The "HemoQuant" Test: A Specific and Quantitative Determination of Heme (Hemoglobin) in Feces and Other Materials

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We describe a new, specific, quantitative method for fecal blood, based on conversion of nonfluorescing heme to fluorescing porphyrins, that obviates serious deficiencies inherent in currently used tests. A two-reagent system is used to determine the two hemoglobin-related fractions that are found in feces. The hot citric acid extract includes only the variable fraction of porphyrins that have been preformed from heme in the intestinal tract; this is often the major fraction. Total hemoglobin is indirectly determined by reaction with heated oxalic acid:FeSO4 reagent, which converts the remaining heme to porphyrin without loss of the preformed porphyrins. A three-step purification procedure eliminates interfering materials. Analytical recovery of added hemoglobin is linearly related to concentration over a several-thousand-fold range. The assay is equally applicable to whole blood or to sub-microgram amounts of hemoglobin in the 8-μg (wet weight) fecal sample tested. Quality control by liquid chromatographic and fluorometric analysis documents fluorescence specificity of the heme-derived porphyrins.

Additional Keyphrases: heme-derived (in the gut) porphyrins and unaltered heme distinguished · liquid chromatography (of porphyrins) compared · effects of diet, other exogenous gut contents gastrointestinal bleeding, normal and abnormal

For detection of fecal (occult) blood, we have long relied primarily on the relatively simple and inexpensive guaiac test, as exemplified by the widely used "Hemoccult" test (SmithKline Diagnostics, Sunnyvale, CA) and other such leuco-dye tests. However, all such tests clearly have serious deficiencies related to their nonspecificity and uncertain quantitative interpretation (1–6); these lead to costly false-positive and false-negative reactions. Less well known is the observation that most of the hemoglobin heme entering the gastrointestinal tract may be converted, presumably by bacteria, to porphyrins (7–13). These do not react with leuco-dyes and thus contribute to the false-negative reactions obtained with Hemoccult and similar tests. In an age of precise measurements, these tests have remained among the few nonquantitative assays in clinical medicine. Quantitative isotopic methods (14–17) are an advantageous alternative, but these methods are invasive, costly, and poorly suited to widespread use.

We proposed here a new specific, quantitative, and noninvasive approach, based on the removal of iron from nonfluorescing heme to convert it to fluorescing porphyrin (Figure 1). Such conversion has been achieved during the past century by several methods that involve reducing conditions and low pH (18–22). None of these, however, has been applied to the quantitative assay of heme in feces. Hot oxalic acid (a reducing acid), has been used recently (23–25) in the quantification of hemoglobin in blood and other tissues, but only at hemoglobin concentrations of less than 4 to 15 mg/L of the final heated solution.

The relatively simple and specific fecal test described here measures not only the heme content of feces, but also the total quantity of hemoglobin heme that enters the gastrointestinal tract. It includes three essential features:

• One aliquot of feces is heated in oxalic acid:FeSO4 reagent to convert remaining fecal heme to porphyrins while retaining those porphyrins that were formed from heme in the intestinal tract. This fraction is used to assay the total equivalent concentration of hemoglobin represented in the feces.

• A second aliquot of feces is heated in citric acid reagent to solubilize and assay only those porphyrins that were formed from heme in the intestinal tract. This reagent converts negligible amounts of heme to porphyrin.

• The heated samples are purified by three extraction steps. These result in fluorescence specificity by removing interference from materials in feces. This method, especially in its anticipated automated version, has wide applications in clinical and investigative medicine (26–29).

The name of the test, "HemoQuant," is intended to identify the procedure and to emphasize its unique quantitative nature.

Materials and Methods

Sampling of Feces

Place 1 to 3 g of fresh feces in a plastic tube with screw-on cap and store at −15 °C until assayed.

Reagents

Aliquots of feces are heated in each of two reagent systems.

The first reagent (the "oxalic acid reagent") contains, per liter, 2.5 mol (315 g) of oxalic acid, 90 mmol (25 g) of FeSO4·7H2O, and 500 mg of an acid-soluble cationic deter-

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1 Patent number 4,378,971 was issued by the United States Patent Office. The University of Minnesota is the owner of record.
2 The reagent compositions described in this section (termed our "110 °C system") have been used for the assays discussed herein, as well as for all clinical studies completed to date (26–29 and others). While these studies were in progress, use of a lower temperature and a modified composition of each reagent was found to have significant advantages, to be detailed elsewhere. These compositions, which we plan to use in the future (termed our "100 °C system"), are as follows:

Oxalic acid reagent: per liter, 2.5 mol of oxalic acid, 90 mmol of FeSO4·7H2O, 50 mmol (8.4 g) of uric acid, and 50 mmol (9.1 g) of mannitol.

Citric acid reagent: 1.5 mol/L citric acid (only).

The only changes in the new system, thus, are: Cetramide is omitted; free radical scavengers (uric acid and mannitol) are added to the oxalic acid reagent, and samples are heated for 30 min at 100 °C (use a water bath or other constant-temperature unit instead of an autoclave).
gent, Cetramide (hexadecyltrimethylammonium bromide; Eastman Organic Chemicals, Rochester, NY 14650). The detergent was included to facilitate fecal dispersion and solubilization of the porphyrin. This reagent mixture, prepared freshly each day, is heated at 80 °C for at least 1 h, then maintained at this temperature with constant mixing until it is added to feces. Most of the oxalic acid is solubilized at this temperature.

The second reagent (the "citric acid reagent") contains, per liter, 1.5 mol (288 g) of citric acid and 500 mg of Cetramide.

Procedures

**Heating.** Weigh 8.0 mg of well-mixed feces into each of two polystyrene screw-cap tubes. Add 2 mL of the first reagent to one, 2 mL of the second to the other. Cap them, and place them in a water bath set at 80 °C for 1 h, then vortex-mix for 15–30 s to facilitate mixing and loosening of feces from the tube wall. Then heat the samples for 90 min at 110 °C in an autoclave.

**Extraction.** Vortex-mix the autoclaved samples for 15 s and then place them in the 80 °C water bath for about 2 min, to allow insoluble materials to settle. No centrifugation is required. The three-step purification procedure (Figure 2) is done next.

**Step A:** Place 250 μL of the supernate in a 12 × 75 mm borosilicate tube. To this solution, add successively 1500 μL of a 10/1 (by vol) mixture of ethyl acetate/acetate acid (EtOAc:HOAc) and 500 μL of a 3.0 mol/L (294 g/L) solution of potassium acetate (KOAc). Vortex-mix for 15 s after each of these additions. (Potassium salts are used because sodium oxide is relatively insoluble in water.) The porphyrin analytes are extracted into the organic (upper) solvent phase.

**Step B:** Add 625 μL of the upper-phase EtOAc:HOAc from Step A to 250 μL of n-butanol (BuOH) and 1.9 mL of a 3 mol/L solution of KOAc in 1 mol/L (56 g/L) KOH. Vortex-mix for 15 s. Coproporphyrin and other porphyrins that are not derived from hemoglobin heme (they contain more than two carboxyl groups) are removed, along with other impurities, into the alkaline aqueous (lower) phase, which is discarded.

**Step C:** Add 250 μL of the EtOAc:BuOH extract to 750 μL of a mixture of 2 mol/L H₃PO₄ and HOAc (9/1, by vol) in a 10 × 75 mm borosilicate tube, and vortex-mix for 15 s. Chlorophylls are retained in the organic (upper) phase; the desired 2-carboxyl porphyrins are extracted into the H₃PO₄·HOAc.

This extract represents a total dilution of the fecal sample of about 6300-fold: a 251-fold dilution in the heated sample (2008/8), and about a 25-fold further dilution in the three-step extraction procedure.

**Fluorescence assay.** The vortex-mixed sample from Step C, with both phases included, is used directly for assay. We use a Perkin-Elmer MFP-44B fluorescence spectrophotometer with a no. R298 red-sensitive photomultiplier tube. Slits for incident (In) and emitted (Em) light are set to accommodate only the aqueous (lower) phase in the tube; the upper phase, therefore, need not be removed. A xenon lamp (cat. no. N350-1646; Perkin-Elmer Corp., Norwalk, CT 06856) is used for excitation, with the primary monochromator set at 402 nm. The secondary monochromator is set at 653 nm, with a nonfluorescing yellow Corning filter (no. CS 3-144; Corning Glass Works, Corning, NY 14830) added to ensure that blue and violet light are excluded. Both wavelength slits are kept quite wide (at 20 nm), because the several porphyrins present differ by as much as 5 nm in their In and Em maxima.

**Fluorescence standards.** Compare the measured fluorescence with that of a standard 50 μg/L solution of coproporphyrin in 1.5 mol/L HCl. Coproporphyrin (may be purchased from Porphyrin Products, Logan, UT 84321) is used as the reference standard because it is much more stable than the porphyrins derived from heme. Adjust sensitivity settings to yield a consistent total fluorescence reading. The calculated fluorescence set with our apparatus was 33 300 (arbitrary units). Corresponding fluorescence of extracted solvent blanks in the two systems has ranged from about 20 to 50, while that of fecal extracts is about 3000 at a concentration of 1 mg of hemoglobin per gram of feces.

For purposes of quality control, standard solutions of cyanmethemoglobin are also assayed with the fecal samples. These standards (30) in Drabkin's solution (Sigma Chemical Co., St. Louis, MO 63178) are more stable than blood diluted with saline and kept for several weeks at 4 °C. Add 20 μL of a 1 g/L solution to 2 mL of the oxalic acid:FeSO₄ reagent and assay this with each batch of unknowns.

**Calculations.** Calculated values for both reagents refer to equivalent concentrations of hemoglobin; i.e., they are directly equal to the amount of hemoglobin required to yield the amounts of porphyrin found, rather than to the actual amount of hemoglobin present as such in the fecal sample. Calculated values obtained with the citric acid reagent divided by values obtained with the oxalic acid reagent, multiplied by 100, give the "intestinal conversion fraction," that is, the percentage of total hemoglobin that has already been converted to porphyrin in the excreted feces.

**Liquid chromatography of heated samples.** We have modi-
fied the urinary porphyrin method of Ford et al. (31) to make it applicable to the fractionation and assay of these fecal porphyrins. This analysis is not part of the HemoQuant assay, but has proven to be of great value for quality control. This analysis may be done immediately after heating (and without further purification) or after keeping the heated samples at -15 °C for several months or more.

Maintain the samples at 40 °C for 5-10 min, then filter them through a Millex filter, 0.45-μm nominal pore size (Millipore Corp., Medical Products Division, Bedford, MA 01730). We used a Waters Associates MBondapak C18 column (3.9 x 300 mm, 10-μm nominal particle size) in a Hewlett-Packard Model 1084 B liquid chromatograph in tandem with a Perkin-Elmer Model 650-10s fluorescence spectrophotometer equipped with a 20-μL quartz flow cell and red-sensitive photomultiplier tube. Because of the altered emission spectrum at the eluent pH, this fluorescence is assayed at In 398 nm, Em 625 nm, and 15/15 nm slits. Our modified two-solvent system includes equal volumes of methanol ("AR") and 0.1 mol/L (11.5 g/L) NH4H2PO4 (pH 3.5) in bottle A, and methanol only in bottle B. Prepare the aqueous buffer with de-ionized, glass-distilled, and column-purified water (LiChroprep™ RP-8, 40-63 μm; EM Labs, Elmsford, NY 10623). Filter solvents through a 0.45-μm pore-size nylon 66 membrane filter (Rainin Instrument Co., Woburn, MA 01801) before use.

Appropriate injection volumes vary from 5 to 200 μL, depending on fluorescence intensities found by the present assay. The flow rate is 1 mL/min; solvent and oven temperatures are maintained at 40 °C.

The proportion of bottle B is increased linearly from 50% to 100% during 25 min. In the interval from 26 to 32 min, the proportion from bottle B is returned to its initial 50% value. The integrator printout ignores the nonspecific fluorescence, which is eluted during the first 10 min, as illustrated in the following section. Calculation of hemoglobin-equivalent values is based on comparisons with similarly treated standards of known hemoglobin concentration.

Results

Fluorescence Spectra of Blood and Fecal Fractions

We studied fluorescence properties in many ways, including recorded excitation and emission spectra and their second derivatives. These spectra illustrated to us the need for purification and other features (Figures 3, 4).

Only a minor part of the total red fluorescence of normal feces is due to porphyrin derivatives of hemoglobin in either reagent system. Nonspecificity is especially great in the case of the citric acid reagent (Figures 3, 4). Chlorophyll may account for more than 90% of the total red fluorescence in some unpurified samples, even though its fluorescence intensity is markedly decreased in the oxalic acid solution. The presence and amount of chlorophyll is best determined in the heated citric acid sample and in its ethyl acetate extract. (The excitation maximum of about 410 nm for fecal chlorophyll is close to the 398-408 nm maxima for porphyrins derived from hemoglobin.) The relatively pure emission spectrum of heme-derived porphyrins, especially at about Em 650 nm, always seen in the final H3PO4 extract (Figure 4C), confirms that interfering materials are removed by the purification method.

Effect of Added Ferrous Iron

Of the numerous reducing compounds we tested with oxalic acid, ferrous iron was by far the most effective in eliminating the fluorescence plateau found by Morrison (23) and others (24, 25) at high concentrations of hemoglobin.

Fig. 3. Fluorescence spectra of unpurified blood and feces
Both heated samples were diluted with 0.5 mol/L oxalic acid. A, blood only; B, normal feces; C, similar dilution of pooled two-day feces from the same normal subject after ingestion of 15 mL of his blood (note changed scale). Emission maxima are at about 600 and 655 nm.

The addition of 1 to 30 g (3.6 to 108 mmol) of FeSO4·7H2O per liter to a 2 mol/L solution of oxalic acid sufficed to yield similar recovery of fluorescing porphyrin when tested with hemoglobin concentrations as great as 1200 mg/L in this reagent (Figure 5). At the latter concentration, the added iron increased the fluorescence by 10-fold. Feces differs somewhat from blood in this respect, requiring higher concentrations of FeSO4.

Validation of the Three Extraction Steps

Table 1 summarizes our findings concerning the essentially complete analytical recovery of heme-related porphyrins, and the differential removal of two major interfering compounds by each of the three purification steps. We assayed blood and purified solutions of coproporphyrin and of chlorophyll, along with fecal samples that contained large amounts of these compounds. Analytical recovery was determined by analysis of fluorescence spectra of both aqueous and organic solvent phases at each step. Concentrations of coproporphyrin in each phase were also assayed by liquid chromatography.

Comparison of the Present Assay and Liquid Chromatography

Blood heated with citric acid yields no discernible fluorescence peaks by liquid chromatography at the sensitivity...
setting used (Figure 6). On heating hemoglobin with the oxalic acid:FeSO₄ reagent, one minor and three major elution peaks were found under the same conditions. Materials represented by these peaks are eluted in the interval from about 10 to 21 min. Based on their successive elution times, we have tentatively identified them as hematoporphyrin, two isomers of monovinylmonohydroxyethyl deuteroporphyrin, and protoporphyrin. Fecal porphyrins derived from heme in the intestinal tract show different ratios of these peaks, as well as several additional peaks. A major fecal nonporphyrin fluorescence peak is eluted at about 3 min, and coproporphyrin is eluted at 9 min. The computer analysis of the elution pattern, therefore, includes only those areas of fluorescence that are ascribable to the heme-derived 2-carboxyl porphyrins, which are eluted from 10 to 25 min. After blood ingestion (28), even the citric acid reagent yields large increases in total porphyrins eluted in the interval from 10 to 20 min. As many as 12 significant peaks contribute to this total in some samples; these compounds are the heme-derived, intestinally converted fraction. Chlorophylls (emission maximum, 670 nm) are not detected or eluted by this chromatographic program.

Results of liquid chromatography and analyses quantitatively validate the purification procedure. In one series, we assayed 150 consecutive fecal samples by both the present oxalic acid:FeSO₄ procedure and liquid chromatography; the correlation coefficient for this relationship was 0.97 (Figure 7). A similar correlation (not shown) was found with the corresponding citric acid-treated samples.

**Analytical Recovery of Hemoglobin Added to Feces**

Blood added to normal feces or to saline homogenates of feces yields recovery data similar to that shown in Figure 5 for blood alone treated with the oxalic acid:FeSO₄ reagent. Additionally, 60 fecal samples submitted by an out-of-state laboratory, to which had been added three different amounts of whole blood or of hemin to feces from each of five patients, were analyzed in a blinded design. The added hemoglobin-equivalent values ranged from 0.5 to 24.3 mg per gram of feces; equivalent hemoglobin concentrations in the five untreated samples ranged from 0.4 to 4.8 mg per gram. Triplicate coded tubes were submitted for each of the 20 samples thus prepared.

As seen in Table 2, the average analytical recovery was 99.1% of theoretical values.

**Heme Specificity of the Present Method**

*Variable heme sources.* Fluorescence yields from protoheme compounds such as hemin, hemoglobin, myoglobin, and catalase, as well as from non-protoheme compounds such as cytochrome c and horseradish peroxidase, are shown in Figure 8. Fluorescence in this study was evaluated after direct dilution of heated samples and without purification, because the more water-soluble porphyrins derived from the nonprotohemes are removed by the purification procedure. All compounds showed a linear relationship between the concentration of heme added and the fluorescence yield recovered. Because of the unknown purity of several samples, comparison by molar heme content was not feasible. Fluorescence yields of heme in hemin, hemoglobin, and myoglobin, however, all agreed within 10% of one another when calculated in terms of moles per liter.

**Peroxidase-rich vegetables that react with leuco-dyes.** Homogenized 100 g/L suspensions of turnips, radishes, arti-
Table 2. Analytical Recovery of Hemoglobin (Hb) Added to Feces

A. Detailed results from one of five patients

<table>
<thead>
<tr>
<th>Added</th>
<th>Found</th>
<th>Av</th>
<th>Recovery, %</th>
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<tbody>
<tr>
<td>None</td>
<td>0.40</td>
<td>0.41</td>
<td>41</td>
</tr>
<tr>
<td>1.0</td>
<td>1.33</td>
<td>1.34</td>
<td>98</td>
</tr>
<tr>
<td>3.0</td>
<td>3.30</td>
<td>3.65</td>
<td>108</td>
</tr>
<tr>
<td>9.0</td>
<td>10.4</td>
<td>9.8</td>
<td>108</td>
</tr>
</tbody>
</table>

B. Summary of all 60 samples from five patients

<table>
<thead>
<tr>
<th>Recovery, %</th>
<th>Mean</th>
<th>Std. error from average</th>
</tr>
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<tbody>
<tr>
<td>Triplicates, % difference</td>
<td>5.4</td>
<td>0.4</td>
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*Citric acid test values increased negligibly, from 0.10 to 0.20 mg/g in the four series of samples.

Liquid-chromatographic analyses (n = 26) averaged 96% (std. err 1.9%) of the corresponding values by the present method.

Reproducibility

Reproducibility was compared in duplicate weighings from 64 consecutive 2- to 4-g fecal samples submitted to us. Individual values for these well-mixed samples differed by an average of 1.7 and 2.0% from the duplicate mean in the oxalic acid:FeSO₄ and citric acid assays, respectively. Standard deviations were 0.9 and 1.6% for these two assays. Duplicate extractions of the same heated solutions showed that about half of the difference found was ascribable to the weighing step, and half to the assay itself. (The reproducibility results included earlier in Table 2 included an additional variable, namely, the outside laboratory's mixing of whole blood with about six to 270 volumes of feces, and submission to us of three samples from each mixture.)

Discussion

We believe the assay described here has many unique and substantial advantages over other methods currently used for the detection or measurement of fecal blood. Most importantly, its specific, sensitive, and quantitative nature allows investigation of both basic and clinical questions that have hitherto been unapproachable because of technical limitations. Some relevant questions, now being investigated further, include the following:

1. Will availability of quantitative data significantly improve the diagnostic distinction between normal and abnormal gastrointestinal bleeding, and what variables must be considered or controlled? Where clinical laboratory values form a continuum, it seems axiomatic that diagnostic interpretation of precise values is much superior to that of imprecise "+" or "0" values. This is especially true of the present test, which yields three values with distinct diagnostic implications, as discussed below: (a) total gastrointestinal heme, (b) the proportion of this heme that is converted to porphyrin in the intestinal tract, and (c) residual heme in the feces. Each may be calculated in terms of milligrams per gram or per day. We are exploring the definition of so-called normal reference intervals for each of these three values in relation to many variables, including diet, exercise, and treatment with aspirin, antibiotics, and other medications. Such a definition is of obvious importance with respect to early diagnosis of colorectal cancer and other gastrointestinal lesions (26-29).

Table 3. Approximate Sensitivity Limits of Test Procedures for Fecal Blood Analysis

<table>
<thead>
<tr>
<th>Approximate detection limits of Hb</th>
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<tbody>
<tr>
<td>mg/g (total body)</td>
</tr>
<tr>
<td>mg/mL</td>
</tr>
<tr>
<td>5Cr,</td>
</tr>
<tr>
<td>3.0</td>
</tr>
<tr>
<td>Hemocult</td>
</tr>
<tr>
<td>Other leuco-dyes⁶</td>
</tr>
<tr>
<td>Immunofluorescence</td>
</tr>
</tbody>
</table>

*Under conditions where blood Hb concentration is 150 g/L and 24-h fecal (wet) weight is 150 g, then 1 ml of fecal blood per day represents a fecal Hb concentration of 1 mg/g.

⁶Values given are approximate, because the observed sensitivity of leuco-dye tests is strongly influenced by water content (26-28) and other variables, in addition to properties of the selected dye. Thus, as will be documented elsewhere, Hemocult results may be positive at 0.2 mg/g and negative at 15 mg/g, depending largely on water content.

Increases sensitivity (if needed) is readily achieved by decreasing the total 6300-fold dilution factor of the present method.
Water content of feces significantly affects the assayed concentration values. We have given this variable special attention with respect to both the present (HemoQuant) and "Hemoccult" testing (26).

2. What proportion of the hemoglobin heme that enters the gastrointestinal tract is excreted as heme in the feces, and what are some implications of this proportion? This important question has not been, and cannot be, addressed by leuco-dye, immunochemical, or isotopic tests, because none will detect that portion of heme that is degraded to porphyrin in the intestinal tract. It is strange that this intestinal degradation of heme has received so little attention, because our present findings were presaged more than half a century ago by Snapper and coworkers (8–10), who first emphasized the finding of negative guaiac reactions in the feces of patients known to have gross intestinal bleeding. Subsequently, such feces were found to exhibit intense red fluorescence on exposure to near-ultraviolet light. The fecal extracts also had absorbance bands characteristic of porphyrins or altered hemes, or both. The precise bacteria and other intestinal factors involved have not been defined (11–13). We have found negligible amounts of porphyrin in the (purified) citric acid-reacted fraction of feces from newborns (29) and in feces of patients who had received antibiotic therapy during the preceding few weeks.

Although the intestinal converted fraction constitutes an unavoidable source of false-negative reactions with leuco-dye tests, it promises to provide a valuable bonus for the present assay, namely, prediction of the approximate site in the gastrointestinal tract at which bleeding is taking place (27). Under ideal circumstances of constant bacterial activity and transit times, the proportion of heme converted to porphyrin logically would increase with the distance of the bleeding site from the anus. Conversely, the calculated value for unaltered fecal heme (oxalic reagent value minus the citric acid reagent value) should be relatively increased in subjects with rectal or lower colon bleeding. We expect that this latter value will improve the distinction of these subjects from normal persons, whose moderate meat ingestion, for example, might lead to slightly increased values for total hemoglobin-heme equivalents (28).

We have not established the chemical nature of the porphyrins formed from heme in the intestinal tract. Neither have we yet explored the likelihood that intestinal bacteria alter the heme side chains (especially the two vinyl groups) before removal of the iron atom. Such altered hemes appear to be present in feces, as indicated by increased amounts of unidentified porphyrins found by liquid chromatography of samples heated in oxalic acid reagent. Snapper and van Creveld (9) and Hulst (13) reported long ago that feces of patients with gastrointestinal cancer contained larger amounts of "deuterohematin" than were found in feces of patients with similar amounts of bleeding due to other diseases. Their identification of "deuterohematin" was based on the finding (in fecal extracts) of hemochromogen maxima at 545 and 517 nm, in contrast to the corresponding maxima of 557 and 527 nm for protohemochromogen.

3. Does the present test accurately reflect the total amount of hemoglobin heme that enters the gastrointestinal tract? The answer to this question seems to be "yes," from three lines of evidence:

- Addition of blood to feces has yielded accurate analytical-recovery data. This indicates accurate analytical recovery of residual (i.e., unaltered) fecal heme.
- As noted in a preliminary report (28), nine normal volunteers drank 10 to 36 mL of their own blood over a period of one to four days. An average of more than 85% of hemoglobin-heme ingested by these volunteers was accounted for in the present assay. From 20% to about 75% of the ingested heme was converted to porphyrins in the subjects' intestinal tracts. This indicates we can well account for all the heme that enters the gastrointestinal tract, regardless of its subsequent degradation. It also explains, in part, the insensitivity of guaiac tests for gastric bleeding (32).
- We know of no evidence that heme is converted to nonporphyrin derivatives in the gastrointestinal tract. It is not yet clear whether heme or its derivatives can be absorbed to any extent, especially from the colon and rectum.

4. Is the present test specific for heme compounds? We have found no substantial nonspecific fluorescence in the assay. However, protoporphyrin has both an anabolic and a catabolic relationship to heme, though the quantitative ratio of these two sources in normal subjects is unknown. The approximately 1 mg of protoporphyrin and related 2-carboxyl porphyrins excreted daily in the feces of normal adults would, if derived entirely from hemoglobin catabolism, represent a blood loss of about 0.2 mL per day. This amount represents 15% to 50% of the total hemoglobin equivalent value found in feces of normal subjects on a meat-free diet (27), and is consistent with the percentage conversion of ingested blood noted above. Daily fecal protoporphyrin values of 20 mg or more in patients with protoporphyria will lead to erroneously high hemoglobin-equivalent values. If essentially all the heme in feces is present as porphyrin, the examiner will be alerted to the possibility of a diagnosis of protoporphyrin. Liquid-chromatographic analysis will confirm the preponderance of protoporphyrin, in comparison with the bacterial degradation products of heme.

Finally, the heme assayed may come from myoglobin, catalase, or other protohemates. We have found increased values after ingestion of large amounts of red meat, but not of fowl, fish, or vegetables (28).

5. How does the sensitivity of the present assay compare with that of other tests? Historically, several techniques have been used to overcome the leuco-dye defects noted earlier. These spectrophotometric (33), isotopic (14–17), or immunochemical (34–35) approaches each have their own problems. The approximate lower usable sensitivity limits of such tests are compared with those of the present test in Table 3. Conversion of heme to fluorescent porphyrin, as in the present test, appears to offer the most sensitive approach to heme detection and quantitative assay yet available. Indeed, full exploitation of this sensitivity is not required for the fecal test, although it may be for other applications, such as the quantitative assay of hemoglobin even in normal urine, where only three erythrocytes per microliter represents a total hemoglobin concentration of approximately 90 μg/L. This level of sensitivity is easily achieved, though appropriate modifications of the present test are required for urinary hemoglobin analysis. (Only the oxalic acid reagent need be used routinely with urine because bacterial degradation of hemoglobin is rarely a problem in the urinary tract. These studies will be described elsewhere.)

6. Can this test be used in mass screening? As yet, all assays by the present method have been done manually, but there are no immediately obvious technical barriers to automation of the essential features of the test.

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