All of the cancer patients were being treated at the time of sampling, but the exact clinical status of each was not available. The low activity of tartrate-resistant acid phosphatase in some patients may have been due to a good response to treatment. We will consider the tartrate-resistant acid phosphatase activity of individual patients at different stages of treatment in future studies.

Alkaline phosphatase is an enzyme marker of osteoblastic activity (11, 12); most of the cancer patients in this study, however, did not have increased serum activities of alkaline phosphatase. The few instances of extremely high activities of alkaline phosphatase, but normal tartrate-resistant acid phosphatase, could be due to damage of tissues other than bone.

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References


Some Factors Affecting Determination of Carotenoids in Serum
Micheline M. Mathews-Roth and Meir J. Stampfer

Because of increasing interest in carotenoid pigments, we conducted a study of the methods of determining carotenoids in serum. We found 1 mol of KOH per liter of absolute methanol to be the most effective saponifying solution. The absorbance of beta-carotene in petroleum ether, the extraction solvent, is proportional to dilution up to an absorbance of 0.85 at 450 nm. Beta-carotene in petroleum ether solution is not impracticably sensitive to ambient light at room temperature. However, if vitamin A is also to be measured in these serum specimens or petroleum ether extracts, exposure to light should be minimized. We found that serum may be shipped either in cold packs or at ambient temperature (tested up to five days) without significant change in carotenoid concentration. Serum samples for carotenoid determination are best stored at −70 °C; samples stored at −20 °C deteriorate substantially over several months.

Additional Keyphrases: sample handling, variation, source of beta-carotene is prescribed to ameliorate the photosensitivity associated with erythropoietic protoporphyria and other light-sensitive diseases (1). Recently, much interest has been generated by reports that beta-carotene or vitamin A may offer protection against the development of some forms of cancer (2). As a result, more laboratories probably will be measuring carotenoids in serum. We therefore closely examined the traditional method of determining serum carotenoids, as well as the effect of different specimen-handling procedures on carotenoid concentrations.

Materials and Methods
Apparatus. We used a Cary Model 14 recording spectrophotometer (Varian Instruments, Palo Alto, CA) in determining absorption spectra of carotenoid-containing solutions. Specimens were shaken during the extraction procedure on a Kraft Model 5-500 "Shaker in the Round" fitted with a Model RD-20 head and three Model SR 815 centrifuge tube racks, which can be turned while attached to the shaker so that the tubes can be shaken with their length parallel to the table top, thus affording efficient extraction (Kraft Instrument Co., Mineola, NY). In this apparatus, 24 tubes can be shaken at one time.

Reagents. Two reagents are needed for the extraction of carotenoids from serum. For saponification, a 1 mol/L solution of KOH in absolute methanol is used (dissolve 56 g of KOH in ACS Certified reagent-grade methanol and dilute to 1 L), and for extraction of the saponified carotenoids, petroleum ether (boiling range 35–60 °C, ACS Certified reagent).

Carotenoid extraction procedure. We have used the method described here since 1969 in our studies of carotenoid treatment of photosensitivity in various diseases. In the present study, however, blood was obtained from normal

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volunteers, not patients with photosensitivity.

To measure carotenoids in serum, place 0.1 to 1.0 mL of serum or plasma in a graduated, stoppered centrifuge tube. Add 1 mL of the methanolic KOH reagent, shake the tube, and place in a 60 °C water bath for 30 min. Then remove the tube from the bath and allow it to cool for 10 min. Add 2 mL of petroleum ether, shake the tube by hand to equilibrate the phases, then shake it in the Kraft shaker for 5 min. Allow the layers to settle for 5 min, and then transfer the petroleum ether layer to a fresh 5-mL graduated, stoppered centrifuge tube. Repeat the extraction procedure, including shaking, and combine the second 2-mL portion of petroleum ether with the first; mix. Take care not to include any of the methanol/KOH layer. If necessary, use centrifugation to separate the phases and (or) to clarify the petroleum ether layer. Record the absorbance of the combined extracts from 300 to 600 nm.

As noted later, we sometimes varied the reagents used, to study how this would affect the results. In all cases, we measured the absorbance of the extracts in the Cary spectrophotometer over the stated wavelength range.

Results

Extraction procedure. With the standardized procedure described above, including use of a sensitive spectrophotometer, and scanning the spectrum rather than reading absorbance at a single wavelength, the results of multiple analyses of a given blood sample fall within 7% of each other; about 20% of the time identical values were obtained. Also, the absorbance of crystalline beta-carotene in petroleum ether and of carotenoids contained in the petroleum ether extract of blood is proportional to dilution up to an absorbance of 0.85.

Effect of alkali concentration on carotenoid determination.

In surveying the literature on carotenoid determination, we noticed that various workers used different concentrations of alkali in the saponifying solution. Most used a final KOH concentration of 0.1 mol/L; others, such as Oliver (3) and we, used 1 mol/L KOH. Because the more concentrated solution is required for bacterial extractions, it was more convenient for us to use it for all work in the laboratory. Oliver found that the lower concentrations of alkali did not totally extract the serum carotenoids (personal communication). When we tested this by extracting aliquots of the same batch of serum with 0.1 and 1.0 mol/L solutions of KOH in absolute methanol, we confirmed that the lower concentration of alkali indeed does not extract all the carotenoids in 1 mL of serum (the volume most often used). For volumes of serum greater than 0.5 mL, the lower concentration of alkali will extract only about 56% of the amount of carotenoids extracted by the 1 mol/L KOH solution. Thus, we recommend that a final KOH concentration of 1 mol/L in absolute methanol be made standard.

Effect of varying the extraction and saponification reagents. Some workers (4, 5) use absolute ethanol to extract serum for carotenoids and omit the saponification step. We found that extracting serum with absolute ethanol or extraction and saponification with 1 mol/L KOH in absolute ethanol removes the same amount of pigment from serum as does extraction and saponification with 1 mol/L KOH in absolute methanol. However, extraction of serum with absolute methanol alone, omitting saponification, extracts only about 70% of the pigments. Saponification also is usually needed for extracting carotenoids from bacteria or plant or animal (including human) tissues (6, and Mathews-Roth, unpublished observations). We also found that hexane is a good solvent for extracting beta-carotene after saponification.

Effect of the volume of serum extracted on the reproducibility of the assay. We extracted 0.1-, 0.25-, 0.5-, 0.75-, and 1-mL portions of serum from the same specimen from each of two subjects, using the optimized extraction method. Duplicate determinations of each portion agreed within 4%.

Absorbance was linearly related to dilution under the range studied, which indicates that with careful pipetting small sample volumes will give accurate results.

Stability of carotenoids on handling. We studied the effects of systematically altering sample exposure to light and temperature. First, crystalline beta-carotene dissolved in petroleum ether in a graduated centrifuge tube was exposed to ambient light (no direct sunlight) and temperature (22 °C). The absorbance of the solution did not decrease during the first 10 h of light-exposure and decreased by only about 10% after a week of these conditions (during this time the volume of the petroleum ether decreased by <1%). The absorbance of a similar solution kept at −20 °C in the dark did not change during this period.

We then studied the effect of light and temperature on the carotenoid content of plasma. In one experiment, plasma from each of three different individuals was divided into two portions, one of which was protected from light from the moment the blood was drawn, the other being exposed to ambient light in the laboratory. An aliquot of each portion was immediately analyzed for carotenoid content, another was left at room temperature for 3 h, another in ice for 3 h, and a fourth in a freezer at −20 °C for 24 h before carotenoid was determined. We found that these various handling conditions affect the results of carotenoid analysis minimally (at most, less than 5%).

To determine the effect of long-term freezer storage, we re-assayed the carotenoids in specimens of plasma from carotenemic and non-carotenemic individuals, obtained for a study on beta-carotene intake and sunburn prevention. The subjects in this study were healthy men, ages 21 to 49 years. The plasma samples had been stored in a freezer at −20 °C for approximately 10 years. We found that most of the carotenoids were lost—especially in the carotenemic subjects, where the loss was 97%. Storage for even six months at −20 °C may decrease carotenoid content by 15%.

To determine the effects of storing plasma at colder temperatures on carotenoid content, we re-assayed carotenoids in plasma that had been stored at −70 °C for a year. Carotenoid values were essentially unchanged, there being a small, statistically insignificant increase in the mean value.

In clinical studies, samples for carotenoid determination may be collected at distant locations and shipped to a central laboratory for analysis, so the effect of various shipping conditions on the carotenoids in plasma needs to be known. We conducted the following pilot study before collecting baseline samples from some 16 000 physicians enrolled in our large clinical trial of the effects of beta-carotene on cancer incidence (7). Blood obtained from each of 10 normal volunteers was divided into three portions. One portion was frozen immediately and kept protected from light. The second was put in a "cold pack" ("Ice Cold" brand; GAM Industries, Stoughton, MA) for 24 h, and then frozen. "Cold packs" at first keep the specimen at −5 °C, but by 5 h the temperature has risen to 16 °C, and reaches room temperature by 22 h. We studied the effect of 24-h storage as the typical time it would take for specimen delivery by air express. The third aliquot was kept at room temperature in the dark for five days, considered the maximum time ordinarily required for delivery of specimens by first-class U.S. mail or a parcel delivery service. Values for carotenoids in the specimens were virtually identical by each of the
three sample-handling methods. The logistics of collecting, handling, and storing more than 16 000 blood samples will be described elsewhere.

Discussion

Our studies indicate that, for the determination of carotenoids in serum, the traditional method of extraction with an organic solvent and spectrophotometric quantification is adequately precise and sensitive for routine clinical use. However, for complete extraction of carotenoids, we recommend use of a 1 mol/L solution of KOH in methanol for saponification.

This method works well with sera from normal individuals as well as with sera from patients with erythropoietic protoporphyria and other diseases of photosensitivity. Carotenoids in sera from patients with diabetes and hypothyroidism have been determined by this method with no reported difficulty, and there is no reason to suspect that it would not work well on sera from patients with other diseases.

It is important that the absorption spectrum of the petroleum ether extract be measured over the range of 300–600 nm, rather than at a single wavelength (e.g., 450 nm), especially for samples containing small amounts of carotenoids. Occasionally, factors other than carotenoids in the petroleum ether extract cause a slight turbidity that is not removable by centrifugation; the possibility of obtaining a falsely high reading can be avoided only by examining a complete spectrum.

If one uses the extraction method given here, accurately measures the volume of specimen to be extracted, and determines the absorption spectrum of the petroleum ether extract over 300–600 nm, the reproducibility (CV) of the method is within 10% and the carotenoid content of even small volumes of serum (0.1 mL) can be determined accurately.

Careful exclusion of light during the extraction process evidently is not needed. Neither protein-bound carotenoids nor pigments dissolved in petroleum ether are as sensitive to light as was once thought. Moreover, specimens can be validly shipped either under refrigeration or at room temperature. Thus, precautions for keeping specimens cold or dark will mainly depend on what other components (e.g., vitamin A) are also to be measured in a given specimen.

We do not yet know how long carotenoids are stable at −70 °C, but specimens stored at −20 °C lose significant amounts of carotenoids within weeks, and certainly by 10 years have deteriorated almost completely.

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