Antioxidant prevention of Heinz body formation and oxidative injury in cats

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Objective—To determine the effectiveness of 3 antioxidants in preventing Heinz body anemia in cats.

Design—Prospective study.

Animals—44 specific-pathogen-free healthy cats.

Procedure—Cats were housed individually, divided randomly into 4 groups, and given the following orally every 12 hours: empty gelcaps (control cats), N-acetylcysteine (NAC, 100 mg/kg of body weight), vitamin E (d,l-α-tocopherol; 400 IU), or ascorbate (250 mg). After 2 weeks, Heinz bodies were induced by dietary onion powder (OP; 1% or 3% of dry matter) or propylene glycol (PG, 8% wt/vol in drinking water) for an additional 3 weeks. Intake of treated water or food was recorded daily. Body weight, PCV, Heinz body and reticulocyte percentages, reduced glutathione concentration, and total antioxidant status were measured twice weekly in all cats.

Results—Heinz body percentage and degree of anemia did not differ significantly among cats receiving antioxidants and control cats except in cats that ingested water containing PG, in which antioxidant supplementation was associated with a decrease in water intake. Of cats that were fed a diet that contained OP, cats that received NAC had significantly higher reduced glutathione concentrations, compared with other cats in the experiment. Total antioxidant status did not consistently correlate with antioxidant supplementation or type of oxidant administered (ie, OP or PG).

Conclusions and Clinical Relevance—Although the effect of antioxidant supplementation on Heinz body anemia in cats was minimal, antioxidants may have subclinical biochemical effects such as GSH sparing that may be important against milder forms of oxidative stress. (*Am J Vet Res* 2001;62:370–374)

Feline hemoglobin is uniquely sensitive to oxidative damage resulting primarily from its high number of free sulfhydryl groups.[1] As a result, **Heinz body (HB)** formation is a common hematologic abnormality in cats.[2] Heinz bodies are observed microscopically as clumps of denatured oxidized hemoglobin and result in shortened RBC survival and hemolytic anemia. Heinz body formation can be induced by dietary ingredients such as fish or onion products, drugs with oxidant properties such as acetaminophen, and diseases causing oxidative stress such as diabetic ketoacidosis.[1-3] In clinical practice, veterinarians are sometimes presented with cats having a high percentage of circulating Heinz bodies but no known history of exposure to oxidative diets or pharmaceuticals. When the primary cause is untreated or cannot be determined, Heinz bodies may persist and increase in size and number. If the bone marrow has insufficient time to compensate for shortened RBC survival, the resulting RBC destruction can exacerbate anemia in a cat already compromised by illness or injury. Current treatment for HB anemia consists of removing the offending cause (if known) and supportive care.[1] It is unknown whether antioxidants would be beneficial in preventing or ameliorating HB anemia in cats.

Antioxidants are being researched widely in human medicine for prevention and treatment of oxidative damage associated with coronary artery disease, reperfusion injury, diabetes, and more than 100 other diseases.[4,5] One common preventive approach is the enhancement of intracellular, cell membrane, or plasma concentrations of antioxidants. Except for their use in treating acetaminophen toxicosis,[6] there has been little research into the effectiveness of antioxidants in cats, despite the sensitivity of cat hemoglobin to oxidative stress, and the frequency with which cats develop oxidative anemia.

The purpose of the study reported here was to test the effectiveness of 3 antioxidants in the prevention or amelioration of feline HB formation and anemia. **N-Acetylcysteine (NAC)** is a cytosolic sulfhydryl donor used to treat acute acetaminophen toxicosis in cats.[6] Vitamin E, a common ingredient in high quality commercial pet foods, acts within the RBC membrane, primarily to prevent lipid peroxidation.[4,7,8] Ascorbate is available in nutritional supplements as well as some commercial pet foods; it scavenges free radicals in the cytosol and in plasma and regenerates α-tocopherol from the α-tocopheroxy radical form.[4,8,9] Exogenous antioxidants sometimes work through their effect on endogenous antioxidants such as **reduced glutathione (GSH)**, an important cytosolic antioxidant involved in RBC ascorbate recycling and critical for preventing plasma and RBC lipid peroxidation.[4,10] We tested the efficacy of these 3 antioxidants, using different methods and degrees of HB induction, and compared differences in anemia, GSH concentration, and **total antioxidant status (TAS**; as measured by a commercial kit). The results of our study lay the groundwork for ascertaining the rational use and selection of antioxidants in the prevention of HB anemia in cats.

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Materials and Methods

Cats and experimental groups—Specific-pathogen-free 3- to 4-kg cats were housed individually in metabolism cages. Room temperature was controlled at 21 ± 2 C, with 50% humidity and a 12-hour light/dark cycle. Cats were determined to be healthy on the basis of results of a physical examination, CBC, and blood biochemical analysis. All cats received water ad libitum and were fed a fish-free canned diet[a] prior to beginning the experiments. Quantitative analysis of vitamins or cysteine compounds in the diet was not done.

Three experiments were conducted to induce different amounts and types of oxidative stress. In the first experiment, 24 near-adult cats (12 male, 12 female), 6 to 8 months old, were fed 3% **onion powder**[b] (**OP**; 3% OP experiment) added to the canned diet as a percentage of dry matter.[11] In the second experiment, 20 male cats, 6 to 8 months old, were fed 1% OP in the diet (1% OP experiment). In the third experiment, the 24 cats from the first experiment (12 male, 12 female), now 8 to 10 months old, received **propylene glycol**[c] (**PG**; 1,2-propanediol; 8%, wt/vol; PG experiment) in drinking water.[12] The 2-month washout period allowed complete turnover of RBC mass. The CBC results confirmed there were no residual hematologic abnormalities, and HB percentages were < 2%. Food intake of cats was recorded daily in the 3% and 1% OP experiments. Cats with PG in their drinking water were acclimated over a 3-week period to drink water exclusively from bottles hung on the cages, prior to addition of PG[c]; water intake was measured daily throughout the PG experiment. Body weight of all cats was measured twice weekly.

Prior to beginning oxidant challenge, cats in each experiment were randomly divided into 4 antioxidant groups consisting of 5 cats (1% OP experiment) or 6 cats (3% OP and PG experiments) each. Cats received gelatin capsules orally twice daily, containing NAC[d] (100 mg/kg of body weight), vitamin E[e] (d,l-α-tocopherol; 400 IU), ascorbate[e] (250-mg tablet), or no antioxidant (control group). The dosage of NAC was determined on the basis of that recommended for acetaminophen toxicosis.[6] The ascorbate dose was determined on the basis of those used previously in a cat with acetaminophen toxicosis (200 mg, q 8 h, SC)[13] and in horses with red maple leaf toxicosis (50 mg/kg, q 12 h).[14] The vitamin E dose was determined on the basis of those used to treat HB anemia in people[4,15] and oxidative spinal cord injury in cats.[16] After 2 weeks of antioxidant supplementation, oxidant challenge was begun (1% or 3% OP diets or 8% PG in drinking water) and continued for 3 weeks. Cats continued to receive oral antioxidants at the same dosages during the 3-week period of oxidant challenge. Cats receiving the 1% OP diet also were given antioxidants for 2 weeks after removal of OP from the diet.

Analytic methods—Prior to beginning antioxidants, prior to beginning oxidant challenge, and twice weekly to completion of the experiments, 2 ml of blood was drawn from each cat by jugular venipuncture into heparinized tubes. Samples were obtained approximately 2 hours after administration of antioxidant or placebo and prior to feeding. The PCV, HB percentage, total reticulocyte percentage, and concentration of GSH were determined on whole blood. The TAS was determined from serum obtained after centrifugation.

The PCV was determined by microhematocrit centrifugation. Supravital new methylene blue stain was used to view HB and total (aggregate and punctate) reticulocytes, which were calculated as percentages of 1,000 RBC counted.[17] Reduced glutathione concentration was determined spectrophotometrically, using 5,5'-dithiobis-(2-nitrobenzoic acid), and expressed as μmol/ml of RBC.[18] The TAS was measured, using a commercial kit[f] adapted to an autoanalyzer.[g] The colorimetric TAS assay measures the ability of serum to inhibit hydrogen peroxide/metmyoglobin-mediated formation of the radical cation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate). Degree of inhibition is proportional to absorbance measured at 600 nm, and results are expressed as mmol of antioxidant/L of serum. Data were analyzed, using 2-way repeated measures ANOVA, with oxidant and antioxidant treatments as variables over time. The Fisher protected least significant difference test was used for posthoc group comparisons. Significance ($P < 0.05$) was determined, and results were expressed as mean ± SD, except where indicated.

Results

3% OP Experiment—Cats in this experiment had a significant decrease in food intake, from 63.2 ± 40.2 to 23.3 ± 14.2 g/kg in the first few days of antioxidant supplementation; however, food intake returned to baseline amounts (69.9 ± 16.3 g/kg) by the end of week 1, prior to initiating the OP diet. Body weight also slightly decreased in the first few days but then increased gradually for the duration of the 3% OP experiment, consistent with typical growth. Cats receiving ascorbate had higher food intake and body weight than cats in other antioxidant groups, but these differences were not significant.

Cats developed nearly 100% HB within 1 week of beginning the 3% OP diet, regardless of antioxidant supplementation (**Fig 1**). The PCV in cats receiving the 3% OP diet decreased significantly ($P < 0.001$) from 35.1 ± 3.1 to 27.9 ± 2.7% by week 2. There was a concomitant increase in total reticulocyte percentage in all cats, from 6.5 ± 3.8 to 18.0 ± 19.6% at 2.5 weeks and peaking at 47.3 ± 13.5% at 3.5 weeks ($P < 0.001$). Essentially all of the reticulocytes were punctate reticulocytes. There was no significant difference in PCV or reticulocyte percentage among control or antioxidant groups. Heinz body percentage, anemia, and reticulocytosis were more severe in cats in the 3% OP experiment, compared with cats in the 1% OP and PG experiments ($P < 0.001$).

Reduced glutathione concentration decreased significantly ($P < 0.001$) in all cats fed the 3% OP diet (**Fig 2**). Cats receiving vitamin E or ascorbate had sig-

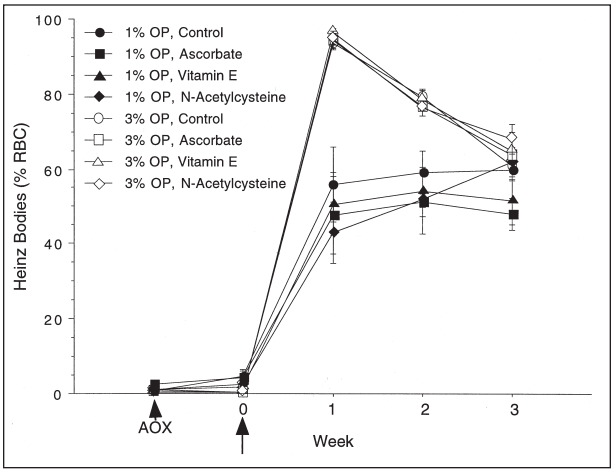

Figure 1—Heinz body formation in cats fed diets containing 3% or 1% onion powder (OP; arrow). There was no significant ($P < 0.05$) difference in Heinz body percentage in control cats, compared with cats that received antioxidants (AOX, arrowhead).

nificantly ($P = 0.003$) lower GSH concentrations than control cats. Cats receiving NAC had significantly ($P = 0.003$) higher GSH concentrations, compared with cats in all other groups. There was no significant difference in TAS concentration in cats over time or with antioxidant supplementation (data not shown).

1% OP Experiment—Mean food intake was significantly lower in the first 2 weeks in cats fed the 1% OP diet (53.0 ± 3.0 g/kg), compared with before OP was added (61.0 ± 2.8 g/kg), particularly in cats receiving NAC (46.0 ± 5.0 g/kg). After 2 weeks, food intake returned to before 1% OP diet amounts in all cats (58.2 ± 2.7 g/kg), including cats receiving NAC (61.2 ± 4.1 g/kg). Body weight increased slightly and significantly ($P < 0.001$) throughout the course of the 1% OP experiment, consistent with typical growth.

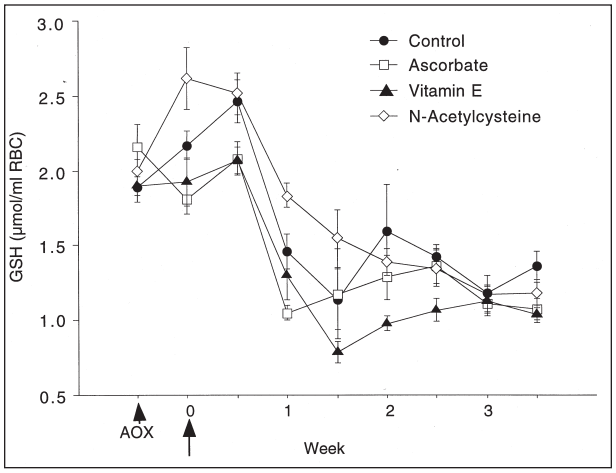

Figure 2—Reduced glutathione (GSH) concentration in cats fed diets containing 3% onion powder. Cats that received N-acetylcysteine had significantly ($P < 0.05$) higher GSH concentrations than cats that received other AOX (arrowhead) and control cats prior to and during oxidative challenge (arrow). Cats receiving vitamin E or ascorbate had significantly ($P < 0.05$) lower GSH concentrations than control cats.

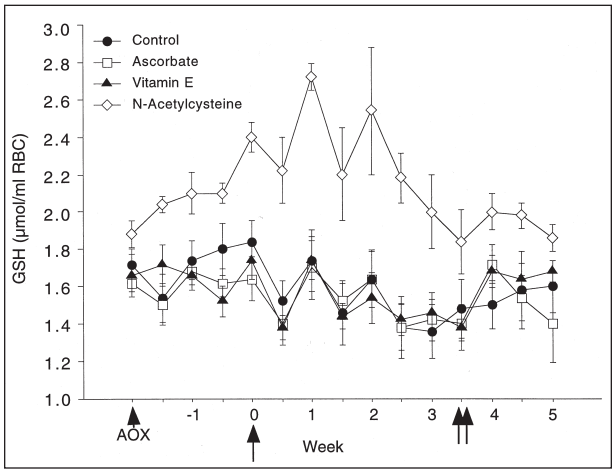

Figure 3—Reduced glutathione (GSH) concentration in cats fed diets containing 1% onion powder (arrow) was significantly ($P < 0.05$) higher in cats that received N-acetylcysteine, compared with cats that received other AOX (arrowhead) and control cats. Double arrows indicate the time at which onion powder was removed from the diet.

Cats receiving antioxidants, particularly NAC, developed slightly fewer Heinz bodies, compared with control cats; however, differences were not significant (Fig 1). Disappearance of Heinz bodies in cats when OP was removed from the diet was unaffected by antioxidant supplementation. All cats developed a mild decrease in PCV (38.4 ± 3.5 to 35.1 ± 4.2%) and reticulocytosis (6.0 ± 4.4 to 23.2 ± 15.4%) while ingesting the 1% OP diet, with no significant differences among control cats and cats receiving antioxidants. Cats receiving NAC had significantly higher GSH concentrations prior to, during, and after the 1% OP diet ($P = 0.009$; **Fig 3**). There was no significant difference in TAS concentration in cats over time or with antioxidant supplementation (data not shown).

PG Experiment—Cats receiving antioxidants drank significantly ($P = 0.014$) less water, compared with control cats, when PG was added to the water (**Fig 4**). Body weight increased slightly but significantly in all cats for the duration of the experiment, consistent with typical growth. Consistent with decreased PG intake, cats receiving antioxidants had significantly lower HB percentages ($P < 0.001$), compared with control cats. The difference in water intake and HB percentage was greatest in the first 2.5 weeks of PG administration. When

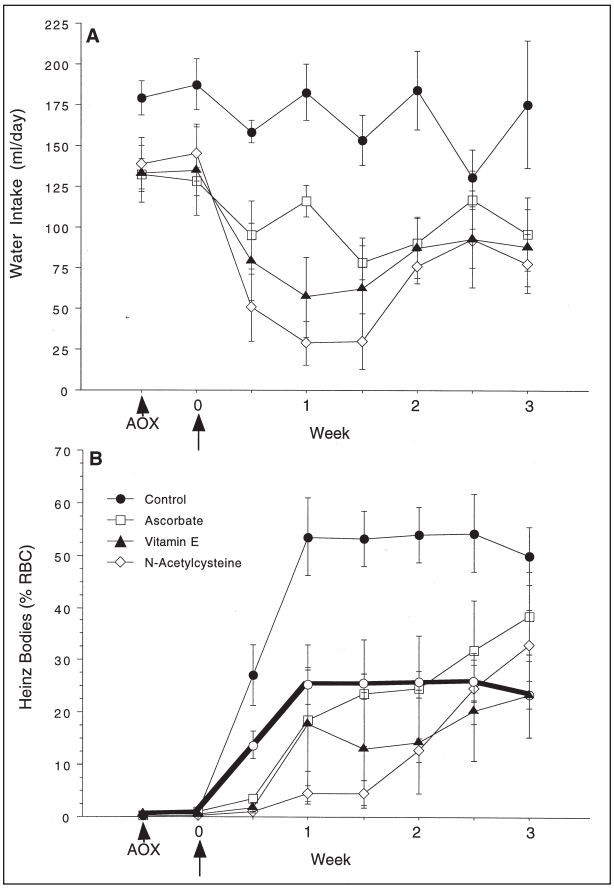

Figure 4—Water intake (A) and Heinz body percentage (B) in cats receiving propylene glycol (arrow) in their drinking water. The significant difference in Heinz body percentage between control cats and cats that received AOX (arrowhead) was likely all or mostly attributable to water intake. The bold line indicates control data normalized on the basis of water intake.

water intake of control cats was normalized to that of cats that received antioxidants (on a group-by-group basis), HB percentage was significantly lower only in cats that received NAC, compared with control cats ($P = 0.034$). There was wide variation in HB percentage in cats receiving ascorbate or vitamin E, probably because of individual variation in water intake.

The PCV decreased significantly ($P < 0.001$) from 38.0 ± 3.7 to 34.8 ± 3.6% in all cats receiving PG, with no difference among control cats and cats that received antioxidants. Reticulocyte percentage increased significantly ($P < 0.002$) only in control cats, from 2.2 ± 2.1 to 21.5 ± 7.4%. In cats receiving PG, there was no significant difference or change over time in GSH concentrations in control cats or cats receiving antioxidants (data not shown). The TAS decreased slightly in control cats on day 4 of PG ingestion; however, the change was not significant. The TAS increased significantly in cats that received PG, particularly in control cats, between 1 and 3 weeks of PG ingestion.

Discussion

On the basis of findings in our study, the oral individual administration of vitamin E, ascorbate, or NAC for 2 weeks prior to oxidant challenge had no or minimal effect on the rate of formation, peak percentage, or disappearance of HB induced by ingestion of OP or PG in cats. The degree of regenerative anemia also was unaffected by antioxidant supplementation; rather, it was closely associated with HB percentage. The lack of antioxidant effect was especially apparent with strong oxidant challenge (cats receiving the 3% OP diet), compared with milder oxidant challenge (cats receiving the 1% OP diet or PG in their drinking water), in which small antioxidant effects were detected. Antioxidant supplementation indirectly decreased HB percentage in cats receiving PG because of decreased water intake. The reasons for water avoidance were not clear but may have been associated with altered taste or smell. Cats that received NAC had a significantly lower HB percentage when control data were normalized for water intake; however, interpretation of these data is complicated by the variability in water intake.

Depletion of GSH was likely secondary to marked HB formation in cats fed the 3% OP diet. Onions are thought to cause oxidative damage through thiosulfate compounds that form disulfide bonds with hemoglobin or GSH,[19] such that the OP also may have had a direct negative effect on GSH concentration. No change in GSH concentration was detected when similar concentrations of OP were fed to cats in a previous study; however, mean peak HB percentage was approximately half of those achieved in our study (attributable to differences in cat age).[11]

N-Acetylcysteine had a slight but significant sparing effect on GSH concentrations in cats in the 3% OP experiment and substantially increased GSH concentrations in cats fed the 1% OP diet. One would predict that an increased concentration of GSH, detected prior to oxidative challenge, would protect hemoglobin from disulfide bond formation, similar to the manner in which NAC is effective against acetaminophen toxicosis. Despite GSH concentrations up to 3 times greater in cats that received NAC, however, there was no significant inhibition of HB formation or anemia. N-Acetylcysteine previously has been shown to have a sparing effect on GSH and methemoglobin percentage; however, to our knowledge, an effect against HB has not been evaluated.[20,21] It is possible that increased GSH concentrations are protective only when the oxidizing agent acts specifically by depleting GSH or when it causes primarily methemoglobinemia rather than HB formation. We did not measure methemoglobin in our study, because neither OP nor PG have been associated with methemoglobinemia.[11,12]

Although ascorbic acid has been used and recommended for treatment of acetaminophen toxicosis in cats and red maple leaf toxicosis in horses (also a HB anemia), controlled studies are lacking, and there is no evidence to indicate that ascorbate is efficacious in diminishing oxidative damage in cats.[13,14] In addition, feline (and equine) RBC are less able to incorporate ascorbate intracellularly, compared with human RBC, possibly because cat RBC have lower dehydroascorbate-reducing activity.[22] Because of this, ascorbate does not substantially stimulate the pentose phosphate shunt in feline RBC. Decreased uptake of ascorbate may explain the lack of effect of ascorbate against hemoglobin oxidation in cats. Indeed, cats receiving ascorbate had lower GSH concentrations in cats fed the 3% OP diet, suggesting a possible prooxidant effect, which can develop with high concentrations of ascorbate.[4,22]

Because vitamin E acts primarily at the level of the RBC membrane, and cats with HB do not typically have an increase in lipid peroxidation products, the lack of effect of vitamin E against HB formation was not surprising.[7,23] The reason for lower GSH concentrations in cats that received vitamin E in the 3% OP experiment was not determined. Neither vitamin E nor ascorbate concentrations were measured in the serum of these cats, and the length of time of supplementation may have been too short to result in an increase in serum or tissue concentrations.

Several methods have been developed to assess the antioxidant capacity of serum or plasma as an indicator of systemic oxidative stress or oxidative status.[24,25] We selected an automated method that, if it proved useful in differentiating cats that received antioxidants from control cats, may have been useful in the clinical laboratory. The method we used measured the inhibitory effect of serum or plasma on the reaction of metmyoglobin and peroxidase. Results of TAS tests in cats were similar to values reported for healthy human beings (1.26–1.41 mmol of antioxidant/L of serum).[24] According to the manufacturer, antioxidants that contribute to inhibition of the reaction include vitamin E, ascorbate, superoxide dismutase, glutathione peroxidase, transferrin, and ferritin. In a recent study, however, vitamin E and ascorbate were found to account for only 1.75% to 3.08% of TAS, whereas albumin contributed 28%, and unknown or other substances contributed 47%.[24] It is unlikely the serum concentration of vitamin E or ascorbate increased sufficiently to impact TAS results. In cats that received NAC, we would, however, have expected TAS to increase with an increase in GSH concentration, because there is a good

correlation between TAS and GSH concentrations. It is possible the oxidative effect of the OP offset the increased GSH concentration observed in cats ingesting the 1% OP diet. Although TAS decreased slightly in control cats receiving PG, the difference was not significant, probably because of wide variability in TAS values prior to starting antioxidant treatment. The gradual increase in TAS in the PG control cats may have indicated an increased in synthesis of endogenous antioxidants in compensation for the oxidative stress. The TAS assay, in summary, was not useful in monitoring plasma concentrations of these particular antioxidants but may have detected a mild increase in endogenous antioxidants induced by oxidative stress.

In conclusion, short-term oral supplementation with NAC, ascorbate, or vitamin E did not substantially prevent or diminish HB formation in cats, although some effect on GSH concentration was observed, particularly in cats that received NAC. Future investigations into the effects of long-term antioxidant supplementation may be useful, particularly against milder forms of oxidative stress, as may develop in viral, neoplastic, metabolic, and inflammatory diseases. In these disorders, biochemical markers of DNA, lipid, and protein damage or changes in antioxidant enzyme activity may elucidate subtle beneficial effects of antioxidants. Heinz body formation may be too acute or severe of an oxidative endpoint to expect a response to short-term antioxidant supplementation.

CNM UR-formula, Ralston Purina Co, St Louis, Mo.
[b]Gerber Products Co, Freemont, Mich.
[c]Fisher Scientific, Fair Lawn, NJ.
[d]Sigma Chemical Co, St Louis, Mo.
[e]Dayton-Hudson, Minneapolis, Minn.
[f]Total Antioxidant Status kit NX2332, Randox Laboratories, San Francisco, Calif.
[g]Hitachi 912 autoanalyzer, Boehringer-Mannheim, Indianapolis, Ind.

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