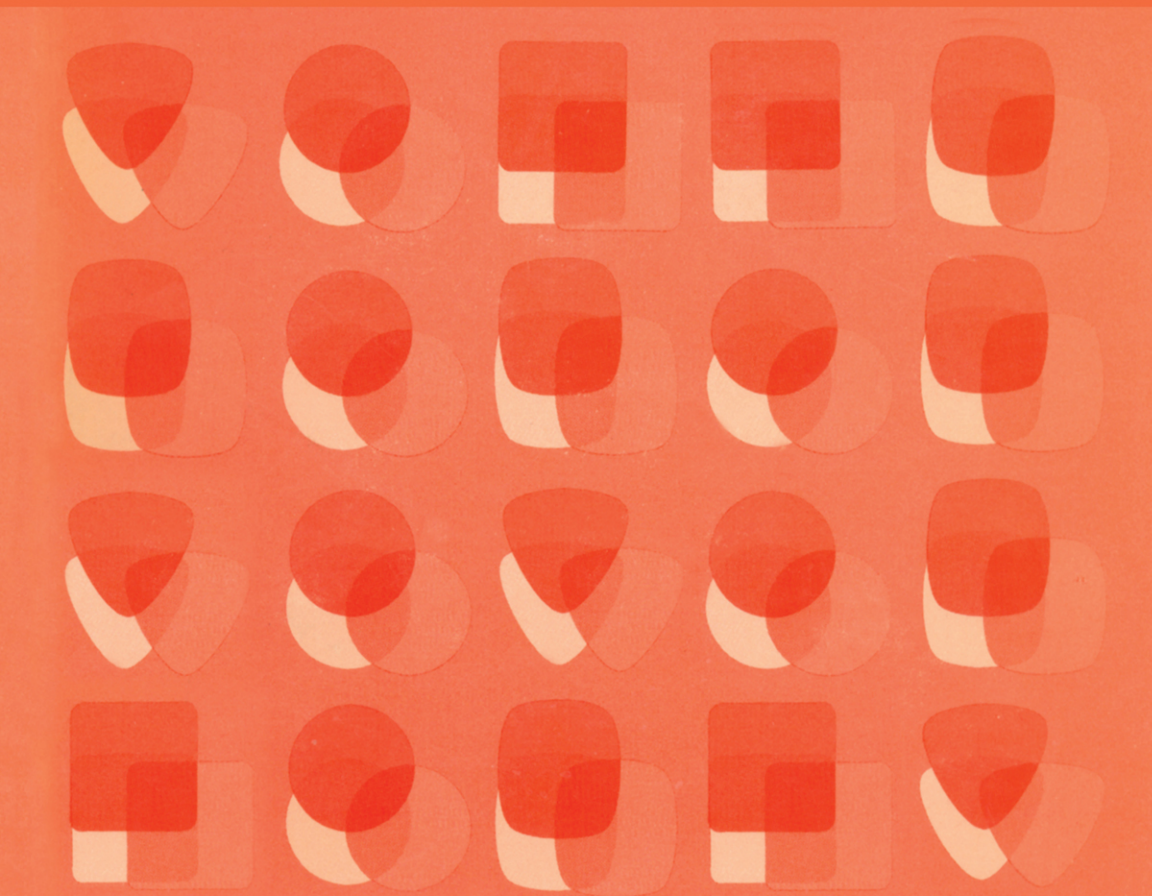
THE VITAMINS

Chemistry, Physiology, Pathology, Methods

volume VI second edition

Edited by Paul György and W. N. Pearson



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THE VITAMINS

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SECOND EDITION

VOLUME VI

VOLUME I—VOLUME V

Edited by

W. H. SEBRELL, JR. and ROBERT S. HARRIS

VOLUME VI and VOLUME VII

Edited by

PAUL GYÖRGY and W. N. PEARSON

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SECOND EDITION
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Preface

The first edition of "Vitamin Methods" edited by P. György appeared more than 15 years ago. Shortly thereafter, the three-volume treatise entitled "The Vitamins" edited by H. Sebrell and R. Harris appeared and filled the gap that occurred in the literature at that time. When it developed that both publications were scheduled for revision at approximately the same time it seemed appropriate that "Vitamin Methods" should become part of this comprehensive work.

The appearance of "Vitamin Methods" as Volumes VI and VII of this edition necessitated certain changes both in format and in subject matter. The overall effect is that of a new publication rather than a revision of the first edition. The presentation of the material is organized around the various vitamins with the physical, chemical, microbiological, and animal assays for each vitamin being discussed in a single chapter. This change reduced much of the overlapping seen in the first edition, and, we trust, will make this one more convenient to use. Another innovation—insertion of the references as page footnotes—gives the reader easy access to the literature without the annoyance of having to refer constantly to the end of the chapter.

The shifts in technology that have occurred during the past 20 years are evident. Intermingled with the standard procedures that were methods of choice 20 years ago and which are still methods of choice today, the reader will find new chromatographic procedures, enzymatic methods, and isotope dilution techniques. Some of the latter are suitable for routine analyses while others are principally research techniques. Some of the other techniques described here have not yet had the benefit of testing in many laboratories. They have been included because of their potential value in special analytical situations. Although in many cases continued emphasis on animal tests seemed justified, many of them detailed in the first edition have been eliminated. Included are those animal assays which, in the opinion of the authors, were considered "best." The chapter on statistical treatment of assay data was retained from the previous edition but in a re-edited form. The editors and authors invite comments, suggestions, and recommendations, particularly in regard to the new methods.

Finally, the cooperation and patience of the contributors and the publishers should not go unrecognized.

August, 1967

Paul György
W. N. Pearson

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CHAPTER 1

ANIMAL ASSAYS FOR VITAMINS

C. I. BLISS AND PAUL GYÖRGY

Introduction

Assays with animals have been indispensable for the development of our present knowledge of the vitamins. Often their purpose has been to test whether particular preparations could cure deficiency symptoms. In the isolation of individual vitamins from natural sources, much research has been guided by the results of such animal assays. As the final criterion for identification of vitamins, animal assays are not likely to be superseded by microbiological techniques and even less by physical and chemical methods of analysis. For purposes of control or for routine clinical-chemical studies, however, assays with animals have been largely replaced by microbiological or chemical and physical techniques.

Despite their importance in research and for validating alternate methods of assay, the animal assays for the vitamins have been needlessly inexact. One objective of the present chapter is to emphasize the methods that lead to quantitative estimates of potency and of their reliability. Precautions developed for some vitamins seem to have been overlooked in similar assays of other vitamins, so that many opportunities exist for improving the assay of one vitamin from knowledge gained with another. Some of these possibilities are suggested in the present chapter. The design

and statistical analysis of vitamin assays are developed systematically in Chapter 2 on "Statistical Methods in Biological Assay of the Vitamins." For general aspects of small animal experimentation such as breeding of animals, establishing animal laboratories, etc., see Guerrant.[1]

Several methods have been developed for assay of each vitamin, each with its own advantages and limitations. The technical skills required differ widely. A laboratory that has the necessary equipment and experience may prefer an assay that is inherently more complex but makes use of its existing facilities and personnel. The animal assays for a given vitamin are included in the chapter dealing with the vitamin in question. They have been restricted to the most commonly used and most reliable techniques.

I. Precision and Its Measurement

There are two approaches to the problem of ensuring reproducible results from animal biological assays. One is to specify its exact conduct in meticulous detail. This implies that if everyone were to follow exactly the same procedure, each should arrive at the same result. If in fact a given technique measures the vitamin activity of an unknown preparation, an alternative technique for measuring the same activity should lead to the same answer. If not, one would question the validity of one or both methods or the applicability of the results of either to a different species. A more realistic approach is to allow greater flexibility in experimental detail, but to determine the standard error or confidence limits of each determination as an integral part of the assay. A method that works well in one laboratory may be quite unsatisfactory in another, but its exact details are of little consequence if we know the inherent precision of an estimated potency and the general method by which it was obtained.

Much of the precision of an assay is determined by its design and also by the method of evaluating the results. A basic principle is to administer at least one vitamin preparation in each assay at two or more dosage levels, in order to provide a quantitative estimate of the change in response that corresponds to a known change in dose. A second principle is that an assay must be comparative, with concurrent observations on the test material or unknown and on the reference preparation or standard. Given these two prerequisites, the activity of the unknown can be expressed in terms of an equivalent quantity of the standard rather than of units of response. Living material is subject to so many variations, both inherent and environmental, that considerable ingenuity may be required to keep

[1] N. B. Guerrant, *in* "Vitamin Methods" (P. György, ed.), Vol. 2, pp. 1–40. Academic Press, New York, 1951.

them from invalidating an assay or destroying its precision. This is a function of experimental design. To avoid bias it is essential to assign animals to treatments or doses with some element of randomization. This is assumed in the statistical procedures for measuring either potency or its experimental error, and it should be done by an objective physical process and not by "thinking up" a "random" arrangement. Finally, each assay should carry a measure of its inherent precision to guide the experimenter in determining its reliability.

For a given method, an assay is most precise when the average response on the unknown equals that on the standard. The larger the difference between these averages, the greater is the error of the estimated potency. Since this discrepancy cannot be predicted in advance, any estimate of the inherent precision of a method assumes necessarily that the difference in mean response departs negligibly from zero. In most animal assays for vitamins, some function of the response can be plotted linearly against the logarithm of the dose. In the assay of a single unknown two such curves are determined, one for the standard and one for the unknown. If the two preparations are qualitatively alike, these lines should be parallel within the experimental or sampling error, and the horizontal distance between them estimates the relative potency of the unknown in logarithmic terms.

The precision of this estimate depends upon the standard deviation λ, as measured on the log-dose scale; λ, in turn, is equal to the ratio of s, the standard deviation of the response about the dosage-response line, to b, the slope of the line, or $\lambda = s/b$. This is the most generally useful single term for comparing the precision of several alternate assay techniques. Since λ is not an absolute term but a statistic computed from experimental data, it is as subject to experimental error as an assayed potency and this is measured by its standard error.

The precision of a given assay may be reported in terms of its standard error or, more exactly, of its fiducial (or confidence) limits, and both may be expressed as percentages of the assayed potency. These depend upon the number of responses observed with the standard and the number with the unknown preparation. They give a range that should include the true potency of the unknown in a predetermined proportion of tests. The experimenter may require, for example, that the limits computed from the experimental data should bracket the true potency, which is being estimated by each assay, in 19 of 20 cases. In comparison with the standard error of an assay, these fiducial (or confidence) limits are less likely to encourage a feeling of false security as to an assay's precision.

Both the standard error and the fiducial (or confidence) limits of the assayed potency are proportional to their λ's. The error of an estimated potency can always be reduced, by increasing the number of observations