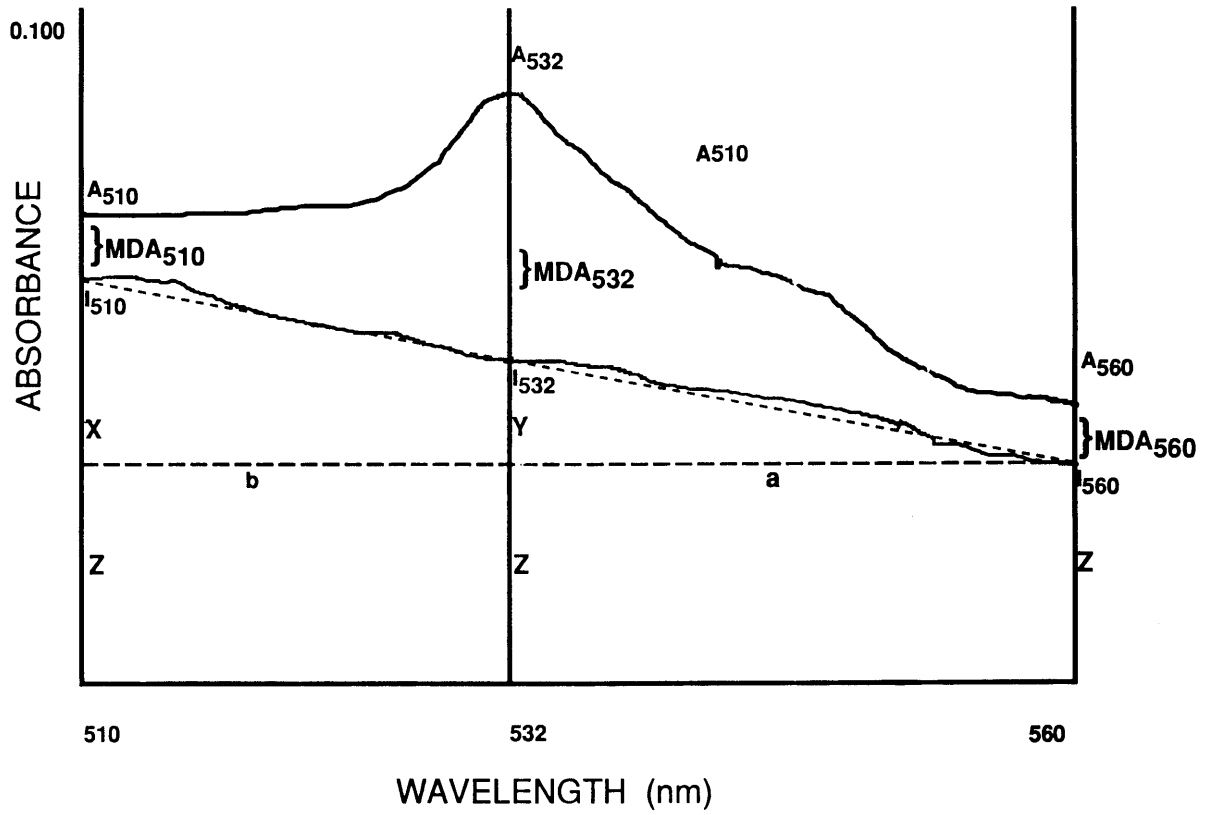

Figure 1. Composition of a plasma TBARS spectrum. A diagram of a possible spectrophotometric scan is presented. Absorbance is displayed on the ordinate. Wavelength in nm is presented on the abscissa. The solid line represents the absorbance spectrum of the sample. The dashed line represents the absorbance due to interfering substances in the sample. Note that points I_{510}, I_{532}, and I_{560} (x, y, and z) are colinear.

ume of 1.0 ml in different phases of the experiment. Four milliliters of TBA reagent were added to each sample in 16- × 80-mm glass test tubes. One hundred milliliters of reagent consisted of 40.5 ml of 0.8% TBA (Sigma), 40.5 ml of 20% acetic acid (glacial acetic acid-Malinckrodt) buffered to pH 3.5 with 1 *N* NaOH (Fisher), 13.2 ml of 8.2% sodium dodecyl sulfate (Sigma), and 8.8 ml of commercially distilled H_2O (Glenwood). Reagent components were freshly combined daily. Test tubes were then covered with glass beads to exclude additional air and incubated at 95°C for 80 min in a covered water bath. After incubation, test tubes were cooled in an ice water slurry. An extraction solution (4 ml) consisting of an *n*-butanol (Fisher), distilled water (Glenwood), pyridine (Sigma) mixture in a ratio of 15:3:1 was added to the incubate under a vapor hood. Test tubes were covered with Parafilm and vortexed for 10 sec to facilitate extraction, and the separation was effected by centrifugation at 3000 rpm (10-cm rotor radius) for 15 min. Aliquots (1.5 ml) of the extract were placed in 2-ml semimicro plastic cuvettes (Fisher Scientific). The reproducibility readings from plastic cuvettes were assessed by measuring aliquots of the same sample of TMP standard in multiple cuvettes and no appreciable difference was noted. Furthermore, a standard curve of samples containing only the MDA-TBA chromagen, formed from TMP, was analyzed with each day's experiments. Absorbances at 510, 532, and 560 nm were simultaneously determined using a Beckman model DR 6 spectrophotometer. Spectrophotometric scans were also performed on the same instrument. All assays were performed in triplicate. The spectrophotometer was zeroed using a blank of the extraction solution.

Experiments. *Scanning spectroscopy.* Absorbance spectra of TBARS incubates from the following sources are shown: (i) newborn plasma prior to ECMO, (ii) newborn plasma after 30 min of ECMO, (iii) TMP standard, (iv) lamb myocardium homogenate in 10% KCl, (v) human plasma with added hemolysate and bilirubin, and (vi) red blood cell hemolysate. Samples were scanned at a rate of 150 nm/min through a range of at least 200 nm centered about 532 nm.

Heme and bilirubin interference. An initial experiment consisted of adding varying amounts of red cell hemolysate, bilirubin, and TMP standard to 0.3-ml plasma samples for a total of 64 combinations of plasma, hemolysate, bilirubin, and TMP to determine the effect of added interfering substances on 532 nm absorbance. Normal human plasma and red blood cell hemolysate was obtained from a volunteer donor. Blood was drawn into heparinized syringes by periph-

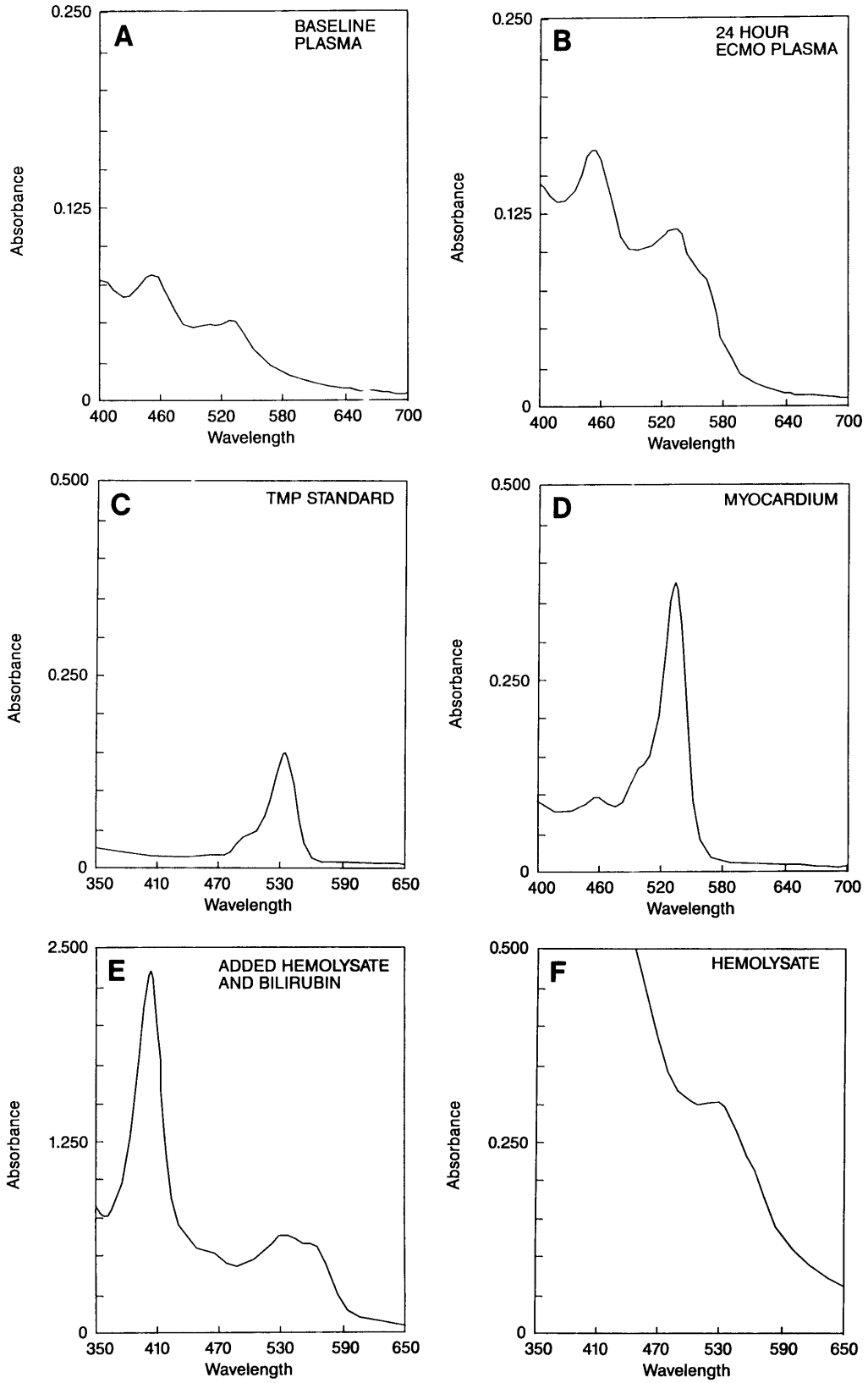

Figure 2. Experiment A (TBARS incubated spectra). (A) Baseline plasma spectrum: Spectrophotometric scan of arterial plasma sample from a newborn prior to ECMO. Absorbance is displayed on the ordinate (0.250 maximum) as a function of wavelength on the abscissa (400–700 nm). Calculated TBARS level is 0.40 nmol/ml of plasma. (B) ECMO plasma: Spectrophotometric scan of arterial plasma sample from a newborn 24 hr after initiation of ECMO. Absorbance is displayed on the ordinate (0.250 maximum) as a function of wavelength on the abscissa (400–700 nm). Calculated TBARS level is 1.48 nmol/ml of plasma. (C) TMP standard: Spectrophotometric scan of 1,1,3,3-tetramethoxypropane standard. Absorbance is displayed on the ordinate (0.500 maximum) as a function of wavelength on the abscissa (350–650 nm). The 532-nm absorbance is 0.148 absorbance units. Sample was 1 nmol/ml at final concentration TMP reacted with TBA reagent. (D) Lamb myocardium:

eral venipuncture after alcohol preparation. Red blood cell hemolysate was prepared by diluting the red cell fraction of centrifuged whole blood with deionized water and ultracentrifugation at 35,000 rpm (10-cm rotor radius) for 0.5 hr and then diluting the supernatant 1:10 with 0.9% NaCl. Plasma hemoglobin levels were determined by the method of Cripps (13) by the clinical laboratory. Bilirubin solution was prepared by dissolving bilirubin (Sigma) in 0.1% Na_2HCO_3 and then diluting in 0.9% NaCl in 4% bovine serum albumin (Sigma). Bilirubin was initially placed in solution at pH 11, but was adjusted to pH 3.5 with 1.0 *N* HCl without producing precipitation. The concentration of the bilirubin solution was 20–25 mg/dl as measured at 453-nm absorbance using the published molar extinction coefficient, 60,700 cml/mole (14). Plasma, hemolysate, and bilirubin were each prepared on the day of experimentation.

Alternate methods. A method of measuring red blood cell TBARS was proposed by Gilbert *et al.* (15) that involved subtracting interference due to hemoglobin by subtracting a portion of the measured absorbance at 453 nm. We attempted to use this method to correct for interference due to hemoglobin and bilirubin and applied this method to the samples described above. Samples with 64 combinations of hemoglobin, bilirubin, and TMP were analyzed by measuring 453-, 490-, 510-, 532-, and 560-nm absorbances. Concentrations of added hemoglobin, bilirubin, and TMP were reported as if they were contained in the 0.3-ml sample.

Subtraction coefficient. The value for the term $1/1 - \{[(0.56)\ (MDA_{510}) + (0.44)\ (MDA_{560})]/MDA_{532}\}$ was determined by reacting the TBA reagent as described above with known amounts of TMP standard. Absorbance was determined at 510, 532, and 560 nm and the results were inserted into Equation 14 to produce the coefficient for Equation 15.

Desferoxamine. Desferoxamine was added to samples with varying amounts of hemolysate to determine the relationship between *in vitro* potentiation of the TBARS reaction and amount of added hemoglobin. Desferoxamine concentration in the 1-ml sample prior to addition of TBA reagent was varied between 0 and 4.2 m*M*. Added hemolysate provided a plasma hemoglobin concentration between 0 and 178 mg/dl. TBARS levels were calculated using the subtraction method to find MDA_{532}.

Test of subtraction method. The final experiment consisted of varying bilirubin, hemolysate, and TMP in 48 combinations, performed in triplicate, for a total of 144 samples, in samples containing 1.0 m*M* desferoxamine and employing the subtraction formula (Equation 15). Aliquots of added hemolysate, bilirubin, and TMP were added to give 16 combinations of hemolysate and bilirubin with 0, 1, or 2 nmol of TMP added to the sample.

Results

Scanning Spectroscopy. Comparison of the absorption spectrum of incubated newborn infant plasma before and after ECMO readily identified the problem of interfering substances (Fig. 2, A and B). Total 532-nm absorbance was obviously not due completely to TBARS production either before or after ECMO support. This becomes apparent by comparing these spectra with that of an MDA standard prepared by reacting 1,1,3,3-tetramethoxypropane with TBA and the spectrum from lamb myocardium (Fig. 2, C and D). Figure 2E is a representative spectrophotometric scan of a sample with added hemolysate (220 mg/dl) and bilirubin (18 m/dl). Note that the peak absorbances in Figure 2E are similar to those of Figure 2B. Figure 2F presents the spectrum from the red blood cell hemolysate used for subsequent experiments. Note that the red blood cell hemolysate contained TBARS reactivity.

Heme and Bilirubin Interference. Figure 3 presents the results of Experiment C. In Figure 3A, 532-nm absorbance is presented as a function of added hemolysate, given as a concentration of plasma hemoglobin. Note that the addition of bilirubin to the samples produces approximately a 3-fold increase in the 532-nm absorbance, yet spectra of bilirubin blanks (not shown) had no specific 532-nm peak. The linear regression line for the data points without added bilirubin (bilirubin = 0.3 mg/dl) shows an acceptable coefficient ($R^2 = 0.948$). However, careful scrutiny reveals that a nonlinear increase in 532-nm absorbance occurs between 0 and 38 mg/dl of plasma hemoglobin. Figure 3B presents data for samples with 0 and 115 mg/dl of added hemoglobin. Again, with 0 mg/dl of added hemoglobin (and thus only one additive) the increase in 532-nm absorbance is linear. With 115 mg/dl of added hemoglobin, the increment with increasing bilirubin is not linear at higher concentrations of added bilirubin. Thus, interactive effects of bilirubin and hemoglobin

Spectrophotometric scan of sample of myocardium prepared by homogenizing with 10% KCl. Absorbance is displayed on the ordinate (0.500 maximum) as a function of wavelength on the abscissa (400–700 nm). (E) Plasma with added hemolysate and added bilirubin: Spectrophotometric scan of plasma sample with added hemolysate (300 μl, to give plasma hemoglobin of 220 mg/dl), bilirubin (300 μl to give plasma bilirubin of 18 mg/dl), and desferoxamine (1 m*M*). Absorbance is displayed on the ordinate (2.50 maximum) as a function of wavelength on the abscissa (350–650 nm). Calculated TBARS level is 5.88 nmol/ml of plasma. (F) Red blood cell hemolysate: Spectrophotometric scan of sample of red blood cell hemolysate. Absorbance is displayed on the ordinate (0.500 maximum) as a function of wavelength on the abscissa (350–650 nm). Plasma hemoglobin concentration is 200 mg/dl. Calculated TBARS level is 2.85 nmol/ml of plasma. No desferoxamine is present.

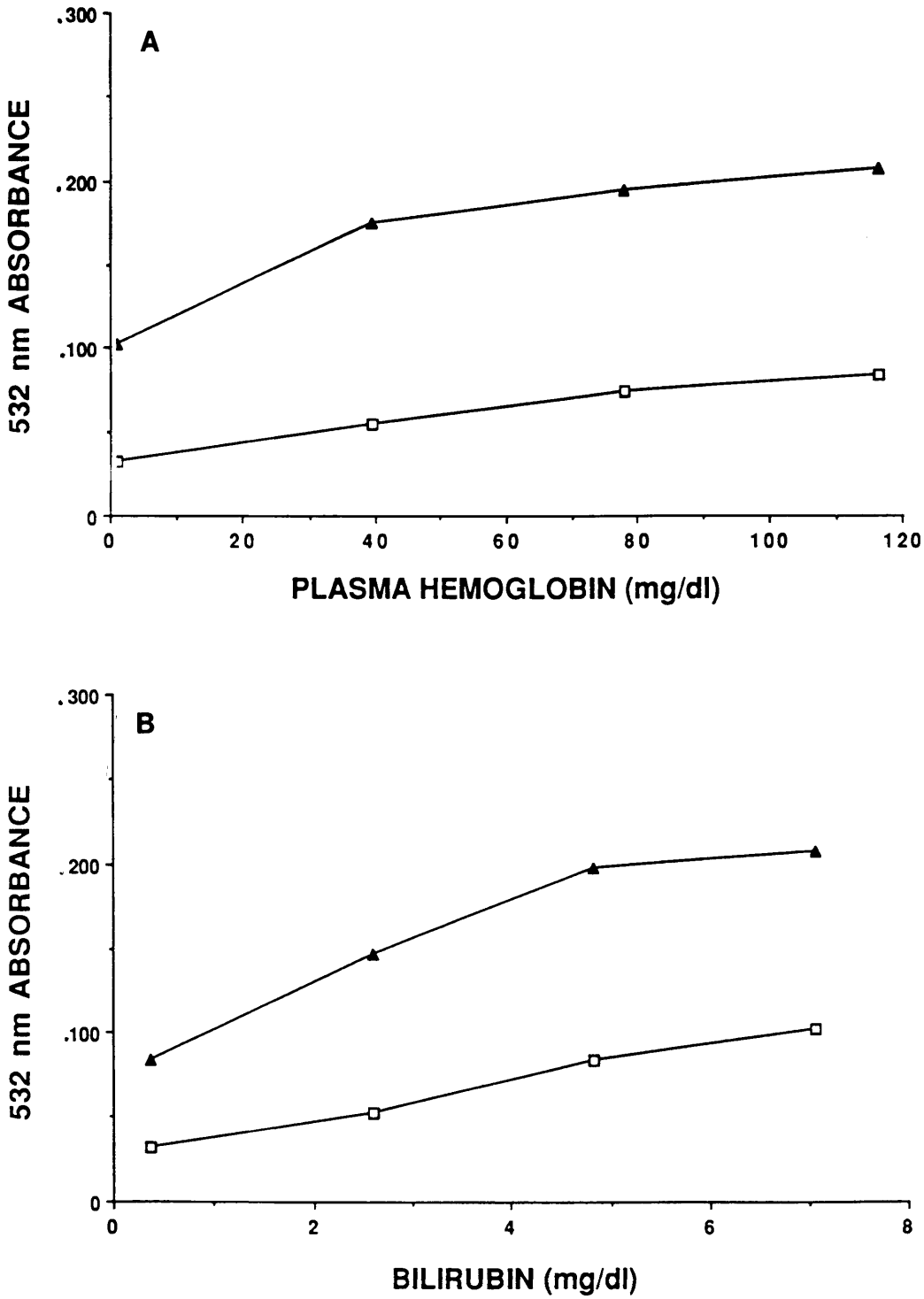

Figure 3. Experiment B (raw 532-nm absorbance). (A) Measured 532-nm absorbance is presented on the ordinate as a function of plasma hemoglobin in hemolysate added to samples (abscissa). Bilirubin in samples varies from 0.3 mg/dl of bilirubin present (donor's serum bilirubin level) (□) to 7.0 mg/dl of bilirubin (▲). (B) Measured 532-nm absorbance is presented on the ordinate as a function of added bilirubin (abscissa). Samples without added hemoglobin (□) and with 115.5 mg/dl of added plasma hemoglobin (▲) are displayed.

appear to result, suggesting perhaps both spectral interference and effects on oxidative status.

Alternate Methods. In Figure 4, TBARS levels obtained by correcting 532-nm absorbance by subtracting 20% of 453-nm absorbance as described by Gilbert *et al.* (15) are presented. There would appear to be a linear increase in TBARS with increasing bilirubin but no added hemoglobin. However, in the presence of both hemoglobin and bilirubin, the increase in corrected 532-nm absorbance is irregular. Additionally, we

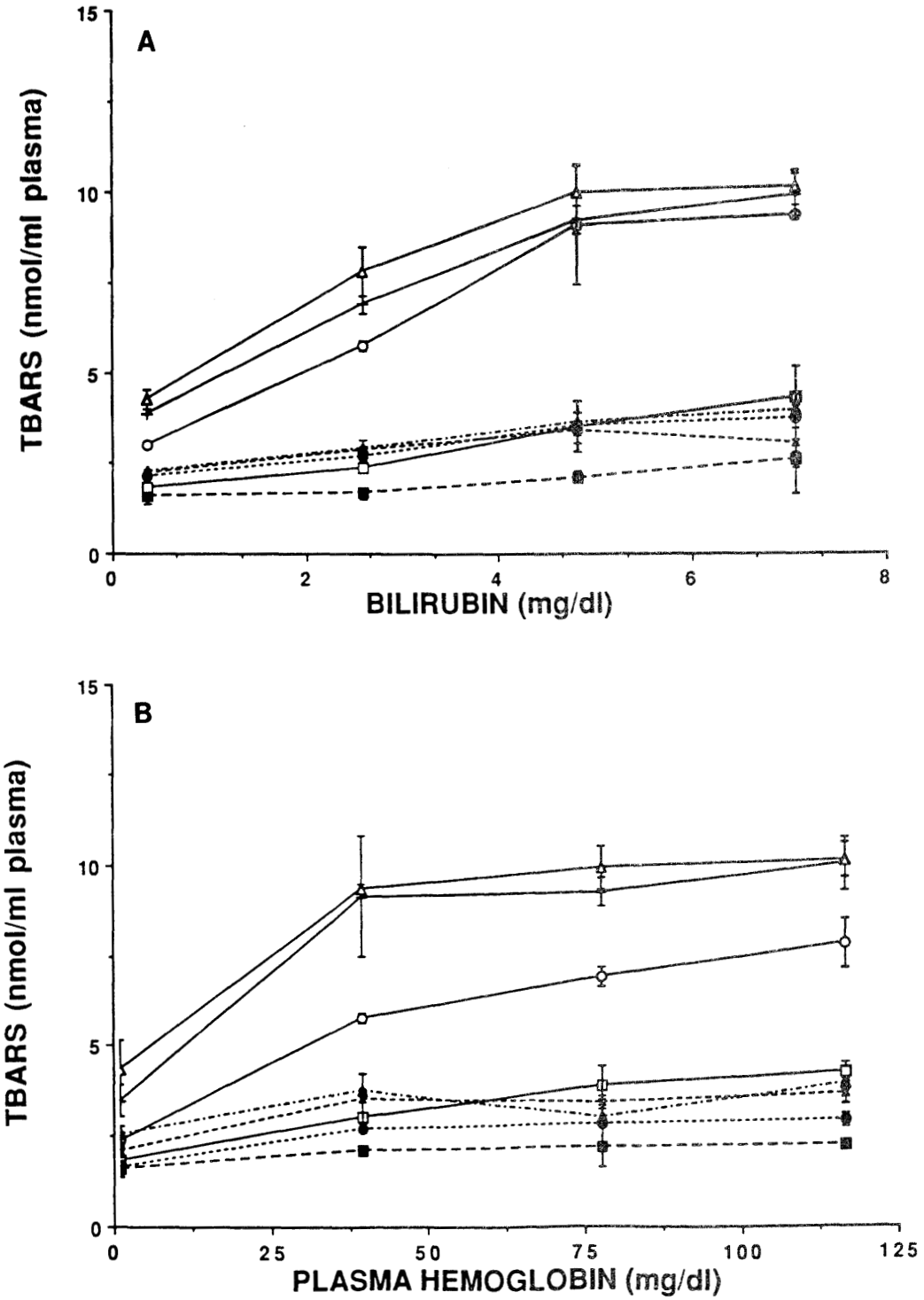

Figure 4. Experiment C (Gilbert's method). (A) Variability of TBA-reactive substances levels as measured by Gilbert's method (—) with regard to bilirubin concentration is displayed on the ordinate. TBARS levels (dashed line, filled symbols) converted from MDA_{532} values (Equation 15) are also shown. Bilirubin is displayed on the abscissa. Four separate groups of data, calculated by each method, are displayed for hemoglobin: 0 mg/dl (□, ■), 38.5 mg/dl (○, ●), 77 mg/dl (+, ×), and 115.5 mg/dl (△, ▲). (B) Variability of TBARS as measured by Gilbert's method (—) and values from MDA_{532} (–, filled symbols) are given as a function of added hemolysate (listed as plasma hemoglobin level). Four separate groups of data for bilirubin are shown: 0 mg/dl (□, ■), 38.5 mg/dl (○, ●), 77 mg/dl (+, ×), and 115.5 mg/dl (△, ▲).

were unable to show TBA reactivity for the bilirubin solution. Thus, in the presence of bilirubin and hemoglobin, the 453-nm correction proposed by Gilbert *et al.* (15) for red blood cell TBARS level measurement would not appear to be valid.

Subtraction Coefficient. Multiple samples containing 1, 2, and 4 nmol of TMP were analyzed by measuring 510-, 532-, and 560-nm absorbance. Absorb-

ances at the different wavelengths ($\times 10^3$ and values for $1/1 - \{[(0.56)\,(MDA_{510}) + (0.44)\,(MDA_{560})]/MDA_{532}\}$ are given in Table I. The coefficient for subsequent subtraction calculation (Equation 15) is shown to be 1.22 ± 0.01 (mean ± SD).

Desferoxamine. The irregular increase in 532-nm absorbance with the first increment of added plasma hemoglobin, presented in Figures 3 and 4, led to the next experiment in which variable amounts of desferoxamine and red cell hemolysate were added to plasma samples. Figure 5 presents data from this experiment, in which desferoxamine was varied between 0.1 and 4.2 m*M*. In Figure 5A, TBARS levels decrease exponentially with increasing desferoxamine up to a concentration of 1 m*M*. This concentration of desferoxamine has been chosen for subsequent experiments as TBARS variability with regard to [desferoxamine] appears to be small in this range. As shown in Figure 5B, $R^2 = 0.99$ for the correlation between TBARS level and added hemolysate (displayed as plasma hemoglobin concentration) with 1.0 m*M* desferoxamine present. With 1.0 m*M* desferoxamine, the TBARS level increased by 0.0067 ± 0.0006 nmol/l mg/dl of plasma hemoglobin. The standard deviation was lowest for 1 and 2 m*M* desferoxamine and highest for 0 and 5.0 m*M*. These data suggest that the inductive catalytic effect of varying amounts of iron is held most constant when 1 m*M* desferoxamine is added.

Test of Subtraction Method. Figure 6A presents the results of the test of the subtraction method for samples with added hemoglobin and bilirubin but no added TMP spike and shows TBARS levels calculated from MDA_{532} (Equation 15) and from measured 532-nm absorbance. TBARS levels calculated from MDA_{532} are markedly lower and do not vary appreciably with increasing hemolysate. Hemolysate, incubated alone, showed TBARS reactivity (Fig. 2F). TBARS levels calculated from measured 532-nm absorbance show larger variability, which is in part related to the increments of added bilirubin. Figure 6B presents the same TBARS level data as a function of bilirubin concentration.

Figure 7 presents the results of Experiment F, in which increments of hemolysate, bilirubin, and TMP standard were added to single donor plasma samples (in triplicate). Corrected 532-nm absorbance (MDA_{532}, nmol/ml plasma) is presented as a function of plasma hemoglobin (mg/dl) and TMP (nmol/ml) concentrations. Desferoxamine concentration is held constant at 1 m*M*. The three-dimensional plot demonstrates three groupings of points representing TMP standard added to samples to give an effect of 0, 2.7, and 5.5 nmol/ml of plasma on TBARS reactivity. Regression analysis comparing MDA_{532} with the value of plasma TBARS plus the absorbance expected from the TMP spike showed 86.6% recovery of the TMP spike when hemoglobin and bilirubin were factored into the determination, as follows:

$$\begin{aligned} MDA_{532} = {} & 0.58 + 0.866 \times \text{TMP spike} \\ & + 0.0045 \\ & \times \text{plasma hemoglobin (mg/dl)} \\ & + 0.015 \\ & \times \text{bilirubin (mg/dl)} \\ & \cdot (r^2 = 0.0996). \end{aligned} \qquad (16)$$

The hemolysate showed TBA reactivity (Fig. 2F). The contribution from hemolysate to the measured MDA_{532} value is significant in the higher range, although the contribution from bilirubin remained negligible (e.g., for hemoglobin 238 mg/dl, plus bilirubin 11.7 mg/dl, plus TMP spike of 2 nmol [5.25 nmol/ml in 300-μl sample], $MDA_{532} = 0.58 + (0.866)(5.25) + (0.0045)(238) + (0.015)(11.7)$ or 6.32 nmol MDA/ml plasma; the measured value of MDA for this combination was 6.23 nmol/ml of plasma). In actual practice in patients, the hemoglobin and bilirubin levels were not of this magnitude. Analysis of variance of the effects of hemoglobin, bilirubin, TMP, and their additive effects revealed that most of the variance of the model (94.6%) was related to the TMP spike. The effect of hemoglobin accounted for 4.8% of variability. The effect of bilirubin was statistically significant as well, but accounted for only 0.2% of the variability. TMP/bilirubin and TMP/hemoglobin interactions accounted for similar amounts of variation. Thus, bilirubin and interactive effects were statistically significant but not clinically important.

In summary, the various experiments demonstrate the utility of the proposed subtraction method by demonstrating 86.6% recovery of a sample spike and no spuriously high values for samples with added hemoglobin and bilirubin interfering substances. Furthermore, the effect of desferoxamine on *in vitro* production of MDA-TBA chromagen is demonstrated at various

Table I. Determination of Coefficient for Subtraction Equation

560 nm	532 nm	510 nm	Constant[a]
6	140	40	1.218
6	140	40	1.218
7	141	42	1.233
7	140	40	1.222
4	137	37	1.196
5	140	40	1.213
6	141	41	1.222
6	142	41	1.220
5	140	40	1.213
10	146	45	1.254
			1.22 ± 0.01[b]

[a] $1/\{1 - [(0.56)\,(MDA_{510}) + (0.44)\,(MDA_{560})]/MDA_{532}\}$.
[b] Mean ± SD.

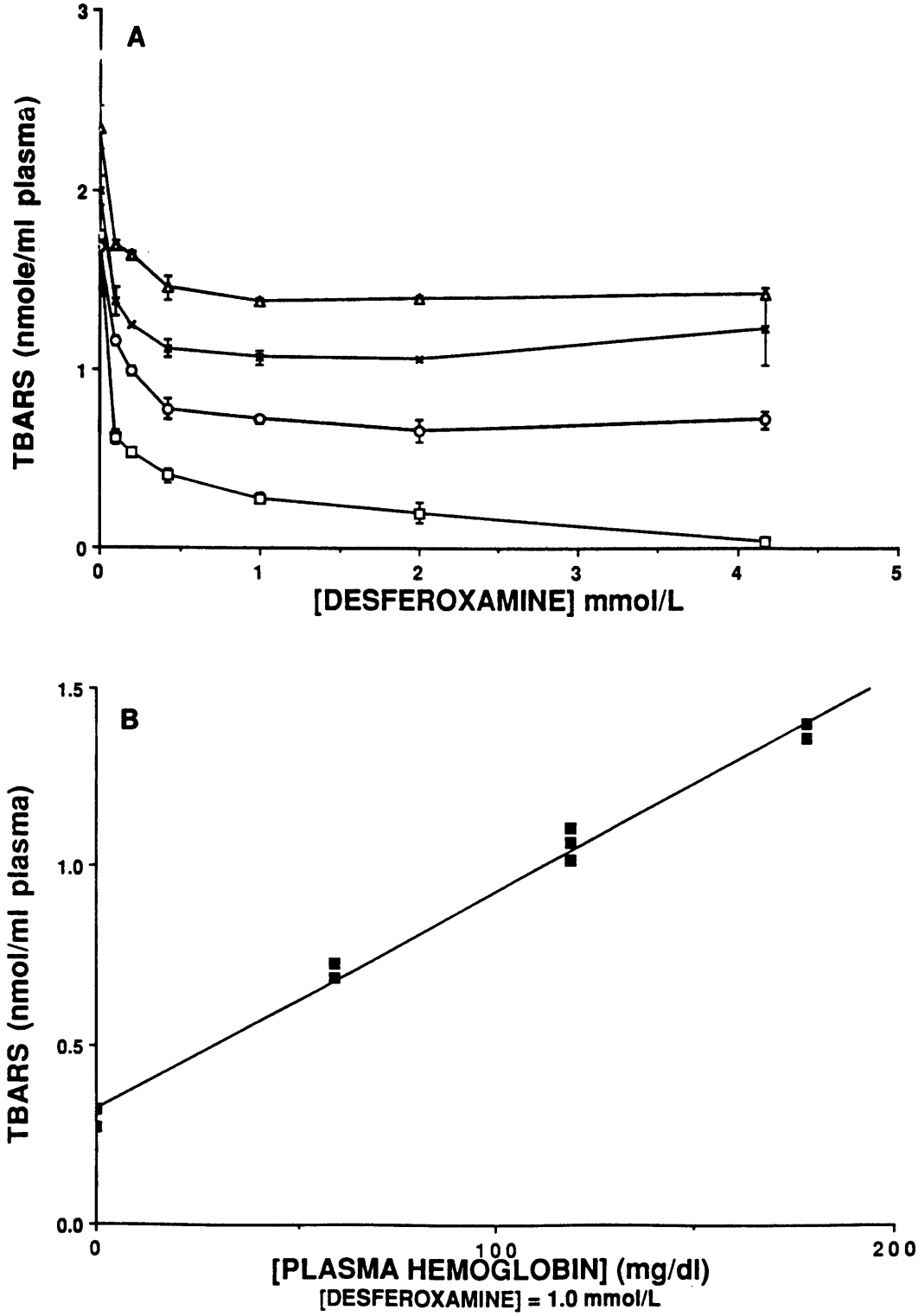

Figure 5. Experiment E (desferoxamine tests). (A) TBA-reactive substances levels (nmol/ml plasma) are displayed on the ordinate (converted from MDA_{532} values). Desferoxamine concentration is presented on the abscissa. Data points represent an average ± SD of three samples with each combination of desferoxamine and added plasma hemoglobin. Four separate groups of data for hemoglobin are displayed: 0 mg/dl (□), 59 mg/dl (○), 119 mg/dl (×), and 178 mg/dl (△). (B) TBA-reactive substances levels (nmol/ml plasma) are displayed on the ordinate (converted from MDA_{532} values). Hemoglobin concentration of sample with added red cell hemolysate is displayed on the abscissa. Data from Figure 5A is displayed for desferoxamine concentration of 1.0 m*M*. The correlation coefficient (R^2) of 0.990 was the highest for any of the desferoxamine concentrations.

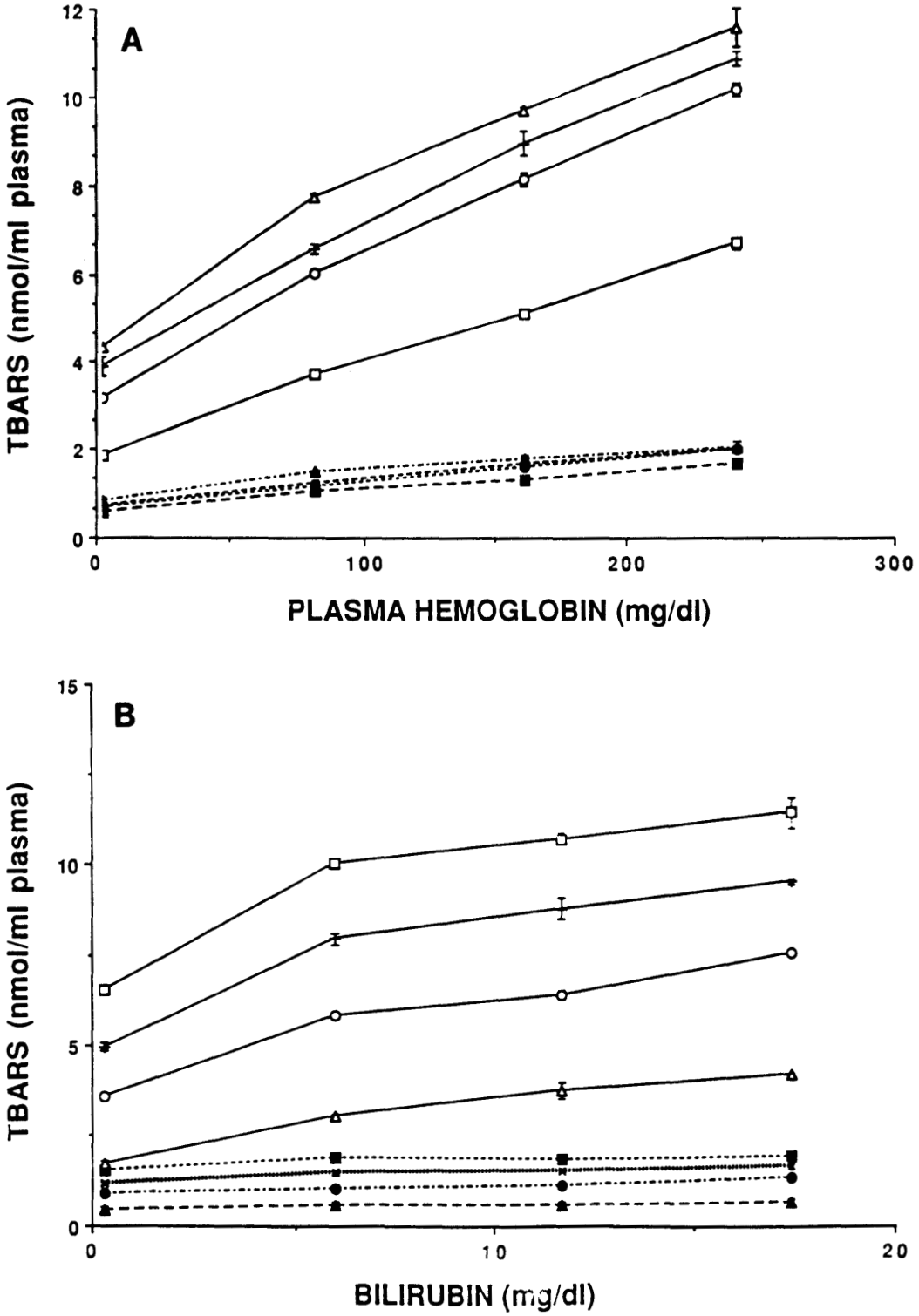

Figure 6. Experiment F (raw versus corrected absorbance). (A) Data from samples without TMP spikes from Experiment F are presented. TBA-reactive substances levels (nmol/ml plasma) from raw 532-nm absorbance (—) and levels obtained from MDA_{532} (Equation 15) values (–, filled symbols) are displayed on the ordinate. Added plasma hemoglobin concentration is displayed on the abscissa. Samples have 0.3 (△, ▲), 6.0 (○, ●), 11.7 (+, ×), or 17.4 (□, ■) mg/dl of bilirubin present, representing 0, 50, 100, or 150 μl of added bilirubin solution. (B) TBA-reactive substances levels (nmol/ml plasma) from the same experiment are displayed on the ordinate as a function of bilirubin. Raw 532 nm (—) and MDA_{532} (–) values are graphed for plasma hemoglobin values of 0 (△, ▲), 79 (○, ●), 159 (+, ×), and 238 (□, ■) mg/dl, representing 0, 50, 150, and 150 μl of added hemolysate.

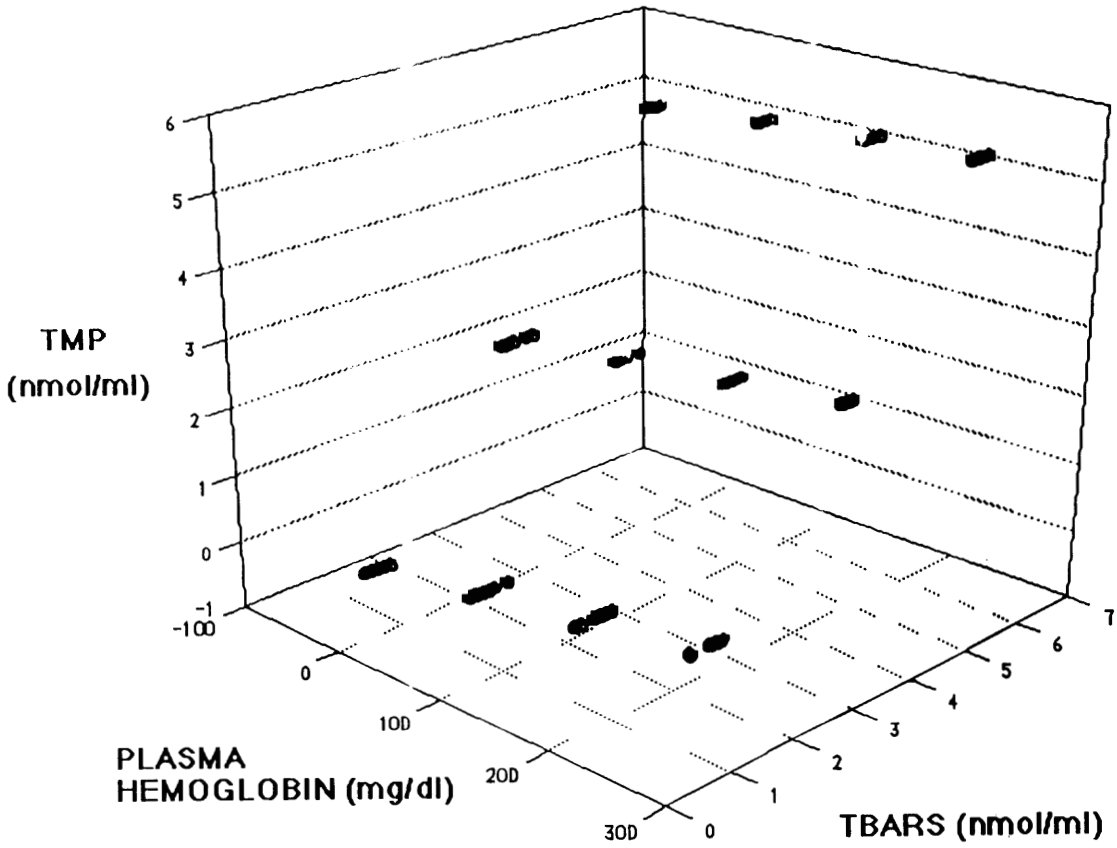

Figure 7. Experiment F (TMP spikes). Data points representing 48 combinations of hemoglobin, bilirubin, and TMP standard are presented in a three-dimensional array. Each point represents an average of a triplicate determination. TBA-reactive substances levels (nmol/ml plasma), displayed on the x-axis (converted from MDA_{532} values) are shown as a function of hemoglobin (y) and TMP (z) concentrations.

hemoglobin concentrations and the 1 m*M* concentration shown to be most suitable for plasma TBARS measurements in the presence of hemolysis. As stated previously, the utility of an arithmetic subtraction method depends upon the colinearity of the points *x*, *y*, and *z* (referring to Fig. 1), which denote the absorbance of the interfering substances at the various wavelengths. We were unable to obtain an appropriate spectrum of interfering substances without TBARS reactivity. Therefore, an additional scanning spectroscopy experiment was performed in which a hemolyzed sample was run against a blank containing an amount of TMP to give a 532-nm absorbance equal to the calculated MDA_{532} value for the sample. Figure 8 gives the resulting spectrum. In this spectrum, which represents I_i for the original sample, absorbances at 510, 532, and 560 nm are colinear.

For any given biologic system to be tested, the spectra generated by samples should be visually inspected to determine colinearity of points *x*, *y*, and *z* (Fig. 1). It should be especially noted that if a different TBA system is used (different incubation time, pH, and different acid used, especially trichloroacetic and phosphotungstic), not only will MDA or TBARS production possibly be different, but other pigments present in the

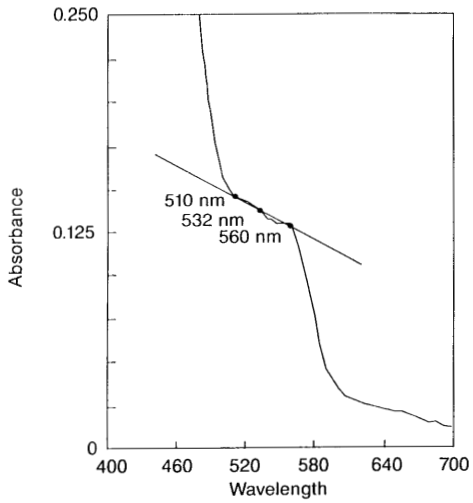

Figure 8. Spectrum of I_{532}. A spectrophotometric scan is obtained by calculating MDA_{532} for the sample from Figure 2E and reading absorbance from the sample from Figure 2E against a TMP standard possessing the calculated amount of 532-nm absorbance. Note that absorbance values at 510, 532, and 560 nm are colinear and correspond to points *x*, *y*, and *z* in Figure 1.

reaction mixture may also be retained and preserved to different degrees. It may be necessary to choose different wavelengths for subtraction depending upon the system under study.

Discussion

Little mention of spectral interference can be found in the literature of malondialdehyde assays using 2-thiobarbituric acid (16). Spectra showing the presence of obvious interfering substances as well as experiments detailing large increases in raw 532-nm absorbance in the presence of added hemoglobin and bilirubin are presented. A method of subtracting background interference due to heme and bile pigments in the measurement of TBARS levels is presented. Several methods have been described previously to remove interference from one or the other class of compounds, but none has addressed the simultaneous problem. Gilbert was able to correct for spectral interference from hemoglobin because hemoglobin decomposes to hematin with an absorbance peak in the region of 453 nm, in addition to the usual peaks in the region of 520–560 nm and the Soret band peak at 405 nm. However, in our hands, in the presence of bilirubin (with an absorbance peak at 453 mm), this method was not valid. Yagi (17) described a fluorometric method of avoiding bilirubin interference in which fluorescence from the TBA-MDA chromagen was determined at an excitation wavelength of 515 nm and emission wavelength of 553 nm. Excitation and emission spectra similar to those reported by Yagi (17) were obtained, but results were poorly reproducible in our experience and that of other investigators as well (18). Wong and co-workers (19) described a chromatographic method to avoid interference from various drugs, but stated that hemolysis was to be avoided. In our subtraction method, reproducible, accurate assessments of TBARS were obtained because the multiple hemoglobin species (oxyhemoglobin, deoxyhemoglobin, and methemoglobin) all decompose to hematin during the 95°C incubation. Since the hematin and bilirubin peaks are both far removed from the region of interest, they can be considered as one interfering substance. Testing of multiple spiked samples has shown this to be a reasonable assumption. The assay results were not compared with a different, independent method of removing interfering substances such as high-pressure liquid chromatography because of Wong's (19, p. 217) notation that "addition of erythrocyte hemolysate to plasma specimens caused positive interference in plasma lipoperoxide measurements," as well as the notation that all of the high-performance liquid chromatography methods that we reviewed involved differences in incubation conditions such that a strict comparison was not possible (16, 19, 20).

In this proposed system of measuring TBARS levels in the presence of variable amounts of hemolyzed red blood cells by subtraction of spectral interference, an excess of desferoxamine was added to the incubate to chelate free iron. Although heme iron by itself can catalyze malondialdehyde formation, Graf and coworkers (21) have shown that desferoxamine-chelated iron from hemoglobin is prevented from potentiating the TBARS reaction. Analysis of the effect of desferoxamine is obscured by the presence of TBA reactivity in the red cell hemolysate preparation that was added to samples. In Figure 5A, an incremental increase in TBARS level is demonstrated with each level of added hemolysate. The effect of varying desferoxamine concentration between 0.2 and 2 mmol/liter on TBARS level appears to be equivalent for all concentrations of added hemolysate. We have proposed that desferoxamine be added in this concentration range to the assay when dealing with variable amounts of iron and, in fact, that 1.0 m*M* desferoxamine be used in all assays.

Finally, the TBARS level may be affected by levels of endogenous antioxidant protective substances. This would seem trivial except that vitamin E depletion has been reported during conventional cardiopulmonary bypass for cardiac surgery and overall plasma antioxidant levels have been noted to vary with age (22–24). In addition, bilirubin may be a physiologically important antioxidant (25). Thus, careful assessment of the *in vitro* effect of bilirubin was necessary.

Conclusion

A method of subtracting spectral interference from measurements of TBARS levels is presented. Despite the pitfalls described by various investigators, measurement of TBARS levels remains a widely employed means of assessing lipid peroxidation, MDA formation, and effects of oxygen radical damage in biologic systems (26). The validity of this test for measurement of plasma TBARS levels is greatly improved by employing this subtraction method and by recognizing the importance of plasma iron and antioxidants in the interpretation of the values obtained. Most importantly, this study highlights the importance of visually inspecting TBARS spectra prior to interpreting the results of the spectrophotometric TBARS assay.

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