ASSESSMENT OF FOLATE METHODOLOGY USED IN THE
THIRD NATIONAL HEALTH AND NUTRITION
EXAMINATION SURVEY (NHANES III, 1988-1994)

November, 1994

Prepared for
CENTER FOR FOOD SAFETY AND APPLIED NUTRITION
FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH AND HUMAN SERVICES
WASHINGTON, D.C. 20204

under
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Task Order No. 4
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edited by
Daniel J. Raiten, Ph.D.
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FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments on topics in the biomedical sciences. Reports are based upon comprehensive literature reviews and the scientific opinions of knowledgeable investigators engaged in work in relevant areas of biology and medicine.

This report was developed for the Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration (FDA), in accordance with the provisions of Task Order No. 4 of Contract No. 223-92-2185. It was edited by Daniel J. Raiten, Ph.D., Senior Staff Scientist, LSRO and Kenneth D. Fisher, formerly Director, LSRO, on the basis of discussions of, and materials evaluated by, an ad hoc Expert Panel convened by LSRO. The members of the Expert Panel were chosen for their qualifications, experience, and judgment, with due consideration for balance and breadth in appropriate professional disciplines. Members of the Expert Panel and others who assisted in the preparation of this report are listed in Chapter VII.

The study was initiated on January 31, 1994. In a notice in the Federal Register of June 8, 1994 (59 FR 29611), the Food and Drug Administration (1994) announced that FASEB was inviting data, information, and views bearing on the topic under study. Accordingly, FASEB provided an opportunity for public oral presentations in an Open Meeting held on July 15, 1994 and for written submissions. Two (2) individuals made oral presentations at the Open Meeting. Three (3) organizations provided written submissions for consideration by the Expert Panel (FDA Docket No. 94N-0169). The LSRO wishes to express its appreciation to all individuals and organizations who have contributed materials for this study. Names of individuals and organizations who contributed to this study are listed in Chapter VIII.

The final report was reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent Society of FASEB) under authority delegated by the FASEB Board. Upon completion of these review procedures, the report was approved and transmitted to FDA by the Executive Director, FASEB.

While this is a report prepared by a Panel convened by an office of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of FASEB, its constituent Societies, or the individual members of each constituent Society.

November 30, 1994
Date

Sue Ann Anderson, Ph.D.
Acting Director
Life Sciences Research Office
EXECUTIVE SUMMARY


As part of the survey, blood samples were collected from persons aged one year and older and analyzed for numerous biochemical and nutritional indicators including levels of folate in serum and red blood cells (RBC). Serum and RBC folate have been analyzed from the approximately 15,000 individuals who participated in NHANES III (1988-1991) using a commercially available radioassay (RA) kit (Quanta-Phase™ manufactured by Bio-Rad Laboratories, Hercules, California). In December, 1993 (approximately halfway through NHANES III, 1991-1994) the NHANES Laboratory of the Centers for Disease Control and Prevention (CDC) began using the modified RA kit, Quanta-Phase™ II®, a combined vitamin B₁₂ and folate RA.

Both kits use pteroylglutamic acid (PteGlu) as the standard. Bio-Rad Laboratories calibrated the standard in 1975 for the first generation RA kit Quanta-Count™. All standards for subsequent kits through the Quanta-Phase™ kit were calibrated based on the original standard. The Quanta-Phase™ II® kit differs from the Quanta-Phase™ kit only in the values assigned to the PteGlu reference standard. Throughout the history of use of the RA kits, the CDC had performed a series of quality control tests to evaluate the precision of the kits. These test had confirmed both the between and within run consistency of these methods. In anticipation of the switch in 1993 to the combined folate/vitamin B₁₂ RA kit, CDC performed its own internal comparison study to determine the consistency of analyses with both kits. These latter tests again confirmed the consistency of the folate assay and a lack of influence from the addition of the vitamin B₁₂ assay.

Recently, investigators have reported a discrepancy in the values obtained from the Quanta-Phase™ kit when using the kit standard compared with using a freshly prepared standard. Levine (1993) reported that folate data derived from Quanta-Phase™ kits were about 30 percent higher than values obtained using freshly prepared standards and suggested that the difference was due to the values assigned to the kit standard originally established in 1975. Results from a multinational round-robin study and studies by the CDC have confirmed these findings.

The original Quanta-Count™ kit was calibrated against the Lactobacillus casei microbiological assay. Subsequently, all PteGlu standards used in the Bio-Rad RA kits were assigned the same values as those assigned to the original standard in 1975. Bio-Rad Laboratories have attributed the original problem to the standard materials used to calibrate the original kits, rather than the adjustment of the RA results to the microbiological results. Bio-Rad Laboratories have confirmed the discrepancies resulting from the erroneously calibrated standards and have corrected the problem with the development of the new Quanta-Phase™ II® RA kits. The PteGlu concentrations of the new standards used in the Quanta-Phase™ II® have been confirmed gravimetrically.

STUDY APPROACH

This report provides an assessment by an Expert Panel of several issues relative to the analytical problems associated with folate status assessment data from NHANES III (1988-1994) including:
(1) the analytical and biochemical bases of the discrepancies associated with serum and RBC folate values derived from the use of the Bio-Rad Laboratories kits used in NHANES III (1988-1991); (2) an evaluation of the scientific basis and validity of the procedure proposed by CDC to make corrections to these folate values; (3) the utility of current cutoff values used for estimation of folate status; (4) the appropriateness of the application of the adjustment factor to existing cutoff values; and (5) the comparability of the folate data derived in NHANES III (1988-1991), NHANES III (1991-1994), and NHANES II (1976-1980). The Expert Panel accomplished these assessments through a review of previous comparative studies, the multinational round-robin studies performed by the European Community Food-Linked Agro-Industrial Research (FLAIR) Program, and studies by the CDC and Bio-Rad Laboratories.

COMPARATIVE STUDIES

With regard to studies that have compared commercially available RA kits, including those manufactured by Bio-Rad Laboratories, to the L. casei microbiological assay, results for serum folate from the former have differed from those of the latter. The performance of the various iterations of the kits produced by Bio-Rad Laboratories has been inconsistent in terms of comparability to the microbiological assay and in the ability to assess RBC folate. Historically, although variability between commercial kits in the ability to assess serum folate has been reported repeatedly, the performance characteristics within kits has been consistent with relatively low coefficients of variation within and across studies.

The FLAIR studies confirmed the existence of several issues reflecting on the accuracy and precision of commercially available RA kits, including those produced by Bio-Rad Laboratories. The results of the FLAIR report indicated that most RA kits tended to "overestimate serum folate content due to improper standard calibration and the use of PteGlu rather than MTHF as the standard." Both fresh PteGlu and MTHF standard solutions produced values about 30 percent lower than PteGlu standards provided with the kits, suggesting that assigned values for kit PteGlu standards were too high. In addition, one of the studies performed confirmed that the use of the Quanta-Phase II® kit with the recalibrated standards resulted in lowered whole blood folate values.

CDC STUDIES AND PROPOSED ADJUSTMENT FACTOR

Based on information received from the NHANES Laboratory of CDC, the Expert Panel made the following observations:

• The CDC, beginning in 1978 with its use of the Quanta-Count™ folate kit for NHANES II (1976-1980) and through Hispanic Health and Nutrition Examination Survey (HHANES 1982-1984), followed by the use of the Quanta-Phase I® folate-only kit beginning in 1985, and through the introduction of the Quanta-Phase II® folate/B12 kit in 1993, has performed both comparative testing and exhaustive testing of the performance characteristics of these kits. The quality control data presented to the Expert Panel are demonstrative of high precision reflected by the excellent reproducibility of the results from all of the folate kits used by CDC. Similarly, comparative data revealed no differences between the three methods or from the introduction of the combined folate/B12 Quanta-Phase II® kit.

• The consistent excellence of the quality control data from the CDC tests confirms that the error in the Bio-Rad Laboratories kits was systematic and not related to the performance of the assays.
• Testing performed by the CDC confirms that the addition of the B₁₂ assay in the Quanta-Phase II² RA kit did not affect the performance of the kits in the assessment of serum and RBC folate.

Based on discussions with several investigators, the NHANES Laboratory at CDC undertook a comparison of serum and RBC folate values using the PteGlu reference standards included in the Quanta-Phase I² ra kit versus fresh PteGlu reference standards with folate concentration confirmed spectrophotometrically. In mid-1993, the NHANES laboratory, CDC, analyzed in duplicate 1833 NHANES III (1991-1994) blood samples (selected as received) and batches of laboratory quality control samples by using "old" (i.e., microbiological assay performance matched as in the Quanta-Phase I² RA kits) versus "new" spectrophotometrically verified folate standards (as in Quanta-Phase II² kits). The standards were provided by Bio-Rad Laboratories and the "new" standards were spectrophotometrically confirmed by CDC.

Analyses of these samples resulted in the creation of the following correction factor:

\[
\log_{10}(\text{corrected value}) = -0.1956 + 1.0199 \times \log_{10}(\text{uncorrected value})
\]

This may also be expressed as follows:

Corrected value = 0.6374 × uncorrected value\(^{1.0199}\)

On a linear basis, the following equation may be used:

Corrected value = -0.1411 + 0.6849(uncorrected value)

The Expert Panel noted that both the \(\log_{10}\)-based equation and the inverse log equation (corrected=0.6374 × uncorrected value\(^{1.0199}\)) are equivalent. The third or linear equation is an approximation of the first two. All corrected data used in this report were generated using the \(\log_{10}\)-based equation.

### IMPACT OF APPLICATION OF THE ADJUSTMENT FACTOR

After consideration of the impact of the application of the adjustment factors to available data sets, the Expert Panel concluded that due to historical considerations (changes in dietary habits, use of dietary supplements, etc.) and questions about the validity of the data related to the problems between the various iterations of the kits used to assess both serum and RBC folate, comparisons between survey data sets from NHANES II (1976-1980), HHANES (1982-1984), and NHANES III (1988-1991) are not advisable at this time. Moreover, the application of the adjustment factor to data from NHANES II (1976-1980) and/or HHANES (1982-1984) cannot be justified at this time.

With regard to data from NHANES III (1988-1994) derived from analyses with the Quanta-Phase I² kit, the Expert Panel concluded that rather than using existing cutoffs with adjusted data, the more appropriate approach would be to apply an adjustment factor to the previously established cutoffs. While the Expert Panel recognized that the data used to establish the existing cutoffs were derived from not only RA assays but also microbiological and other assays, the Expert Panel concluded that some adjustment is required.
UTILITY OF CURRENT CUTOFF VALUES

Considerable uncertainty exists in regard to the definition of folate deficiency based solely on cutoff values for serum and RBC folate. Lacking a consensus among authoritative experts of what serum and RBC folate cutoff values constitute "normalcy," "at-risk," "low," "indeterminate," or "deficiency," interpretation of the NHANES III (1988-1991) adjusted or unadjusted data appears premature and open to question. Irrespective of what cutoff values are used, the Expert Panel concluded that there is an inherent inadequacy in the reliance of single indices as sole determinants of inadequate folate nutriture. Indeed, the use of one clinical or biochemical measure as a sole criterion of either nutritional deficiency or toxicity is increasingly being recognized as scientifically inappropriate.

With regard to the application of the adjustment factor to current standards, the Expert Panel emphasized that such adjustments are relevant only to the data derived by CDC using the Bio-Rad Laboratories Quanta-Phase I® RA kit, i.e., NHANES III (1988-1991) and part of NHANES III (1991-1994). The application of the adjustment to the cutoff values is a mathematical manipulation only; the broader issue of defining clinical folate deficiency remains. Neither adjustment of the serum or RBC folate data from NHANES III (1988-1994), the use of the data without adjustment, nor use of either data set with adjusted criteria for normalcy and deficiency, by themselves, can predict the prevalence of inadequate folate nutriture of the United States population. As indicated by previous authoritative groups, corroborative and confirmatory measures in addition to serum and RBC folate are essential for the accurate determination of prevalence of folate deficiency.

To determine the validity of using NHANES III (1988-1991) and NHANES III (1991-1994) folate data as components of an approach to prediction of folate status of the United States population, additional evaluation of parameters of folate status other than serum and RBC folate levels in NHANES III (1988-1991) and NHANES III (1991-1994) subjects is required. Functional parameters include serum or plasma homocysteine levels, neutrophil hypersegmentation, and various measures of iron status. Additional factors to be integrated into this evaluation include the effects of dietary intake of folate, age, socioeconomic level, and various life-style factors such as smoking, alcohol consumption, dietary supplement use, and oral contraceptive use.


Finally, comparison of NHANES III (1988-1991) data with NHANES II (1976-1980) data on serum and RBC folate would be inappropriate at this time. Similarly, application of the adjustment factor to NHANES II (1976-1980) data would be inappropriate. The issue of methodological consistency and standardization of PteGlu reference solutions must be addressed first. Application of an adjustment factor to NHANES III (1988-1991) data, as proposed by CDC, may be acceptable; however, the appropriateness of the approach should be based on carefully planned reanalyses of a statistically valid number of NHANES III (1988-1991) and NHANES III (1991-1994) blood samples using the same RA kits, the same protocols, and externally calibrated PteGlu and/or MTHF reference standards.
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I. INTRODUCTION

A. BACKGROUND INFORMATION

The Life Sciences Research Office (LSRO) of the Federation of American Societies for Experimental Biology (FASEB) is under contract with the Center for Food Safety and Applied Nutrition (CFSAN) of the Food and Drug Administration (FDA) to review and evaluate current knowledge and technology in selected areas of nutrition and health sciences. Because of its responsibility for ensuring the safety and adequacy of the American food supply, FDA has a continuing interest in data that bear on nutrient-disease relationships in the United States population. Thus, CFSAN has an ongoing concern about the folate content of the United States diet and the folate status of the United States population. For this reason, CFSAN has a need to examine serum and red blood cell (RBC) folate data from the current National Health and Nutrition Examination Survey (NHANES III, 1988-1994) that may be used to evaluate the folate status of the United States population. NHANES III (1988-1994) was conducted in two phases. Phase 1 was conducted from 1988 to 1991 and Phase 2 from 1991 to 1994. Samples for both phases are nationally representative samples. The majority of data discussed in this report are Phase 1 data and referred to as NHANES III (1988-1991); Phase 2 will be referred to as NHANES III (1991-1994).

In late 1993, FDA became aware of a methodological problem associated with the radioassay (RA) kits used in NHANES III (1988-1994) that affected serum and RBC folate values and, consequently, data interpretation. The method by which the RA kits used in NHANES III (1988-1991) and part of NHANES III (1991-1994) were standardized appears to affect interpretation of folate status of subjects included in this national survey. Therefore, CFSAN requested that LSRO, under its contract with FDA, review the issues and present its findings in a documented report to CFSAN.

A tentative report was prepared that outlined the issues and presented preliminary conclusions of an ad hoc Expert Panel which met at LSRO/FASEB on April 15-16, 1994. This Tentative Report was made available publicly as background material for an Open Meeting held at FASEB on July 15, 1994. Oral and written presentations made prior to and at the Open Meeting were considered by the ad hoc Expert Panel in its deliberations. A listing of those parties who contributed materials can be found in Chapter VIII.

B. STATEMENT OF THE PROBLEM

The National Nutrition Monitoring and Related Research Program (NNMRRP), authorized by Public Law 101-445 in 1990 (U.S. Congress, 1990), provides for periodic monitoring of the nutritional status of the United States population. As a part of this effort, as described in the Ten-Year Comprehensive Plan for the NNMRRP (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 1993), the National Center for Health Statistics (NCHS) has conducted the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1994).

Approximately 30,000 persons were examined in NHANES III (1988-1994). As part of the survey, blood samples were collected from persons aged one year and older and analyzed for numerous biochemical and nutritional indicators. Levels of folate in serum and in RBC were among the clinical measures of nutrient status assessed in these samples. Serum and RBC folate have been analyzed from the approximately 15,000 individuals who have participated in NHANES III (1988-1991).
In NHANES III (1988-1991) and part of NHANES III (1991-1994), analyses of serum and RBC folate were performed using the Bio-Rad Laboratories Quanta-Phase I® Kit #192-6002 (Bio-Rad Laboratories, Hercules, California), a radioassay (RA) using 125I folate as the tracer. Folate values were evaluated using pteroylglutamic acid (PteGlu) standards calibrated by the manufacturer in 1975 when this RA kit, then known as Quanta-Count™, was first made available commercially.

The Centers for Disease Control and Prevention (CDC) began using the modified RA kit (Quanta-Phase II® Kit #192-1002) developed by Bio-Rad Laboratories in December of 1993, approximately halfway into NHANES III (1991-1994). Both the Quanta-Phase I® and Quanta-Phase II® RA kits are available as combined 57Co-vitamin B₁₂ and folate kits. The CDC used the folate-only Quanta-Phase I® in NHANES III (from 1988 to late 1993) and only began to use the combined kit when the switch was made to Quanta-Phase II® in December, 1993. With regard to the folate assay, Bio-Rad Laboratories has indicated that the new Quanta-Phase II® RA kit differs from Quanta-Phase I® only in the values assigned to the PteGlu reference standard.

Recently, Levine (1993) reported that, based upon comparisons with internal PteGlu standards prepared in his laboratory, the folate data derived from Quanta-Phase I® RA kits were about 30 percent higher than expected. Levine (1993) suggested that the higher values were associated with the standard values provided in the Quanta-Phase I® RA kits which were originally established for the Quanta-Count™ RA kit by Bio-Rad Laboratories in 1975. The CDC and others (van den Berg et al., 1994) have confirmed the difference (i.e., higher values) between results obtained using standard values contained in the Bio-Rad Laboratories kits and certain other RA folate kits and those derived using fresh PteGlu standards. If these observations are correct, then the serum and RBC folate values derived from use of the Quanta-Phase I® RA kits in NHANES III (1988-1991) and NHANES III (1991-1994) may also be higher by a factor of 30 percent.

Similarly, a multinational survey in Europe has also documented higher serum and RBC folate values with several commercial RA kits, including those of Bio-Rad Laboratories (van den Berg et al., 1994). Efforts to identify the basis of these differences were conducted over a 2 to 3 year period in several laboratories. However, results of these studies were not widely distributed in the United States until 1993.

According to Edwards (1993), the original Quanta-Count™ B₁₂/Folate Assay Kit introduced in 1975 was calibrated against the Lactobacillus casei microbiological assay which was the accepted standard at that time. Subsequently, the PteGlu standards used in the modified RA kit (Quanta-Phase I® Kit) introduced in 1981 were assigned the same values as those PteGlu standards calibrated in 1975 in the development of the original Quanta-Count™ kit.

Bio-Rad Laboratories scientists have attributed the original problem to the standard materials used to calibrate the original Quanta-Count™ kit in 1975 rather than the adjustment of the RA results to the microbiological results (Bussell, 1994). According to Bussell (1994), as a result of the lack of written documentation or available samples of materials used in the standardization process, the reasons for the discrepancies leading to the assignment of incorrect values to the internal standards can be hypothesized but cannot be determined with certainty.

In anticipation of the switch in 1993 to the combined folate/vitamin B₁₂ kit (Quanta-Phase II®) from the folate-only Quanta-Phase I® kit, the CDC performed its own internal study to determine the consistency of analyses with both kits. To address this issue, the CDC performed a comparison test (10 runs) of fresh NHANES III (1991-1994) blood specimens for folate using the Quanta-Phase I® folate-only versus the Quanta-Phase I® RA combined folate/B₁₂ kits and found no difference (Gunter and Bowman, 1994).
No universally accepted reference standards for PteGlu exist. However, various RA kit manufacturers, including Bio-Rad Laboratories, prepare their own PteGlu reference standards. Recently, Bio-Rad Laboratories did prepare a series of PteGlu reference sets and confirmed the PteGlu concentrations gravimetrically (Edwards, 1993). Based upon blood samples from normal and clinically established folate-deficient subjects, folate values were about 30 percent lower than would have been expected using 1975 and 1981 calibration curves derived from the PteGlu standards provided with those kits. In essence, Bio-Rad Laboratories confirmed the observations of Levine (1993) and others (van den Berg et al., 1994).

Based on these findings and the historically consistent correlation between and within batches and types of kits, i.e., Quanta-Count\textsuperscript{TM}, Quanta-Phase I\textsuperscript{®}, and Quanta-Phase II\textsuperscript{®} RA kits, CDC has proposed an "adjustment" factor based on corrected reference standard values for use in the recalibration of the folate data derived from the use of Quanta-Phase I\textsuperscript{®} during NHANES III (1988-1994). Use of this adjustment may also require consideration of using new interpretative criteria for evaluating the folate status of the United States population from NHANES III (1988-1994).

C. SCOPE OF THE LSRO STUDY

For the LSRO review, CFSAN requested that LSRO address the following issues in the Tentative Report:


2. Evaluate the scientific basis and validity of the procedures proposed by CDC to make appropriate corrections to serum and RBC folate values obtained in NHANES III (1988-1991).

3. Reexamine current "cutoff" values used for estimation of "deficient," "low status," etc. in light of the need for application of a correction factor.

4. Determine whether these approaches are still useful for estimating the prevalence of inadequate folate nutrure.


To address these issues, LSRO convened an ad hoc Expert Panel (see Chapter VII) which met on April 15-16, 1994 to hear presentations on the issues by representatives of several agencies and organizations that have been involved in production of the RA kits, analysis of serum and RBC samples for NHANES III, and preparation of data on folate status. In addition, other experts familiar with the issues raised by FDA were invited to make presentations to the ad hoc Expert Panel. (For listing of contributors, see Chapter VIII.) A Tentative Report was prepared from the discussions of the ad hoc Expert Panel and presenters and provided a basis for further deliberation and input that occurred at the Open Meeting held July 15, 1994. Subsequent oral and written comments about the Tentative Report were used by the Expert Panel in preparing this report.
II. ANALYTICAL AND BIOCHEMICAL BASES OF DISCREPANCIES

A. REVIEW OF COMPARATIVE STUDIES

The choice of commercial kits utilizing the RA assay over the microbiological assay is based on reproducibility, convenience, and cost. The RA kits have the advantages of ease of use and a lack of interference by antibiotics that make them particularly useful in the clinical setting. Most commercial RA assays are designed to use PteGlu as the standard because of its stability. By controlling the pH at 9.3, the sensitivity of the binder for PteGlu is equilibrated with its sensitivity for 5-methyl tetrahydrofolate (MTHF), the primary circulating form found in serum and RBC. (The RBC are treated with folate hydrolase to deconjugate the polyglutamate derivatives.) In contrast, the microbiological assay responds similarly to all monoglutamate forms of folate (Shane et al., 1980; Tamura, 1990).

It should be noted that the original strain of *L. casei* (ATCC No. 7469) used in the microbiological assay for folate was modified in order to eliminate the need for the sterilization process (Davis et al., 1970). Some studies have used the chloramphenicol-resistant strain of *L. casei* (ATCC No. 27773), others did not, and still other studies did not specify the strain of *L. casei*. It should also be noted that *L. casei* (ATCC 7469) is now referred to as *L. rhamnosus* (Collins et al., 1989). In reviewing the following comparative studies, the strain of microorganism used is noted where that information was provided.

With specific regard to folate/B₁₂ kits manufactured by Bio-Rad Laboratories, the following chronology supplied by Moreton (1994) provides useful background:

mid-1970s: Quanta-Count™ B₁₂ and Quanta-Count™ folate RA kits were released as separate assays.

1977: Quanta-Mate™ dual B₁₂/folate charcoal-based RA kit was released.

1980: Quanta-Mate™ dual B₁₂/folate charcoal-based RA kit was altered to remove interference from R-proteins.

October, 1983: Quanta-Phase I™ B₁₂/folate RA kit was released. This kit differed from earlier kits in its use of "immunobeads" coated with intrinsic factor (for vitamin B₁₂) and folate-binding protein.

March, 1984: "New Improved" Quanta-Phase I™ folate/B₁₂ RA kit was released. This kit had been "reforulated to add additional non-coated beads to make a better pellet for decanting; dye was also added to the microbead for customer convenience; the pH of the tracer was decreased to 9.8 and the single folate and B₁₂ assays were also released."

September, 1993: Quanta-Phase II™ was released. "The folate side of the assay was restandardized to a gravimetric quantitation." No changes were made to the B₁₂ assay.

Numerous studies have been performed in which commercially available RA kits were compared with other conventional folate assessment methodologies (Bain et al., 1984; Dawson et al., 1980, 1987;
Gilois and Dunbar, 1987; Hill and Dawson, 1977; McGown et al., 1978; Raniolo et al., 1984; Shane et al., 1980).

The RA kits developed by Bio-Rad Laboratories and others are based on the principle of saturation analysis. In particular, the Quanta-Count™ kit contained 125I-labeled PteGlu, β-lactoglobulin as the binding agent and standards composed of lyophilized folate-free human serum spiked with a range of PteGlu. It should be noted that β-lactoglobulin was chosen because it contains the milk folate-binding protein as a constituent. Hill and Dawson (1977) compared the Quanta-Count™ kit with a microbiological assay using a chloramphenicol-resistant L. casei (self-adapted). The comparison was made with sera from 72 "normal persons." Hill and Dawson (1977) reported that while the Quanta-Count™ kit had excellent within and between batch reproducibility, the RA results were "usually lower than the microbiological assays."

In their discussion, Hill and Dawson (1977) explored several possible explanations for the differences between the RA kit results and those from microbiological assay. They suggested that even though endogenous serum binders can produce lower values in some noncommercial RA tests, this should not have been a factor because the serum was heated to inactivate the binders. Hill and Dawson (1977) also discussed the effect of pH, the use of ascorbic acid, and the differences in binding affinity of the milk folate-binding protein for MTHF and PteGlu used in the standards. The affinity for the former is greater than that for the latter.

McGown et al. (1978) compared several commercial RA kits, including the Quanta-Count™ and a kit produced by New England Nuclear, to the chloramphenicol-resistant L. casei (ATCC 27773) microbiological assay for serum folate (Scott et al., 1974) and the L. casei (ATCC 7469) whole blood folate assay of Cooperman (1967). Blood samples used in this report were collected from a total of 570 military personnel (528 males and 42 females) after an overnight fast. A comparison of between-run precision was made using pooled composite serum samples. The results of the serum composite comparison revealed lower values for serum folate for the Quanta-Count™ versus the microbiological assay (8.9 versus 10.0 μg/L, respectively) with a similar coefficient of variation (7.8 versus 8.0 percent over 10 and 7 runs, respectively). Both kits tested on whole blood samples differed from the microbiological assay (higher whole blood and calculated RBC folate levels) and from each other. Results from the Bio-Rad Laboratories kit were higher than results from the New England Nuclear kit. In discussing their results, McGown et al. (1978) observed:

"Some attempt was made to redefine new criteria for each...on the basis of difference in means, or by use of the regression lines. Although apparent agreement could be improved somewhat, such arbitrary adjustments cannot be assumed to be valid clinically. Such mathematical exercises are particularly risky for erythrocyte folate values, which do not have qualitatively similar frequency distributions."... "The discrepancies between radioassays and the microbiological method can be attributed partly to variations in concentration of the standards. For example, microbiological assay of Bio-Rad Laboratories standards indicated them to be about 90% of the stated values."

McGown et al. (1978) also suggested that, as with the microbiological assay, autolysis or treatment with folate hydrolase to deconjugate the polyglutamates may also be necessary for RBC folate assessments using RA kits. When they performed such treatment, they consistently obtained lowered RBC values from the kits, albeit still consistently higher than the microbiological assay.

Shane et al. (1980) performed a comparison of several commercial kits including a Bio-Rad Laboratories kit (presumably the Quanta-Count™) with the microbiological assay. A unique aspect of this study was the use of various forms of folate standards rather than human blood samples and
the analysis of the relative responsiveness of the methodologies tested. The focus of the study was on the ability of the various kits to measure different folate mono- and polyglutamates. The standards, used at concentrations equivalent to about 5 ng PteGlu/mL in sodium ascorbate, were assayed according to manufacturers’ specifications. In all cases, standard curves were prepared with folate standards supplied with the kits and then normalized based on the response to MTHF. For the Bio-Rad Laboratories tests, standards were prepared in 7 g/100 mL of human serum albumin solutions containing ascorbic acid. The microbiological assay used L. casei (ATCC 7469).

Shane et al. (1980) determined that (1) the microbiological assay was unsuitable for the measurement of folate polyglutamates but gave accurate results for the assessment of folates provided they are converted to the monoglutationate forms prior to assay; (2) commercial kits gave variable responses to polyglutamates compared with monoglutamates, with an increase in responsiveness to polyglutamates at higher concentrations; (3) an apparent higher affinity for the binding proteins by the polyglutamates compared with monoglutationate was suggested as an explanation of the variable responsiveness of the kits; and (4) a variable responsiveness was also noted in kit assays for different monoglutationate derivatives, which suggested that the kits would be unsuitable for the assessment of a mixture of monoglutationates such as would be found in samples pretreated with folate hydrolase, e.g., RBC.

Shane et al. (1980) concluded that, "Although radioassay techniques are useful for the qualitative assessment of folate status, their use for the quantitative determination of folate derivatives would appear to be limited." In addition, they noted that the variability reported in other comparative studies utilizing commercial kits may result from the binder used in a given kit and/or materials in which the binders are diluted, e.g., buffer solution, serum, or human serum albumin. According to Shane et al. (1980), the latter factor could compromise the affinity of the binders for folate.

At the request of the Laboratory and Development Advisory Group of the Department of Health and Social Security of the United Kingdom, Dawson et al. (1980) undertook the task of testing the reliability and reproducibility of five available RA kits for folate (including the Quanta-Count™ and six kits for vitamin B₁₂. The microbiological assay used was that of Davis et al. (1970) which employed the chloramphenicol-resistant strain of L. casei. Reference samples were collected from 80 staff members (including 13 taking oral contraceptives). Clinical samples were collected from 70 patients for assessment of folate and 80 other patients for determination of vitamin B₁₂. An additional 24 samples were collected from patients with pernicious anemia.

As with the previous comparative studies, the results from Dawson et al. (1980) showed a large degree of variability between kits in terms of the assessment of both the reference samples (which resulted in ranges that differed from those supplied with each kit) and clinical samples. With regard to the latter, the kits demonstrated considerable variability in their ability to assess both folate and vitamin B₁₂ in patients with various hematological disorders, including pernicious anemia. The accuracy of the kits in assessing PteGlu and MTHF was limited as reflected by a wide range of average recoveries ranging from 86 to 57 percent for the former and 72 to 36 percent for the latter (Quanta-Count™ recoveries were 77 and 63 percent for PteGlu and MTHF, respectively). Assays for 5 µg/L of PteGlu or MTHF were similarly incongruous (Quanta-Count™ results were 8.5 and 16.8 for PteGlu and MTHF, respectively) compared with the microbiological assay (4.9 and 12.3 for PteGlu and MTHF, respectively). For both serum and RBC folate, results from the Quanta-Count™ kit were highly correlated with the microbiological assay (r=0.90 and 0.86 for serum and RBC, respectively).

However, the results for serum folate derived from the Quanta-Count™ (for folate only) were different from those derived from the Quanta-Mate™ combined vitamin B₁₂/folate kit. Both kits are manufactured by Bio-Rad Laboratories. The former Quanta-Count™ kit produced results that were higher than the microbiological assay (2.7 versus 2.2 µg/L) compared with the latter which produced
lower results (1.6 versus 2.2 μg/L, for the kit and microbiological assay, respectively). A similar relationship was noted between these two kits for RBC folate. After reviewing the totality of evidence, including cost, time, shelf-life, and both clinical and analytical sensitivity, Dawson et al. (1980) concluded that while the microbiological assay had the fewest errors, the Bio-Rad Laboratories kits were preferred for the measurement of both serum and RBC folate.

As part of a continuous quality assurance survey, Dawson et al. (1987) summarized patterns in the results of annual round-robin assays of serum folate and vitamin B₁₂ samples (2 samples issued 11 times annually) and RBC folate (1 sample of whole blood issued 11 times yearly) by participating laboratories. Data from participating laboratories involving six different commercial kits (including those of Bio-Rad Laboratories) for serum folate, two versions of the microbiological assay (L. casei was used by four laboratories; chloramphenicol-resistant L. casei was used by 12 laboratories), and a noncommercial RA method were compared. No separate analyses of the data from the two types of microbiological assays were performed.

When compared with other commercial kits, the Bio-Rad Laboratories assay for serum folate produced results consistent with the L. casei methods (the kit was not identified, but presumably it was the Quanta-Phase I). The mean of 13 sera measured with the Bio-Rad Laboratories kit was 98 percent of values found by the microbiological assays; the Bio-Rad Laboratories assay had 94 percent recovery of 5.0 μg/L folate added to sera. In the assessment of whole blood folate, the kits from Bio-Rad Laboratories detected folate values that were 73 percent of those found with the microbiological assay. Dawson et al. (1987) also noted that, of all kits examined, the Bio-Rad Laboratories kit showed the greatest change relative to the microbiological assay values over a period of three years (a reduction from 157 percent to 73 percent of the microbiological values). No explanation was offered to account for these changes.

Gilios et al. (1986) assessed the performance of four commercially available RA kits (including Bio-Rad Laboratories Quanta-Phase I) by comparing the ability of the kits to measure serum folate concentrations in samples from 276 consecutive patients with neurological disorders. The results from the kits were compared with those achieved with the L. casei assay. An additional comparison was made analyzing 25 samples identified as having low serum folate by one or more of the kits with the L. casei microbiological method; a similar comparison for vitamin B₁₂ was made by analyzing (using the L. leishmannii assay) 23 samples identified as low vitamin B₁₂ by kits. Reference samples were obtained from 84 members of the hospital staff and used to compute normal ranges for each kit which were then compared to the ranges supplied by the respective manufacturers.

Statistically significant differences were revealed between log means for all pairs of methods for both serum folate and vitamin B₁₂. With regard to the ranges, the lower limits of the normal ranges generated in the protocol were in agreement with those supplied by the manufacturers; however, the upper limits were generally lower than those supplied by the manufacturers for both serum folate and vitamin B₁₂. When contrasted with the results from the microbiological assays, the Quanta-Phase I kit misclassified 5 (out of the 25 adjudged to have low serum folate by the L. casei method) samples that were low as being within normal range. An additional 13 were classified as being "indeterminate" or low normal. Only 7 of the 25 microbiologically confirmed low samples were classified as low by the Quanta-Phase I kit. The Corning Medical kit performed similarly to the Bio-Rad Laboratories kit, while the kits by Amersham and Becton-Dickinson provided results more consistent with the microbiological assay (20 correctly classified as low by Amersham kits and 14 by Becton-Dickinson kits).

Gilios and Dunbar (1987) compared serum and RBC folate values derived from five commercial RA kits and the microbiological assay (not further identified). They found differences between methods
for both serum and RBC folate and considerable variation in ability to discriminate low and normal folate values. Serum folate measurements were more variable than RBC folate measurements. They also noted RBC folate values with the Quanta-Phase I® RA kit were "much lower" than values obtained with other RA kits and attributed the difference to storage temperature (-20°C) and absence of ascorbic acid as the preservative during sample storage.

The results of the above studies may be summarized as follows:

- Historically, variability among commercial kits in the ability to assay serum folate has been reported repeatedly.

- In general, commercial assays result in serum folate values that differ from the *L. casei* microbiological method. With regard to the Bio-Rad Laboratories assays, the earlier reports indicated a tendency towards values that were lower than the microbiological assay, while later reports indicated a reversal in the direction of the difference, with kits providing higher values than microbiological assay. Dawson et al. (1980) found that the combined folate/vitamin B₁₂ Quanta-Mate™ kit produced serum and RBC folate results that were lower than the microbiological method while the folate-only Quanta-Count™ kit produced results that were higher than the microbiological method.

- The performance of commercial kits has been shown to be consistent with relatively low coefficients of variation (CV) within and across studies.

- The performance of the various iterations of the kit produced by Bio-Rad Laboratories has been inconsistent in terms of comparability to the microbiological method and in the ability to assess RBC folate.

- Explanations offered for the variability of results from commercial assay kits have primarily focused on assay conditions, e.g., pH, extraction temperature, the condition and type of binders, and the standards used.

**B. THE FLAIR STUDIES**

As part of a multinational effort to evaluate available methodologies for the measurement of micronutrient absorption and status, the European Community Food-Linked Agro-Industrial Research (FLAIR) Program conducted a three-part multilaboratory quality-control comparative study of various methods in folic acid status assessment (van den Berg et al., 1994). The first and third studies were round-robin tests of measures of serum/plasma and RBC folate, respectively, using freeze-dried quality-controlled samples of known folate content; the second test was a recovery study using unknown serum samples "spiked" with either PteGlu or MTHF (van den Berg et al., 1994). Ten laboratories using a variety of methods, including RA kits, competitive protein-binding assay using chemiluminescence, high performance liquid chromatography (HPLC), and microbiological assays (*L. rhamnosus*), was involved in these studies. The first and second tests were completed in the period 1991-1992; the third test was completed in 1993.

In Study 1, all laboratories used duplicate or triplicate assays of the two freeze-dried serum-like unknowns and purified PteGlu standards regardless of the method tested (van den Berg et al., 1994). In addition to several different commercially available RA kits (three used the Bio-Rad Laboratories Quanta-Phase I® kit, five used other kits), one laboratory used the *L. rhamnosus* microbiological
method, one used a chemiluminescent method, and one used an HPLC method. Data from these analyses are provided in Table 1.

Table 1. Results of FLAIR Round-Robin Study 1: Serum Folate (μM).

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of Laboratories</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>range</td>
</tr>
<tr>
<td>Quanta-Phase I®</td>
<td>3</td>
<td>34.8</td>
<td>31.6-38.5</td>
</tr>
<tr>
<td>Other RA Kits</td>
<td>5</td>
<td>26.1</td>
<td>16.8-34.7</td>
</tr>
<tr>
<td>Chemiluminescent assay</td>
<td>1</td>
<td>25.1</td>
<td>-</td>
</tr>
<tr>
<td>Microbiological assay</td>
<td>1</td>
<td>23.0</td>
<td>-</td>
</tr>
</tbody>
</table>

The Quanta-Phase I® and two other RA kits (DPC DualCount® and Baxter's Clinical Assay®) that depend on competitive protein-binding gave results that were about 30 percent higher than the microbiological assay results. In their summary, the authors concluded that problems were identified in both accuracy and precision for serum folate (van den Berg et al., 1994). Considerable variability was observed both between methods and between laboratories using similar methods. Further, the authors noted that the Quanta-Phase I® kits tended to give higher values than other competitive protein-binding assays. The report suggested that the problem probably was associated with calibration of the kits and possibly the buffer pH (van den Berg et al., 1994).

Based upon the conclusions reached in October, 1991, the FLAIR participating laboratories initiated follow-up studies in several laboratories to identify the reasons for discrepancies in serum folate values obtained with various RA kits. In the second study, participating laboratories were asked to prepare "fresh" pooled serum samples spiked with either MTHF or PteGlu using a standardized protocol. All standards used for spiking were calibrated spectrophotometrically, and all samples were assayed immediately or within one month of storage at -20°C.

While some commercial RA kits did give high recoveries (120-130 percent) for externally calibrated PteGlu, both PteGlu and MTHF appeared to give similar responses in spiked samples. Recoveries of both MTHF and PteGlu were greater than 100 percent for all the RA kits except one (Amersham). Recoveries were also less than 100 percent for the HPLC and microbiological assays (van den Berg et al., 1994).

The second round robin study, Study 3, used human whole blood quality control samples in 1 percent ascorbic acid prepared by Bio-Rad Laboratories. These pooled samples were prepared with additional PteGlu and each laboratory analyzed three samples. During the interim period between completion of Study 2 and this study, two of the participating laboratories had switched from the Quanta-Phase I® kit to the Quanta-Phase II® kit containing recalibrated standards. Two laboratories added a microbiological assay utilizing L. rhamnosus. The results of Study 3 are summarized in Table 2.
Table 2. Results of FLAIR Round-Robin-Study 3: Whole Blood Folate (μM).

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of Laboratories</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quanta-Phase I®</td>
<td>1</td>
<td>164</td>
<td>811</td>
<td>415</td>
</tr>
<tr>
<td>Quanta-Phase II®</td>
<td>2</td>
<td>92</td>
<td>(109, 75)(^1)</td>
<td>371</td>
</tr>
<tr>
<td>Other RA kits</td>
<td>2</td>
<td>369</td>
<td>(520, 218)</td>
<td>882</td>
</tr>
<tr>
<td>Microbiological assay</td>
<td>3</td>
<td>206</td>
<td>(147, 248)</td>
<td>814</td>
</tr>
<tr>
<td>(L. rhamnosus; ATCC 7469)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemiluminescent assay</td>
<td>1</td>
<td>150</td>
<td>520</td>
<td>347</td>
</tr>
</tbody>
</table>

\(^1\) Numbers in parentheses represent the data from each laboratory used to generate the mean values.

The large variability of whole blood folate values, both within and between methods, seen in Study 3 is consistent with previous comparative reports (p. 5-9). Values obtained from the Quanta-Phase I® kit were considerably higher than those obtained from the Quanta-Phase II® kit, reflecting the recalibration of the PteGlu standard.

Overall, the FLAIR studies confirmed the existence of several issues reflecting on the accuracy and precision (pH of buffers and samples and use of either PteGlu or MTHF standards) of commercially available RA kits, including the Bio-Rad Laboratories kits. Van den Berg et al. (1994) concluded that their results indicated that most RA kits tended to "overestimate serum folate content due to improper standard calibration and the use of PteGlu rather than MTHF as the standard." Both fresh PteGlu and MTHF standard solutions produced values about 30 percent lower than PteGlu standards provided with the kits, suggesting that assigned values for kit PteGlu standards were too high. The issue of standards was underscored by the results of Study 3, which demonstrated lowered whole blood folate values from the Quanta-Phase II® kit which contains the recalibrated folate standard.

C. CDC AND BIO-RAD LABORATORIES STUDIES

The preliminary findings of the FLAIR studies were brought to the attention of the NHANES Laboratory, CDC, in mid-1992, and subsequently to the attention of Bio-Rad Laboratories, Inc. (Gunter, 1994a). They confirmed that the PteGlu standards in the Bio-Rad Laboratories kits (Quanta-Count™ and Quanta-Phase I®) had not changed significantly prior to 1992. CDC also confirmed that internal quality-control samples associated with NHANES III (1988-1991) analyses exhibited no significant downward trends (Gunter, 1994a).

In late 1992, CDC and Levine (1993) independently confirmed that Bio-Rad Laboratories Quanta-Count™ and Quanta-Phase I® RA kits had PteGlu standards that appeared to produce folate values that were about 30 percent higher than freshly prepared spectrophotometrically
confirmed PteGlu standards. These results were also confirmed by Bio-Rad Laboratories (Edwards, 1994). According to Edwards (1994):

"The original Quanta-Count™ B₂/Folate RA was first introduced in 1975, and the folate assay was calibrated to the Lactobacillus casei microbiological assay that was the accepted reference method at the time. The Quanta-Phase® assay, which was introduced in 1981, was calibrated to the Quanta-Count™ method, and no shift in folate recovery or normal range occurred with the new assay."

The following statements can be made based on information provided by Bio-Rad Laboratories and CDC to the LSRO ad hoc Expert Panel:

- The original Bio-Rad Laboratories Quanta-Count™ Folate RA kit was introduced in 1975 and was first used for NHANES II folate determinations in 1978.

- The PteGlu standards supplied by Bio-Rad Laboratories with the Quanta-Count™ and, subsequently, the Quanta-Phase I® RA kits were "adjusted to make them comparable to results obtained with the microbiological assay." This process was supported conceptually by analyses performed by the Expert Scientific Working Group (ESWG) 1984 review (Senti and Pilch, 1984) which had confirmed an acceptable correlation between the microbiological assay and the Quanta-Count™ RA kit but did not consider the possibility of changes in PteGlu standards over time.

- All subsequent lots of reference standards were "adjusted" to be comparable to the original lot of PteGlu standards used in the original calibration of the Quanta-Count™ kit. Bio-Rad Laboratories prepared reference standard lots every 1 to 2 years and compared each to the original reference standard. However, no spectrophotometric, high-performance liquid chromatographic, elemental analyses, or comparative assays with the microbiological assay were performed on the several lots of reference standards provided with the Bio-Rad Laboratories kits after the original calibration.

- While no data were made available to the Expert Panel to document this practice, other manufacturers of RA folate kits appear to have standardized their kits by use of analogous procedures for preparation and dilution.

- The Quanta-Count™ Folate RA kit introduced by Bio-Rad Laboratories in 1975 was replaced by the Quanta-Phase I® Folate RA kit which was released in 1983. The Quanta-Mate™ kit was produced in the period between the change from Quanta-Count™ to Quanta-Phase I®, however, the CDC never employed the Quanta-Mate™ kit in their analyses. Neither the Quanta-Count® nor the Quanta-Mate® kit is available any longer. Inasmuch as the Quanta-Phase I® RA kit was calibrated against the Quanta-Count™ kit when introduced, the apparent systematic error based on the PteGlu standards may have been perpetuated.

- As noted by Edwards (1994), Bio-Rad Laboratories introduced the Quanta-Phase II® RA kit in 1993, and the original Quanta-Phase I® RA kit is no longer available. The Quanta-Phase II® RA kit contains new PteGlu reference standards that presumably correct the systematic error associated with the previously prepared PteGlu reference standards.
The assessment of serum and RBC folate as part of national surveys conducted by NCHS began with NHANES II (1976-1980). According to CDC (Gunter, 1994a), the following chronological sequence of events occurred:

1976-1978: Folate was assessed via the *L. casei* microbiological assay. This method was used to assess approximately 40 percent of NHANES II (1976-1980) samples.

1978: Because of problems with quality control, CDC switched to the Quanta-Count™ RA charcoal separation kit and continued its use through the conduct of NHANES II (1976-1980) and the Hispanic Health and Nutrition Examination Survey (HHANES, 1982-1984).

1985: CDC switched to Quanta-Phase I® RA solid-phase (replaced charcoal with microbeads) folate kit. CDC performed a small comparative study with 22 samples and quality control (QC). The slope was 1.0045, y-intercept was -0.255, and $r^2$ was 0.9930.

1987: Pilot studies began for NHANES III (1988-1994) with the Quanta-Phase I® folate-only kit as the previously used Quanta-Count™ kits were no longer available. CDC's testing of kit performance and assurances from Bio-Rad Laboratories suggested that the standard materials were formulated from PteGlu in an identical manner for both kits.


September 1992: Discussion began with Dr. Levine, CDC, and Bio-Rad Laboratories about the calibration of the standards.

May-July 1993: CDC received the new standards prepared by Bio-Rad Laboratories and began comparison studies using both "old" and "new" standards.

July 1993: CDC began comparison studies between Quanta-Phase I® folate-only and Quanta-Phase I® folate/B12 combination RA kits.

August 1993: CDC began to run combination Quanta-Phase I® folate/B12 kits on serum samples from NHANES III stand 172 samples and continued to run Quanta-Phase I® folate-only assays for RBC and Quanta-Phase I® B12 only runs for all earlier NHANES III samples.

December 1993: CDC began to use the Quanta-Phase II® folate/B12 kits with the recalibrated standards in stands 175 and 178. This is about the halfway point in NHANES III, 1991-1994.

December 1993: After extensive consultation with NCHS, CDC recommended application of the correction factor to all of the previous NHANES III (1988-1991) and NHANES III (1991-1994) data collected prior to the switch to the Quanta-Phase II® B12/folate kit.
Based on the chronology and other information supplied by CDC, the following summary statements can be made:

- The CDC, beginning in 1978 with its use of the Quanta-Count™ folate kit for NHANES II (1976-1980) and through HHANES (1982-1984), followed by the use of the Quanta-Phase I® folate-only kit beginning in 1985, and through the introduction of the Quanta-Phase II® folate/B₁₂ kit in 1993, has performed both comparative testing and exhaustive testing of the performance characteristics of these kits. The quality control data presented to the Expert Panel are demonstrative of high precision reflected by the excellent reproducibility of the results from all of the folate kits used by CDC. Similarly, comparative data revealed no differences between the three methods or from the introduction of the combined folate/B₁₂ Quanta-Phase II® kit.

- The consistent excellence of the quality control data from the CDC tests confirms that the error in the Bio-Rad Laboratories kits was systematic and not related to the performance of the assays.

- Testing performed by the CDC confirms that the addition of the B₁₂ assay in the Quanta-Phase II® RA kit did not affect the performance of the kits in the assessment of serum and RBC folate.
III. PROPOSED CORRECTIVE PROCEDURES

By late 1993, inasmuch as the only reported difference between the Quanta-Phase I® and Quanta-Phase II® RA kits was the PteGlu reference standards, the CDC was convinced that the change of reference standards appeared to correct the systematic error. Consequently, the CDC designed a study intended to utilize data already collected and to make them comparable with new data derived from the newly calibrated Quanta-Phase II® kits. Specifically, the study was devised to develop a formula that would convert measurements calibrated by the "old" PteGlu reference standards from the Bio-Rad Laboratories Quanta-Phase I® RA kit to the "new" PteGlu standards from the Bio-Rad Laboratories Quanta-Phase II® RA kits, which had been confirmed spectrophotometrically by CDC.

Based on discussions with several investigators, the NHANES Laboratory at CDC undertook a comparison of serum and RBC folate values using the PteGlu reference standards included in the Quanta-Phase I® RA kit versus fresh PteGlu reference standards with folate concentration confirmed spectrophotometrically. In mid-1993, the NHANES laboratory, CDC, analyzed in duplicate 1833 NHANES III (1991-1994) blood samples (selected as received) and batches of laboratory quality control samples by using "old" (i.e., microbiological assay performance matched as in the Quanta-Phase I® RA kits) versus "new" spectrophotometrically verified folate standards (as in Quanta-Phase II® kits). The standards were provided by Bio-Rad Laboratories and the "new" standards were spectrophotometrically confirmed by CDC. The actual process occurred over several months and involved a total of 19 runs. Each run consisted of 6 "old" standards and 6 "new" standards (supplied by Bio-Rad and reflecting the standards in the 2 Quanta-Phase kits), 4 serum quality-control samples, 3 RBC quality-control samples, and NHANES III (1991-1994) specimens.

At the request of the LSRO ad hoc Expert Panel, the NHANES Laboratory reexamined the data and eliminated all serum folate values above 20 ng/mL. Analysis of data by error-in-both-variables (EBV) regression on 1754 of the NHANES III (1991-1994) blood samples indicated the slope and intercept were essentially similar to the values found with all 1833 samples (Smith, 1993).

Linear, least squares regression, and EBV regression were run on the logarithms of the measured concentrations for these sets of samples. Within-sample variation was assumed equal for EBV regression. The squared correlation coefficients for all sample sets were > 0.9978 and regression coefficients were similar. The slope and intercept of the regression line for 1754 blood samples (<20 ng/mL) are given in Figure 1. The correction factor formula derived from these analyses is:

$$\log_{10}(\text{corrected value}) = -0.1956 + 1.0199 \times \log_{10}(\text{uncorrected value})$$

This formula may also be expressed as follows:

$$\text{Corrected value} = 0.6374 \times \text{uncorrected value}^{1.0199}$$

Using the $\log_{10}$ based equation, a sample conversion from uncorrected to corrected for a value of 20 ng/mL is illustrated below:

$$\log_{10}20 = 1.30$$
$$1.30 \times 1.0199 = 1.33$$
$$1.33 - 0.1956 = 1.13$$
$$\text{inv log} 1.13 = 13.53 \text{ ng/mL}$$
Figure 1. Comparison of "Old" Quanta-Phase I® (x-axis) versus "New" Quanta-Phase II® (y-axis) Serum Folate Reference Standards (PteGlu) Prepared by Bio-Rad Laboratories for CDC (ng/mL).

Data from 19 separate runs of 1754 blood samples calibrated against both the "old" Quanta-Phase I® (x-axis) and "new" Quanta-Phase II® (y-axis) PteGlu standards provided to LSRO by the NHANES Laboratory, CDC, May 25, 1994 (Gunter, 1994b).
According to Smith (1993), the \( \log_{10} \)-based conversion factor produces a correction of -35.4 percent at a concentration of 2.0 ng/mL and a correction of -29.6 percent for RBC folate at a level of 150 ng/mL. RBC, the appropriate cutoffs for the determination of inadequate recent dietary intake and low folate stores indicative of megaloblastic anemia, respectively. The calculated mean of the correction factor over the range of 1.0 to 20.0 ng/mL is -33.8 percent.

While both additive and multiplicative bias exist when concentrations from "new" standards and "old" standards are compared, a high degree of linearity between the two methods is evident, suggesting that a linear interconversion is appropriate.

On a linear basis, conversion of data derived using "old" standards is accomplished by use of the formula:

\[
\text{corrected value} = -0.1411 + 0.6849 \times (\text{uncorrected value})
\]

A sample conversion from uncorrected to corrected for a value of 20 ng/mL is illustrated below:

\[
20 \text{ ng/mL} \times 0.6849 = 13.70 \text{ ng/mL}
\]

\[
13.70 - 0.1411 = 13.56 \text{ ng/mL}
\]

Thus all three equations (\( \log_{10} \), inverse log, and linear) yield essentially the same adjusted values.

The Expert Panel noted that both the \( \log_{10} \)-based equation and the inverse log equation (corrected=0.6374 x uncorrected value\(^{1.0199} \)) are equivalent. The third or linear equation is an approximation of the first two. All corrected data used in this report were generated using the \( \log_{10} \)-based equation.
IV. ISSUES RELATED TO THE INTERPRETATION OF NHANES DATA

A. CONSEQUENCES OF UTILIZATION OF ADJUSTMENT PROCEDURES ON DETERMINATION OF FOLATE STATUS

In an effort to examine the impact of using the proposed adjustment procedures on NHANES III (1988-1994) folate data, LSRO requested that NCHS provide preliminary tables of adjusted and unadjusted folate data from a representative sample of the NHANES III (1988-1991) data set. NCHS provided provisional analyses that demonstrated the differences between the "unadjusted" and the "adjusted" serum and RBC folate distributions using the age group 20-39 years. These selected results were derived from weighted sample analyses for persons aged 20-39 years; that is, the various means, percents, and percentiles take into account the survey design. These results were prepared solely to illustrate the use of the proposed corrective factors. Sample sizes (2,987 and 2,953 for serum and RBC folate, respectively) vary because of differential loss of data at the individual and laboratory levels. According to NCHS, for purposes of comparison, the difference in population number is inconsequential.

The application of the CDC-derived equation (Smith, 1993) would result in the following changes in the cutoff values:

<table>
<thead>
<tr>
<th>Early Reporting Cutoff Values</th>
<th>Old</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Folate (ng/mL)</td>
<td>&lt;3.0</td>
<td>&lt;1.9</td>
</tr>
<tr>
<td>RBC Folate (ng/mL RBC)</td>
<td>&lt;100</td>
<td>&lt;68.5</td>
</tr>
</tbody>
</table>

(Centers for Disease Control and Prevention, 1994).

The Division of Environmental Health Laboratory Services, CDC, has proposed the following reference ranges for the assessment of serum and RBC folate status (Gunter, 1994c):

<table>
<thead>
<tr>
<th>Normal Range</th>
<th>Indeterminate Range</th>
<th>Deficient Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old</td>
<td>New</td>
<td>Old</td>
</tr>
<tr>
<td>Serum Folate (ng/mL)</td>
<td>4.0-18.0</td>
<td>2.6-12.2</td>
</tr>
<tr>
<td>RBC Folate (ng/mL RBC)</td>
<td>150-600</td>
<td>103-411</td>
</tr>
</tbody>
</table>
The impact of the adjustment factor on serum and RBC folate levels across various percentiles is shown in Table 3. Figure 2 (serum folate) and Figure 3 (RBC folate) present data from the NCHS provisional analyses of folate values for the NHANES III (1988-1991) 20-39 year-old sample population. Application of the correction factor results in a leftward shift of the distribution for both variables. Tables 4 and 5 demonstrate the differences between adjusted and unadjusted data in the percentage of subjects below the "Early Reporting Values" as defined by CDC (Centers for Disease Control and Prevention, 1994). These tables also demonstrate the effect of applying the adjustment factor to the cutoff values.

Table 3. Unadjusted and Adjusted Serum and RBC Folate Values (ng/mL) for a Sample Population of Individuals Aged 20-39 Years.¹

<table>
<thead>
<tr>
<th>Percentiles</th>
<th>5th</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
<th>95th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Folate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>2.8</td>
<td>4.5</td>
<td>6.2</td>
<td>9.3</td>
<td>16.9</td>
</tr>
<tr>
<td>Adjusted</td>
<td>1.8</td>
<td>2.9</td>
<td>4.1</td>
<td>6.2</td>
<td>11.4</td>
</tr>
<tr>
<td>RBC Folate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>121</td>
<td>176</td>
<td>224</td>
<td>300</td>
<td>445</td>
</tr>
<tr>
<td>Adjusted</td>
<td>85</td>
<td>124</td>
<td>159</td>
<td>214</td>
<td>320</td>
</tr>
</tbody>
</table>


The data in Tables 4 and 5 are difficult to reconcile with data reported on NHANES II (1976-1980) and HHANES (1982-1984) folate status using the same criteria as indicators of "low folate" (Table 6). While the NHANES II (1976-1980) and HHANES (1982-1984) serum and RBC folate data are more or less equivalent in terms of the percentages of the adult population distribution with "low" serum and RBC folate values, the unadjusted NHANES III (1988-1991) data appear to be a closer match than the adjusted data. This is to be expected as these data were obtained using kits (Quanta-Count™ RA kit and Quanta-Phase I®) containing the same standards.

Applying the proposed corrective factors to a representative sample of NHANES III (1988-1991) provides an estimate of the percentage distribution of "normal" and "low" or "deficient" serum and RBC folate values based on generally accepted criteria for quantitative adequacy or inadequacy of folate levels (e.g., 3.0 ng/mL and 140 ng/mL RBC). However, doing so provides estimates of the prevalence of normalcy and deficient status that, by themselves, are suspect because of the manner in which they were generated. That is, the range of unadjusted estimates of low serum folate (<3.0 ng/mL) and the range of unadjusted estimates of the percentage population below the cutoff for normalcy (>140 ng/mL RBC) appear to match more closely the prevalence estimates cited for NHANES II (1976-1980) and HHANES (1982-1984) in Table 6.
Figure 2: Corrected and uncorrected mean serum folate values of persons 20-39 years of age and selected percentiles, NHANES III, Phase 1, 1988-91.
Figure 3: Corrected and uncorrected mean serum red blood cell folate values of persons 20-39 years of age and selected percentiles, NHANES III, Phase 1, 1988-91.
Table 4. Percentage of NHANES (1988-1991) Adult Subjects Age 20-39 Years with Serum Folate Values Below the Unadjusted or "Old" (<3.0 ng/mL) and Adjusted or "New" (<1.9 ng/mL) Early Reporting Cutoff Values.¹

<table>
<thead>
<tr>
<th></th>
<th>&quot;Old&quot; Standard</th>
<th>&quot;New&quot; Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
<td>Adjusted</td>
</tr>
<tr>
<td>Both Sexes</td>
<td>6.5</td>
<td>25.9</td>
</tr>
<tr>
<td>Males</td>
<td>5.9</td>
<td>26.8</td>
</tr>
<tr>
<td>Females</td>
<td>7.1</td>
<td>25.0</td>
</tr>
</tbody>
</table>


Table 5. Percentage of NHANES (1988-1991) Adult Subjects Age 20-39 Years with RBC Folate Values Below the Unadjusted or "Old" (<100 ng/mL RBC) and Adjusted "New" (<68.5 ng/mL RBC) Early Reporting Cutoff Values.¹

<table>
<thead>
<tr>
<th></th>
<th>&quot;Old&quot; Standard</th>
<th>&quot;New&quot;² Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
<td>Adjusted</td>
</tr>
<tr>
<td>Both Sexes</td>
<td>1.9</td>
<td>11.5</td>
</tr>
<tr>
<td>Males</td>
<td>1.2</td>
<td>8.6</td>
</tr>
<tr>
<td>Females</td>
<td>2.6</td>
<td>14.5</td>
</tr>
</tbody>
</table>

² As tabular representation of data is presented in whole digits only, data reflect percentages <80 ng/mL.
Table 6. Percentage of Populations with "Low Folate Status" (unadjusted) in NHANES II (1976-1980), HHANES (1982-1984), and NHANES III.1

<table>
<thead>
<tr>
<th></th>
<th>Serum Folate (&lt;3.0 ng/mL)</th>
<th>RBC Folate (&lt;140 ng/mL RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NHANES II (1976-1980)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males 20-44 yr</td>
<td>18.0%</td>
<td>8.0%</td>
</tr>
<tr>
<td>Females 20-44 yr</td>
<td>15.0%</td>
<td>13.0%</td>
</tr>
<tr>
<td><strong>HHANES (1982-1984)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(females 20-44 yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mexican-American</td>
<td>11.0%</td>
<td>3.5%</td>
</tr>
<tr>
<td>Cuban</td>
<td>9.8%</td>
<td>10.8%</td>
</tr>
<tr>
<td>Puerto Rican</td>
<td>8.3%</td>
<td>9.2%</td>
</tr>
<tr>
<td>Unadjusted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males 20-39 yr</td>
<td>5.9%</td>
<td>6.2%</td>
</tr>
<tr>
<td>Females 20-39 yr</td>
<td>7.1%</td>
<td>12.0%</td>
</tr>
<tr>
<td>Both Sexes</td>
<td>6.5%</td>
<td>9.0%</td>
</tr>
<tr>
<td>Adjusted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males 20-39 yr</td>
<td>26.8%</td>
<td>41.3%</td>
</tr>
<tr>
<td>Females 20-39 yr</td>
<td>25.0%</td>
<td>41.0%</td>
</tr>
<tr>
<td>Both sexes</td>
<td>25.9%</td>
<td>41.2%</td>
</tr>
</tbody>
</table>


If, on the other hand, the adjusted estimates for serum and RBC folate levels in the "low" or "deficient" range determined in NHANES III (1988-1991) are an accurate reflection of the folate status of the United States adult population, then the NHANES II (1976-1980) and HHANES (1982-1984) estimates are suspect. This conclusion is based on the observation that either previous estimates or the current projected estimates of folate deficiency are misleading because other studies of the United States population subgroups during the period 1976-1991 do not suggest that the prevalence of deficient folate status could be as high as 25-41 percent of the free-living adult population. However, prevalence could be higher than predicted from NHANES II (1976-1980) and HHANES (1982-1984) data. The Expert Panel recognized that while reference ranges should be derived from large population studies such as NHANES employing validated methods, discrepancies between such data and those supplied by well designed smaller surveys need to be examined and resolved. Due to historical considerations (changes in dietary habits, use of dietary supplements, etc.) and questions about the validity of the data related to the problems between the various iterations of the kits used to assess both serum and RBC folate, the Expert Panel has concluded that comparisons between survey data sets from NHANES II (1976-1980), HHANES (1982-1984), and NHANES III (1988-1991) are not advisable at this time. Moreover, the application of the adjustment factor to data from NHANES II (1976-1980) and/or HHANES (1982-1984) cannot be justified at this time.
With regard to data from NHANES III (1988-1994) derived from analyses with the Quanta-Phase I® kit, the Expert Panel has concluded that rather than using existing cutoffs with adjusted data, the more appropriate approach would be also to apply an adjustment factor to the previously established cutoffs. While the Expert Panel recognized that the data used to establish the existing cutoffs were derived from not only RA assays but also microbiological and other assays, the Expert Panel has concluded that some adjustment is required.

B. REEXAMINATION OF VALUES FOR NORMALCY AND DEFICIENCY STATUS

In 1983, the LSRO convened an ad hoc Expert Scientific Working Group (ESWG) for the purpose of assessing the folate status of the United States population based on data from NHANES II (1976-1980) (Senti and Pilch, 1984). This review and evaluation study was undertaken, in part, because of the switch in 1978 from the L. casei microbiological assay for serum and RBC folate to the Quanta-Count™ charcoal separation 125I folate kit. In reviewing available literature, the ESWG found a range of values delineating "deficiency range" and "normal range" (Table 7) (Senti and Pilch, 1984). Based on their analysis of extant data at that time, the ESWG concluded:

**Serum Folate:**

- As shown in Table [7], most studies of the distribution of subjects with proven deficiency of folate have found that serum folate values more than 3 or 4 ng/mL will exclude almost all deficient subjects. The most common demarcation value reported is 3 ng/mL (Arroyave, 1971; Colman et al., 1974; Herbert, 1964, 1966; O’Neal et al., 1970; Sauberlich, 1977, Sauberlich et al., 1974; World Health Organization, 1972).

- . . . the study of effect of serum folate on either hemoglobin concentration or mean corpuscular volume (MCV) did not assist in defining a range of [folate] deficiency.

- Correlation between microbiological and radioassay data was sufficiently high with serum folate to suggest that the range of deficiency would be similar by both assays.

- Based on the above considerations, the range of serum folate considered to include the majority of subjects with folate deficiency was taken as below 3 ng/mL.

**RBC Folate:**

- As indicated in Table [7], the upper limits of RBC folate concentration associated with deficiency in three laboratories ranged from 137 to 175 ng/mL RBC. Because of the large coefficient of variation of this determination, the deficient range in some hospital laboratories is permitted to extend higher to include all subjects deficient in folate, thus increasing sensitivity with loss of specificity.

- No effect on median hemoglobin concentration or MCV could be detected in the population studied [NHANES II (1976-1980)] when subjects with RBC folate values less than 140 ng/mL were excluded from the calculation, even after exclusion of subjects with evidence of iron deficiency.

- The ESWG also considered that the deficient range for RBC folate should contain fewer subjects than that for serum folate because serum values fluctuate more rapidly than RBC values.
Table 7. Deficiency and Normal Ranges of Serum and RBC Folate Concentrations Reported by Various Investigators.1

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Methoda</th>
<th>Deficiency Range, ng/mL</th>
<th>Normal Range, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum Folate</td>
<td>RBC Folate</td>
</tr>
<tr>
<td>Baker et al. (1969)</td>
<td>Micro</td>
<td>&lt;1 - 3.3 (8)</td>
<td></td>
</tr>
<tr>
<td>Cooper and Lowenstein (1961)</td>
<td>Micro</td>
<td>&lt;3 (12)</td>
<td></td>
</tr>
<tr>
<td>Cooper and Lowenstein (1964)</td>
<td>Micro</td>
<td>&lt;3.0 (14)</td>
<td>&lt;175 (14)</td>
</tr>
<tr>
<td>Cooper and Lowenstein (1964)</td>
<td>Micro</td>
<td>&lt;2.5 - &lt;3.9* (6)</td>
<td></td>
</tr>
<tr>
<td>Hansen and Weinfield (1962)</td>
<td>Micro</td>
<td>&lt;3.0 (8)</td>
<td></td>
</tr>
<tr>
<td>Hoffbrand et al. (1966)</td>
<td>Micro</td>
<td>0.4 - 4.9 (40)</td>
<td>8 - 143 (40)</td>
</tr>
<tr>
<td>Izak et al. (1961)</td>
<td>Micro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnus (1967)</td>
<td>Micro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McGown et al. (1978)</td>
<td>Micro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McGown et al. (1976)</td>
<td>RA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McGown et al. (1978)</td>
<td>Micro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McGown et al. (1978)</td>
<td>RA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minney et al. (1973)</td>
<td>RA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molin et al. (1962)</td>
<td>Micro</td>
<td>0.2 - 5.8 (121)</td>
<td></td>
</tr>
<tr>
<td>Narayanan et al. (1956)</td>
<td>Micro</td>
<td>0.3 - 2.6 (13)</td>
<td></td>
</tr>
<tr>
<td>Rothenberg et al. (1972)</td>
<td>RA*</td>
<td>0 - 3.7 (22)</td>
<td></td>
</tr>
<tr>
<td>Rothenberg et al. (1972)</td>
<td>Micro</td>
<td>1 - 3.3* (10)</td>
<td></td>
</tr>
<tr>
<td>Rothenberg et al. (1974)</td>
<td>RA*</td>
<td>25 - 137 (12)</td>
<td></td>
</tr>
<tr>
<td>Santini et al. (1966)</td>
<td>Micro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wetters and Molina (1961)</td>
<td>Micro</td>
<td>0.6 - 3.5 (22)</td>
<td></td>
</tr>
</tbody>
</table>

1 Table 14 in Seni and Fisch (1984).
2 Range associated with megaloblastic anemia. Numbers in parentheses indicate number of subjects examined.
3 Micro = microbiological method using L. casei; RA = radioassay based on competitive protein binding.
4 Patients with mild deficiency of folate associated with iron deficiency anemia.
5 Bia-Bad Quinova Coor® folate kit.
6 Nonsensical method.
7 Values taken from Figure 6 in Rothenberg et al. (1972).
8 RBC levels in 1-year-old infants. Formiminoglutamic acid and uracilic acid excretion were similar to that in adults at histidine loads less than 300 mg/kg body weight.
Based on the above considerations, it seemed desirable to select a cutoff value below 150 ng/mL as indicative of low RBC folate. Somewhat arbitrarily, a value of 140 ng/mL was selected to include most subjects with folate deficiency.

The cutoff values for serum and RBC folate developed by the ESWG were subsequently used as criteria for describing prevalences of low values for serum and RBC folate levels in Hispanic women (Life Sciences Research Office, 1989). These same values are reflected in the reporting cutoff values suggested by the NCHS for interpretation of NHANES III (1988-1994) data (in ng/mL) (Centers for Disease Control and Prevention, 1994).

As stated by the ESWG in 1984, its conclusions on use of 3.0 ng/mL for serum and 140 ng/mL for RBC folate were "somewhat arbitrary." The issue of cutoff values continues to be unresolved. The International Council for Standardization in Hematology (ICSH) has established an Expert Panel on Megaloblastic Anemia (Gunter, 1994d). The ICSH Panel has determined that its first task will be the establishment of an International Red Cell Folate Standard. The ICSH Panel, chaired by V. Herbert (USA), is currently preparing test materials and protocols for testing in late 1994 (Gunter, 1994d). Results of the ICSH Panel studies are critical not only to definition of the occurrence of megaloblastic anemia but also to resolution of the definition of folate deficiency.

Considerable uncertainty exists in regard to the definition of folate deficiency based solely on cutoff values for serum and RBC folate values. Lacking documentation, confirmation, and consistency among authoritative experts of what serum and RBC folate cutoff values constitute "normalcy," "at-risk," "low," "indeterminate," or "deficiency," interpretation of the NHANES III (1988-1991) adjusted or unadjusted data appears premature and open to question.

With regard to the application of the adjustment factor to current standards, the Expert Panel emphasized that such adjustments are relevant only to the data derived by CDC using the Bio-Rad Laboratories Quanta-Phase 1® RA kit, i.e., NHANES III (1988-1991).

Irrespective of what cutoff values are used, the Expert Panel concluded that there is an inherent inadequacy in the reliance of single indices as sole determinants of inadequate folate nutriment. Indeed, the use of one clinical or biochemical measure as a sole criterion of either nutritional deficiency or toxicity is increasingly being recognized as scientifically inappropriate (Sauuberlich, 1990).

In summary, neither correction of the serum or RBC folate data from NHANES III (1988-1991), the use of the data without correction, nor use of either data set with adjusted criteria for normalcy and deficiency, by themselves, can predict the prevalence of inadequate folate nutriment of the United States population. As indicated previously (Senti and Pilch, 1984), corroborative and confirmatory measures other than serum and RBC folate are essential to accurate determination of prevalence of folate deficiency.

C. APPROACHES TO COMPARABILITY OF SERUM AND RED BLOOD CELL FOLATE AMONG NATIONAL SURVEYS

and standardization of PteGlu reference solutions must be resolved and procedures codified to ensure that similar problems may be avoided in the future. For the purpose of analysis of NHANES III (1988-1991) and NHANES III (1991-1994) data, the application of the adjustment factor as proposed by CDC appears to be appropriate at this time.

Ideally, the corroboration of the validity of the adjustment factor should have come from a carefully planned analysis of a statistically valid number of NHANES III (1988-1991) and NHANES III (1991-1994) blood samples using both the Quanta-Phase I® and II® RA kits, the same protocols, and externally calibrated standards. In effect, the CDC performed such a comparison with incoming fresh NHANES III (1991-1994) samples. However, the Quanta-Phase I® kit is no longer available, thereby eliminating the possibility of a more comprehensive NHANES III (1988-1991) and NHANES III (1991-1994) comparison. In addition, while aliquots of never-thawed NHANES III (1988-1991) serum samples are available, comparable samples of red blood cells are not.

While reanalyses might have confirmed the comparability of NHANES III (1988-1991) and NHANES III (1991-1994) data, interpretation of these data presents a second, broader issue. The ad hoc Expert Panel recommended that additional study of parameters of folate status other than serum and RBC folate levels in NHANES III (1988-1991) and NHANES III (1991-1994) subjects is essential. Functional parameters include neutrophil hypersegmentation, serum or plasma homocysteine levels, and various indicators of iron status. In addition, the study should consider dietary intake of folate, age, socioeconomic level, and various life-style factors such as smoking, alcohol consumption, dietary supplement use, and oral contraceptive use.

The ad hoc Expert Panel has concluded, on the basis of the preliminary analysis of NHANES III (1988-1991) data, that comparison of NHANES III data with NHANES II (1976-1980) data on serum and RBC folate would be inappropriate at this time. The accuracy and reliability of the NHANES III (1988-1991) data need to be resolved before further critical consideration is directed to comparisons with data from previous national surveys. Questions that remain to be addressed with regard to the comparability of the various data sets include: (1) What purpose would be served by reexamination of extant data if reanalyses of blood samples from these surveys is not possible? (2) Even if possible, would reanalysis of NHANES II (1976-1980) and/or NHANES (1982-1984) blood samples be cost-effective if resources are limited? (3) Would available resources be better focused on the resolution of the issues associated with NHANES III (1988-1991) and NHANES III (1991-1994) data? and, (4) Is there a better model available using national survey data to predict folate status of the United States population? The Expert Panel concluded that the interpretation of serum and RBC folate data in relation to folate status requires a more concise definition of deficiency and the parameters that should be included in that definition.
V. SUMMARY AND CONCLUSIONS

A. SUMMARY

- Overestimation of serum and RBC folate values has been observed with several commercially available RA kits. These observations extend over several years and include the Quanta-Count™ RA kits used in NHANES II (1976-1980) and HHANES (1982-1984) and the Quanta-Phase Ⅰ RA kit used in NHANES III (1988-1991) through approximately the first half of NHANES (1991-1994). These observations have been confirmed independently by several laboratories in North America and Europe.

- The Expert Panel noted differences in the performance characteristics between assays for serum and RBC folate that are related to the nature of the forms of folate being assessed. As MTHF (monoglutamate form) is the predominant vitamer in serum (as compared to various other forms of the vitamin in RBC), the serum assay has demonstrated a more consistent and less variable performance relative to the microbiological assay than the RBC assay.

- The PteGlu reference standards provided with the Quanta-Phase Ⅰ RA kits used in NHANES III (1988-1991) and part of NHANES III (1991-1994) were calibrated against the PteGlu reference standards developed originally for the Quanta-Count™ RA kits in 1975. The original standards were developed based on a comparison with values derived from microbiological assays. All subsequent lots of reference standards were "adjusted" to be comparable to the original lot of PteGlu standards used in the original calibration of the Quanta-Count™ kit. Bio-Rad Laboratories prepared reference standard lots every 1 to 2 years and compared each to the original reference standard. However, no spectrophotometric, high-pressure liquid chromatographic, elemental analyses, or comparative assays with the microbiological assay were performed on the several lots of reference standards provided with the Bio-Rad Laboratories kits after the original calibration.

- Scientists from Bio-Rad Laboratories have attributed the original problem to the standard materials used to calibrate the original Quanta-Count™ kit in 1975, rather than the adjustment of the RA results to the microbiological results (Bussell, 1994). According to Bussell (1994) there was no analytical discrepancy; however, because of the lack of written documentation or available samples of materials used in the standardization process, the actual reason for the discrepancy leading to the assignment of incorrect values to the internal standards can be hypothesized but cannot be determined with certainty.

- The continual use of PteGlu reference standards without reconfirmation by spectrophotometric analysis or comparison with other methods, such as the microbiological assay, led to perpetuation of a systematic error resulting in overestimation by about 30 percent of serum and RBC folate values in NHANES III (1988-1991) and NHANES III (1991-1994) samples assayed with the Quanta-Phase Ⅰ RA kit.

- Analyses of NHANES III (1991-1994) blood samples with RA kits containing the "old" and "new" PteGlu reference standards have been conducted by the
manufacturer and the CDC NHANES Laboratory. Similar studies have been conducted by the European FLAIR collaborating laboratories. These analyses have led to preparation of "new" PteGlu reference standards for inclusion in the Quanta-Phase II® RA kits and to the suggestion that NHANES III (1988-1994) data derived from the Quanta-Phase I® RA kit be adjusted or corrected by means either of the following formulas:

\[
\log_{10}(\text{corrected value}) = -0.1956 + 1.0199 \times \log_{10}(\text{uncorrected value})
\]

or

\[
\text{corrected value} = 0.6374 \times \text{uncorrected value}^{0.999}
\]

or the linear approximation

\[
\text{corrected value} = -0.014 + 0.685 \times \text{uncorrected value}
\]

The adjustment factors appear to be linear over the range of low- to high-normal serum and RBC folate values.

B. CONCLUSIONS

- Based on the available information, the discrepancy associated with Quanta-Count™ and Quanta-Phase I® RA kits is most likely an analytical problem associated with the internal PteGlu standard. The Expert Panel has seen no evidence to support the possibility that the error in the kits is related to binding protein and any other biochemical component of these methods.

- If the adjustment factor is applied to the data set from NHANES III (1991-1994) derived from the use of the Quanta-Phase I® RA kit, the cutoff values should be adjusted as well. The **Expert Panel emphasized that this adjustment should apply only to NHANES III (1988-1994) data derived from the use of the Quanta-Phase I® kits.**

- The application of the adjustment to the cutoff values is a mathematical manipulation only; the broader issue of defining clinical folate deficiency remains. Neither adjustment of the serum or RBC folate data from NHANES III (1988-1994), the use of the data without adjustment, nor use of either data set with adjusted criteria for normalcy and deficiency, by themselves, can predict the prevalence of inadequate folate nutriture of the United States population. As indicated by previous authoritative groups, corroborative and confirmatory measures in addition to serum and RBC folate are essential for the accurate determination of prevalence of folate deficiency.

- To avoid problems with RA analyses of serum and RBC folate samples in NHANES III (1991-1994), no changes in the Quanta-Phase II® RA kits should be made during this period, and PteGlu reference standards provided with the Quanta-Phase II® RA kits should be externally verified on a regular basis by calibration with highly purified PteGlu and/or MTHF or an equivalent internationally acceptable reference standard.

- In order to evaluate the comparability of data sets from different phases of NHANES III, the following comparisons should be made:
- NHANES III (1991-1994) prior to switch to NHANES III (1991-1994) after the switch; and

- Publication of NHANES III (1988-1991) data should be done with both adjusted and unadjusted data.

- Comparison of NHANES III (1988-1991) data with NHANES II (1976-1980) data on serum and RBC folate would be inappropriate at this time. Similarly, application of the adjustment factor to NHANES II (1976-1980) data would be inappropriate. The issue of methodological consistency and standardization of PteGlu reference solutions must be addressed first. Application of an adjustment factor to NHANES III (1988-1991) data, as proposed by CDC, may be acceptable; however, the appropriateness of the approach should be based on carefully planned reanalyses of a statistically valid number of NHANES III (1988-1991) and NHANES III (1991-1994) blood samples using the same RA kits, the same protocols, and externally calibrated PteGlu and/or MTHF reference standards.

- To determine the validity of using NHANES III (1988-1991) and NHANES III (1991-1994) folate data as components of an approach to prediction of folate status of the United States population, additional evaluation of parameters of folate status other than serum and RBC folate levels in NHANES III (1988-1991) and NHANES III (1991-1994) subjects is essential. Functional parameters include serum or plasma homocysteine levels, neutrophil hypersegmentation, and various measures of iron status. In addition, this evaluation should consider the effects of dietary intake of folate, age, socioeconomic level, and various life-style factors such as smoking, alcohol consumption, dietary supplement use, and oral contraceptive use. Comparison of previously collected national survey data on folate nutriture with NHANES III folate data should be deferred until the above issues are addressed.

- While the Expert Panel recognized the need to address the adequacy and validity of commercially available kits for the assessment of folate status in clinical populations, the Expert Panel emphasized that the discussions and conclusions presented in this report apply only to those data from NHANES III (1988-1994) generated from CDC laboratories.

- In light of new data regarding folate metabolism and disease, the question of the functional ramifications of current or future cutoffs defining folate status should be reexamined.

- The criteria used to establish the functional relevance of the cutoff levels developed by previous authoritative groups are insufficient in light of current knowledge about folate metabolism in health and disease. If, however, the previous cutoff levels were to be used for comparative purposes, the proposed adjustment formula must be applied to data derived from use of these RA kits. The Expert Panel suggested that the new, adjusted values may be compared with hematologic data to determine if the relationship between hematologic values and cutoff values that provided the basis for establishing the present cutoff values remains valid.
VI. LITERATURE CITED


Gunter, E.W., Centers for Disease Control and Prevention (CDC), Division of Environmental Health Laboratory Sciences. 1994c. Laboratory protocol: folate/vitamin B₁₂ serum and whole blood radioassay.


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VIII. INDIVIDUALS AND ORGANIZATIONS SUBMITTING INFORMATION ON FOLATES

A. OPEN MEETING PARTICIPANTS

The Open Meeting on the Assessment of Folate Methodology Used in the Third National Health and Nutrition Examination Survey (NHANES III) was held July 15, 1994. Oral presentations were made by two (2) individuals and organizations. The following two (2) individuals participated in the presentations:

Jacob Selhub, Ph.D., Tufts University, Boston, Massachusetts

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B. WRITTEN COMMENTS

The following organizations and individuals submitted written materials for consideration by the ad hoc Expert Panel:

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1 Copies of the Open Meeting transcript available from:

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