plastic surface area around the wells. However, the outside wells (those of rows A and H, and columns 1 and 12) have a larger air-to-plastic surface area. One may speculate that more of the light scattered toward the walls would be lost from the outer wells because of the greater air-to-plastic surface. This cannot happen with black-wall plates.

Comments

Previously, we recommended suspending the bacteria by repeatedly aspirating ~150 μl using a pipette. We have found that this step is not necessary, presumably because the light path is up through the well and, because the bacteria have settled to the bottom, essentially equal light scattering occurs whether or not the bacteria have been resuspended.

We recommend that black-wall plates be used for the microbiological assay of folates using *L. casei* and this would, presumably, also apply to assays using *Streptococcus faecium* and *Pediococcus cerevisiae*, and to all turbidimetric assays.

Acknowledgments

The technical assistance of Rosalind S. Holloway is gratefully acknowledged. This work was supported by the Department of Veterans Affairs and by NIH Grant DK32189.

[5] Microbiological Assay for Serum, Plasma, and Red Cell Folate Using Cryopreserved, Microtiter Plate Method

By ANNE M. MOLLOY and JOHN M. SCOTT

The routine analysis of serum and erythrocyte folate has been the subject of numerous reviews, comments, and controversies. The microbiological methods used originally were difficult to set up, difficult to maintain, and slow in obtaining results. Thus many laboratories changed over to newly developed radioassays when they became available. However, the microbiological method remains for most investigators the "gold standard" and the method of choice. What many investigators do not realize is that it has now been refined and automated to such an extent that it is easy to perform, reliable to maintain, and considerably less costly than the radioassay method, particularly where large numbers of samples are involved. Three important changes in the assay technology have contributed to this. First,

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the development of antibiotic-resistant strains of organisms allows the use of disposable laboratory ware and removes the need for autoclaving. Second, the ability to cryopreserve the inoculum in multiple individual vials results in standardized growth curves that are reproducible for hundreds of assays. Last, automated microtiter plate technology and its associated computerized analysis packages, which were developed for enzyme-linked immunosorbent assays (ELISAs), are ideally suited to measuring the turbidity of microbiological growth.

The microbiological assay performed in our laboratory is adapted from the method published by O'Broin and Kelleher.[1] We use microtiter plates and a chloramphenicol-resistant strain of *Lactobacillus casei* maintained by cryopreservation. This chapter describes the method in sufficient detail that it can be set up *ab initio* by virtually any laboratory. It thus emulates a similar paper by Scott *et al.*,[2] which described in detail the traditional microbiological assay.

Materials

Laboratory Equipment

The assay requires any cabinet-type 37° incubator and a microtiter plate reader with optional attachment to a PC-controlled data-handling package. We use a Labsystems (Helsinki, Finland) Multiscan Plus plate reader attached to a 486 computer with ELISA$^+$ software (Meddata, Inc., New York, NY). For storage of the cryopreserved inoculum, access to a −80° refrigerator or a liquid nitrogen storage unit is essential. Other general laboratory equipment includes refrigerator and freezer facilities, drying oven, bench centrifuge, autoclave, Bunsen burner or hot plate with magnetic stirrer, and test tube racks suitable for the disposable tubes described below and for whatever blood collection tubes are in use. The following pipettes should be reserved exclusively for the assay; one eight-arm multichannel pipette with 50- to 200-μl dispensing range (such as the Finnpipette digital MCP; Labsystems), one adjustable repetitive sampling pipette with attachments to give an overall dispensing range from 10 to 5000 μl (such as the Socorex stepper; Socorex, Lausanne, Switzerland), a set of four adjustable pipettes to give dispensing ranges of 5–50 μl, 50–200 μl, 200–1000 μl, and 1–5 ml (e.g., Socorex). The manufacturers supply disposable pipette tips that are suitable for the pipettes described. A plate-sealing roller (Flow Laboratories, Bioggio, Switzerland) and two metal spatulas are also re-

[1] S. O'Broin and B. Kelleher, *J. Clin. Pathol.* **45,** 344 (1992).
[2] J. M. Scott, V. Ghanta, and V. Herbert, *Am. J. Med. Technol.* **40,** 125 (1974).

tained for the assay. The spatulas are soaked overnight in general laboratory detergent and rinsed thoroughly before each assay.

Glass and Disposable Ware

Two 500-ml conical flasks, one 500-ml beaker (of wide enough diameter to insert the stepper pipette), one 500-ml measuring cylinder, two 100-ml volumetric flasks, and one 1-liter volumetric flask should be reserved for the assay and routinely soaked overnight in a bath of cleaning mixture to remove any residual folate that may adsorb to glassware and cause contamination in the next assay. The cleaning mixture is prepared by adding 2.5 liters of concentrated sulfuric acid to 2.5 liters of water, then adding approximately 100 g of potassium dichromate. This solution is reusable for up to 6 months and is discarded by diluting into at least 20 vol of water before washing down a foul water drain. The soaked glassware should be rinsed thoroughly and oven dried before use. Some disposable glass 20-ml broth tubes are required for autoclaving glycerol solutions (see Preparation of Cryopreserved Inoculum, below). All other laboratory ware consists of disposable polypropylene or polystyrene and is supplied by Sarstedt, Ltd. (Essen, Germany). This includes 5-ml tubes into which serum or plasma can be dispensed after centrifugation, 4-ml tubes for the initial dilution of assay samples, 1.5-ml minitubes for storing whole-blood lysates and quality control aliquots, 1.5-ml cryotubes for *L. casei* aliquots, polystyrene storage boxes, petri dishes, 30-ml sterile universal tubes (107 × 25 mm) and 96-well (flat) microtiter plates. Adhesive microtiter plate sealers are obtained from Titertek (ICN Biomedicals, Costa Mesa, CA).

Chemicals

Ascorbic acid, sodium ascorbate, potassium dichromate, manganese sulfate, sodium hydroxide, glycerol, and Tween 80 are purchased from Sigma Chemical Co. (Poole, UK). Chloramphenicol is obtained from Parke Davis & Co. (Pontypool, Wales, UK). Concentrated sulfuric acid, folic acid standard, and vitamin folic acid test broth are supplied by Merck (Darmstadt, Germany). We monitor our stocks of broth and verify that new batches give backgrounds and growth curves similar to those of our current medium. We then buy in bulk sufficient broth to last for up to 2 years. All routine chemicals are purchased or subaliquoted and stored in multiple small amounts (25 to 100 g) rather than in large consignments such as 500 g or 1 kg. Thus, if contamination of chemicals, media, etc., with folic acid arises, all in-use chemicals are discarded and new bottles are opened. (We never try to eliminate the source of contamination, we simply start again with unopened reagents.)

Water. The quality of water used in the assay is important because it is a major source of high blanks and poor growth curves. All water must therefore be ultrapure [e.g., Millipore (Bedford, MA) Milli-RO Plus reverse osmosis] or double distilled. If the purification standard is not of this quality, purchase purified water from a reputable supplier.

Microorganism

The chloramphenicol-resistant strain of *L. casei* (NCIB 10463) can be obtained initially as a freeze-dried preparation from the National Collections of Industrial and Marine Bacteria, Ltd. (NCIB, Torry Research Station, Aberdeen, Scotland, UK). After purchases of a stock culture and cryopreserved subcultures have been made, these may be used indefinitely in the assay. (We will supply, subject only to the cost of preparation and shipping, samples of the cryopreserved organism for researchers who have difficulty in obtaining or setting up the method from the freeze-dried organism.)

Methods

Separation of Blood

The use of serum is usually recommended for folate analysis; however, in research studies where one is limited to a single blood sample, the blood may be collected in K_3EDTA Vacutainers (Becton Dickinson, Mountain View, CA), mixed carefully, and sampled for erythrocyte folate analysis as described below. The sample is then centrifuged (at 1500 *g* for 15 min, room temperature) to separate the plasma. Plasma and serum samples may be stored at $-20°$ until assayed. For long-term storage 5 mg of solid ascorbic acid per ml of sample can be added to preserve folates, although this is probably not necessary if samples are not repeatedly thawed and refrozen. We analyze K_3EDTA plasma folate as for serum folate, except for the addition of manganese sulfate to the culture medium as described in detail under Assay Procedure.

Preparation of Whole-Blood Sample for Erythrocyte Folate Analysis

Whole blood is mixed carefully, preferably with a blood cell suspension mixer, particularly if tubes are full. Mixing can be by hand but must be thorough. In a 1.5-ml minitube, 100 μl of whole blood is added to 900 μl of 1% ascorbic acid solution, freshly prepared by adding 1 g of ascorbic acid (not sodium ascorbate) to 100 ml of distilled water. No pH adjustment is made. It is essential that prior to assay the mixture be left at room temperature for 30 min to allow serum conjugase to convert folate polyglu-

tamates released from the lysed erythrocytes to the assayable monoglutamate forms. These ascorbic acid lysates may then be stored at −20° until assayed.

The packed cell volume (PCV) is determined from the same whole-blood sample either on a Coulter (Hialeah, FL) counter or by taking a hematocrit, using a capillary tube and hematocrit reader. If it is not possible to determine the PCV in this way, it can be estimated by assaying the hemoglobin concentration in the ascorbic acid lysates and converting the values to PCV values.[3]

Preparation of Folic Acid Standard

All operations using folic acid should be carried out well away from the microbiological workstation. Folic acid (20 mg) is weighed on a balance accurate to at least four decimal places and dissolved in approximately 4 ml of 0.1 *M* NaOH in a sterile 30-ml disposable universal tube. The solution is transferred and the liquid residue washed into a 1-liter volumetric flask and made up to the mark with water. This stock solution is divided into 20-ml aliquots in 30-ml disposable universal tubes and stored at 4°. New stock solutions are prepared at regular intervals (every 8–12 weeks or if the standard curve shows a trend toward flattening at higher concentrations). A standard curve is always run on new standards before the old standard is discarded. The working standard is prepared fresh for each assay from this stock standard (see under Assay Procedure).

Preparation of Medium

It is important that weigh boats or weighing tissues, spatulas, and balance area are free from any possibility of folate contamination. To make 100 ml of medium, which is enough for four plates, we use 5.7 g of folic acid broth (Merck). Although this is less than the quantity recommended by the manufacturers, O'Broin and Kelleher[1] have found this amount to be optimal for the assay medium using the cryopreserved chloramphenicol-resistant *L. casei.* The 5.7 g of broth powder is weighed and transferred to a 500-ml conical flask. Using a measuring cylinder, 100 ml of water is added. Chloramphenicol (3 mg) is then added and the mixture is heated. Before boiling but when the solution is hot, 30 μl of Tween 80 is added. It is then brought to the boil and boiling is continued until all of the broth powder has fully dissolved. The broth is then cooled to room temperature and a petri dish cover is placed over the flask. (The petri dish base is retained for aliquoting medium with the multichannel pipette in the assay.) Just

[3] G. O'Connor, A. M. Molloy, L. Daly, and J. M. Scott, *J. Clin. Pathol.* **47,** 78 (1994).

before use, when the assay is set up, 75 mg of ascorbic acid (not sodium ascorbate) is dissolved in the medium and, finally, 200 μl of thawed *L. casei* cryopreserved suspension is added. The medium is mixed thoroughly. If EDTA plasma is to be assayed instead of serum it is essential also to add a solution of manganese sulfate (15 mg/ml; 89 m*M*) to the medium (1 ml/100 ml of medium) to chelate excess EDTA, otherwise residual EDTA interacts with divalent cations in the medium and inhibits growth.[4] We have not found with Merck medium such well-defined inhibition as Tamura *et al.*[4] described for Difco (Detroit, MI) medium; however, leaving out the manganese sulfate results in poor replication between 50- and 100-μl aliquots of analyticals.

Preparation of Cryopreserved Inoculum

Cryopreserved *L. casei* is stored at −80° as a glycerol suspension. The preparation of this suspension is carried out essentially as reported by Wilson and Horne[5] but with modifications to optimize the growth response by freezing cells in the log phase. This modification was developed by Kelleher *et al.*[6] for cryopreservation of *Lactobacillus leichmannii* and is described in detail by Kelleher and O'Broin[7] for use in a microtiter plate assay for vitamin B_{12}. In this instance, medium is prepared using 7.6 g of folic acid broth and 40 mg of chloramphenicol in 200 ml of ultrapure or double-distilled water. (Note that 7.6 g dissolved in 200 ml is half-strength medium according to the recommendation of the manufacturer. This follows the published method for cryopreserving *L. casei.*[5]). As before, the mixture is heated, then 80 μl of Tween 80 is added and the mixture is boiled. The solution is cooled, then 50 mg of ascorbic acid is added. Finally, 50 ng (113 pmol) of folic acid standard is added to the medium. [This is done by adding 50 μl of stock 20-mg/liter (0.045 m*M*) folic acid standard to 450 μl of 0.5% (w/v) sodium ascorbate and aliquoting 25 μl of the diluted standard to the medium.] The medium is mixed well and 20-ml aliquots are dispensed into sterile 30-ml universal tubes and stored at −20° until required. Glycerol (80%, v/v) is prepared by mixing 32 ml of glycerol with 8 ml of water, dividing into two glass 20-ml broth tubes, autoclaving for 10 min at 121°, and cooling.

To start the culture, one of the containers of stored medium is brought to room temperature and 1 ml of medium is added to a lyophilized vial of *L. casei* obtained from the NCIB. The contents are transferred back into

[4] T. Tamura, L. E. Freeberg, and P. E. Cornwell, *Clin. Chem.* **36,** 1993 (1990).
[5] S. D. Wilson and D. W. Horne, *Clin. Chem.* **28,** 1198 (1982).
[6] B. P. Kelleher, J. M. Scott, and S. D. O'Broin, *Med. Lab. Sci.* **47,** 90 (1990).
[7] B. P. Kelleher and S. D. O'Broin, *J. Clin. Pathol.* **44,** 592 (1991).

the tube of medium. Alternatively, if the assay has already been set up and cryopreserved organism is available, 10 μl of cryopreserved inoculum is dispensed into a 20-ml container brought to room temperature. In either event, the culture is incubated at 37° for 42 hr. It is not necessary to shake the culture during incubation. A 10-μl aliquot is then taken into a second tube of medium thawed to room temperature and cultured for 24 hr. This 24-hr procedure is repeated a further two times, at which stage the cells have achieved a rapid growth rate. At this point, 1 ml of culture is transferred to each of a further two thawed 20-ml tubes of medium and incubated at 37°. After 7 hr the contents of the two tubes (i.e., 40 ml) are transferred to a beaker along with 40 ml of the autoclaved glycerol solution. The suspension is mixed thoroughly, aliquoted (500 μl) into 1.5-ml cryotubes, and frozen to −80° in a polystyrene box. The procedure gives enough inoculum for 160 assays with 8–10 plates per assay. This cryopreserving technique ensures that each assay is carried out with inoculum containing the same number of cells, all at the same stage of growth, thus maintaining day-to-day growth curve reproducibility.

Preparation of Quality Control Samples

Blood is collected from healthy volunteers and pooled to give approximately 200 ml of serum (S), EDTA plasma (P), or whole-blood ascorbic acid lysate (R) depending on whichever of these is to be analyzed. [The whole-blood ascorbic acid lysate fraction is obtained by diluting 20 ml of whole blood to 200 ml with freshly prepared 1% (w/v) ascorbic acid as described in the previous section for preparation of erythrocyte folate.] The pooled samples (designated S1, P1, or R1) are then centrifuged, filtered through Whatman (Clifton, NJ) No. 1 paper, and six aliquots of each are analyzed on three separate days to obtain a baseline mean, standard deviation, and coefficient of variation. These samples usually give folate concentrations well into the normal range. Each of these three pools is then divided in three to give two further pools (e.g., the serum sample is divided into the original S1, plus S2, plus S3; similarly for the plasma and whole-blood lysate samples). S2 and S3, P2 and P3, and R2 and R3 are then appropriately diluted with 0.9% (w/v) sodium chloride to give calculated borderline and deficient levels. Six aliquots from each of these new pools are analyzed on three separate days to obtain means, standard deviations, and coefficients of variation. The three pools for each blood fraction (S1, S2, S3; P1, P2, P3; R1, R2, R3) are then dispensed into 1.5-ml minitubes in 150-μl aliquots, which is sufficient for one duplicate assay, and stored in boxes at −20°. For quality control, depending on the blood fraction to be analyzed, an aliquot from each of the three pools of the relevant blood fraction is run in duplicate with every assay.

Assay Procedure

In the 500-ml beaker a solution of 0.5% (w/v) sodium ascorbate is prepared by dissolving 2.5 g of sodium ascorbate (not ascorbic acid) in 500 ml of water. This solution is used for preparing the working standard and for all assay dilutions. The working standard is prepared in the following way: A universal tube containing stock standard solution is brought to room temperature and a 50-μl aliquot is taken and diluted to 100 ml with 0.5% (w/v) sodium ascorbate in a volumetric flask. This solution is mixed thoroughly, then 5 ml is taken and diluted to 100 ml with 0.5% (w/v) sodium ascorbate in a second volumetric flask to give a final working standard concentration of 500 ng/liter (1.13 nM). Duplicate 50-μl aliquots of serum or plasma or duplicate 25-μl aliquots of whole-blood lysate are pipetted into labeled 4-ml polypropylene tubes. Using the adjustable repetitive sampling pipette the aliquots are diluted to a total of 1 ml with 0.5% (w/v) sodium ascorbate. (Our experience with studies of normal populations is that this level of dilution generally gives a growth reading toward the higher, flatter part of the curve. For studies on nondeficient populations we routinely dilute our samples by a further factor of two; i.e., 50 μl of serum or 25 μl of whole-blood lysate is diluted to 2 ml, to ensure that the sample gives readings on the linear part of the curve.) In addition to unknowns, duplicate samples of the three appropriate quality control pools (e.g., S1, S2, S3) are included with each assay depending on whether serum, plasma, or erythrocyte folates are being analyzed.

For each diluted sample, duplicate aliquots of 100 and 50 μl are transferred to four separate wells of a 96-well microtiter plate, again using the repetitive sampling pipette. Fifty microliters of 0.5% (w/v) sodium ascorbate is added to any wells containing 50 μl of analytical sample to bring the total volume in all wells to 100 μl. This results in an overall use of eight wells for every serum specimen analyzed (Fig. 1a). For example, 50-μl

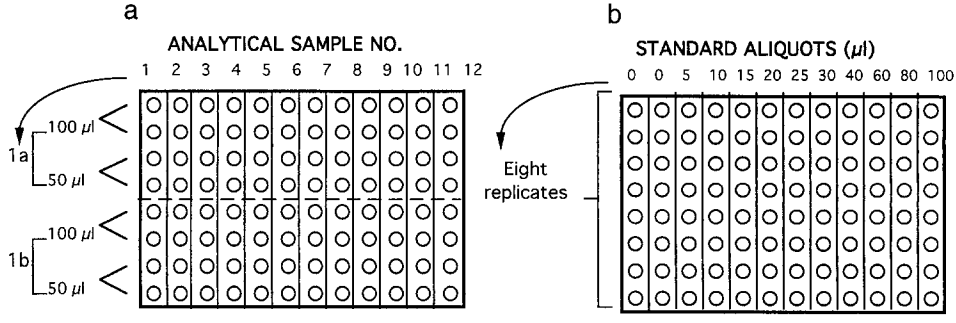

FIG. 1. Diagram of the procedure for aliquoting samples (a) and standards (b) into microtiter plates.

aliquots of serum sample 1 have been pipetted into two polypropylene tubes labeled 1a and 1b and each tube diluted to give a total volume of 1 ml. From each of these tubes, two 100-μl and two 50-μl aliquots are placed in microtiter plate wells and in the latter 50 μl of 0.5% (w/v) sodium ascorbate is also added.

A portion (20 ml) of the working standard is transferred to a 30-ml sterile universal tube and the repetitive sampling pipette is used to aliquot standard amounts into a separate microtiter plate (12 columns with 8 replicates for each standard; see Fig. 1b). Columns 1 and 2 contain 0 μl of standard (i.e., blanks), columns 3 to 12 contain 5 μl (2.5 pg; 5.7 fmol), 10 μl (5.0 pg; 11.3 fmol), 15 μl (7.5 pg; 17.0 fmol), 20 μl (10.0 pg; 22.7 fmol), 25 μl (12.5 pg; 28.3 fmol), 30 μl (15.0 pg; 34.0 fmol), 40 μl (20.0 pg; 45.3 fmol), 60 μl (30.0 pg; 68.0 fmol), 80 μl (40.0 pg; 90.6 fmol), and 100 μl (50.0 pg; 113.3 fmol) of standard, respectively. Appropriate additions of 0.5% (w/v) sodium ascorbate are made to each well, from 100 μl in columns 1 and 2 to 0 μl in column 12, such that the final volume in all wells is 100 μl.

Finally, 200 μl of prepared innoculated folate medium is added to all wells using the eight-arm multichannel pipette. To achieve this it is necessary to pour small amounts of medium into the base of a petri dish and pipette from this. To avoid contamination by folate in the samples and standards the order of addition of medium should be zero standards (columns 1 and 2, standard plate) followed by analyticals (all other plates) followed by remaining standards. This procedure removes the need to change pipette tips for each addition but it is essential that the tips do not touch the liquid in the analytical wells.

Each plate is covered with a plastic plate sealer and a roller is used to ensure that all wells are individually sealed and all air bubbles removed. Plates are incubated at 37° for about 42 hr. After this time each plate is inverted and mixed carefully to produce an even cell suspension and the plate sealer is removed. The plates are read at 590 nm in a microtiter plate reader and a standard curve is drawn from the optical density printout of the plate reader. A typical standard curve is shown in Fig. 2. Alternatively, the plate reader may be linked to a computer using a commercial ELISA program to collect data, draw a standard curve, and calculate the concentration of folate in each well.

Appropriate dilution factors are used to calculate concentrations. Reading the absorbances of analyticals against a growth curve set up as in Fig. 2, the estimated folic acid concentrations per well for the 100- and 50-μl aliquots are multiplied by 200 and 400, respectively, for serum or plasma and by 400 and 800 for whole blood to give results in nanograms per milliliter. (If analyticals have initially been diluted to 2 ml for the assay rather than 1 ml, a further multiplication factor of 2 is applied.) For each

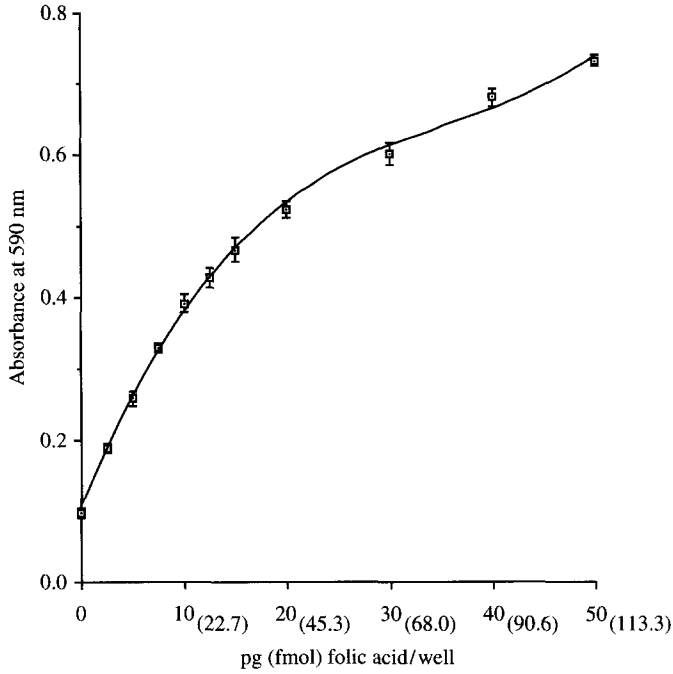

FIG. 2. Standard curve based on the growth response of folic acid to *L. casei.* Values shown are the means ± SD of 8 replicates in the case of folic acid-containing standards and 16 replicates for the zero standard.

sample, the concentration to be reported is calculated by averaging the final results (i.e., ng/ml) from the two 100-μl and the two 50-μl aliquots. The 50-μl average is then checked against the 100-μl average and the result is accepted if there is no more than a 10% difference between the two. The mean of these two averages is then taken as the result for replicate a. The same procedure is carried out on replicate b. Finally, the two results for replicate a and replicate b are checked against each other. If there is agreement within ±5% of the mean the result for that serum or plasma sample is accepted.

The result for each of the three quality control samples is checked against the acceptable limits for each sample on the quality control chart. The limits are ±2 standard deviations from the baseline mean obtained when setting up the quality control pools and the entire assay is repeated if the control samples are outside this range. The quality control charts are monitored for nonrandom trends, such as drifting and a run of results above

or below the mean.[8] In such instances, the assay of routine samples is halted until possible causes are checked and corrected. As a typical example of quality controls, our current plasma and whole-blood ascorbic acid lysate controls have the following values (mean ± SD):

P1: 0.95 ± 0.23 ng/ml (2.15 ± 0.52 pmol/ml)
P2: 2.51 ± 0.55 ng/ml (5.69 ± 1.25 pmol/ml)
P3: 6.10 ± 0.78 ng/ml (13.82 ± 1.77 pmol/ml)
R1: 53 ± 4 ng/ml (120 ± 9 pmol/ml)
R2: 89 ± 9 ng/ml (202 ± 20 pmol/ml)
R3: 176 ± 12 ng/ml (399 ± 27 pmol/ml)

To obtain a value for erythrocyte folates (as concentration per milliliter of erythrocytes) the measured whole-blood value is divided by the PCV obtained from an automated (Coulter) counter or hematocrit, or by an estimate of the PCV derived from analysis of hemoglobin in the ascorbic acid lysate samples (O'Connor *et al.*[3]).

[8] E. Mullins, *Analyst* **119,** 369 (1994).

[6] Determination of Tetrahydrobiopterin Biosynthetic Activities by High-Performance Liquid Chromatography with Fluorescence Detection

By ERNST R. WERNER, HELMUT WACHTER, and GABRIELE WERNER-FELMAYER

Introduction

Tetrahydrobiopterin [6*R*-6(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin] serves as cofactor of phenylalanine 4-monooxygenase, tyrosine 3-monooxygenase, glyceryl-ether monooxygenase, and nitric oxide synthase.[1] Because intracellular tetrahydrobiopterin concentrations affect the amount of metabolites of, e.g., nitric oxide synthase formed by intact cells,[2] regulation of the biosynthesis of tetrahydrobiopterin is of interest. Assays of the three biosynthetic enzymes (Fig. 1) involved in the formation of tetrahydrobiopterin from guanosine 5′-triphospate (GTP) based on high-performance liquid chromatography (HPLC) with fluorescence detection

[1] S. Kaufman, *Annu. Rev. Nutr.* **13,** 261 (1993).
[2] G. Werner-Felmayer, E. R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, and H. Wachter, *J. Exp. Med.* **172,** 1599 (1990).

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