At the time when an increasing number of clinical conditions were recognized as deficiencies of one or another chemical substance, such so-called vitamins were measured by their effect as supplement to a basal diet upon the growth and health of some species of laboratory animals. Vitamins are organic substances of natural origin, required in minute quantities, and their effects are not based on their caloric value, but exclusively on their catalytic nature. Elucidation of their structure and biosynthesis are exciting chapters of organic chemistry, and their identification with the prosthetic groups of vital enzymes is a signal contribution to biochemistry.

The recognition of their structure permits the determination of vitamins by the tools of analytical chemistry, but while such methods are widely used in industrial production, the minute quantities in body fluids and tissues limit the purely chemical approach to a few members of this group present in relatively high concentration; e.g., vitamin C. Microchemical methods are in use for the determination of thiamine, riboflavin, and some of the fat-soluble vitamins, on the basis of the most sensitive colorimetric and, in particular, fluorometric technics. Vitamin D, on the other hand, is determined by animal assay.

Vitamins, especially the water-soluble ones, provide the co-enzymes for the most fundamental cellular reactions. Thus, their presence is
required by vertebrates and invertebrates as well as by monocellular organisms, such as bacteria and other fungi, and by protists. One finds in nature many microorganisms which need those vitamins that they are unable to produce at all or in sufficient quantities. Thus, one has a wide choice of microbes for the assay of vitamins (1).

Nutritional deficiency diseases are relatively rare in the temperate zone. The etiology of numerous other clinical conditions involves vitamin deficiencies, due to faults in absorption, transfer, or utilization. Because of the central position of the vitamins as source of coenzymes, such functional deficiencies are important in malabsorption, where the picture is often complicated by multiple deficiencies; in anemias, where the defect is in general highly specific; and in many other diseases where the deficiency is secondary to other pathologic events, but nevertheless of grave consequences.

Microbiological assays are applicable when a microorganism responds to a metabolite for which physical and chemical determinations are neither sensitive nor specific enough. One must select a microorganism sensitive to the substance under assay: it should be cultivable with ease, the growth response should be easily measurable, and the response should be specific; it should be nonpathogenic. Systems in which the vitamin only stimulates growth are impractical. The culture medium must not support any growth without the vitamin and permit full growth with it.

The growth of the microorganism, measured most simply by optical density (O.D.), should be linearly proportional to the concentration of vitamin over an appreciable range. A graph of O.D. as ordinate, and concentration as abscissa should thus yield a straight line passing through the origin; its slope should be such that it shows, at double concentration, double absorbance. If the log of the concentration rather than concentration itself is used as abscissa, the curve has the well-known sigmoid shape. The O.D. of the unknown is read on the standard curve and the abscissa of this point gives the vitamin concentration of the sample as tested. The vitamin content of the original sample is computed from this value. The titer of the vitamin, tested at three different concentrations, should yield values falling within a range of 10 per cent one from another.

The standard curve will, in some instances, not follow a straight course or will have a "wrong" slope. If the slope is too steep—i.e.,
if the double concentration gives more than double a value of absorbance, one will suspect the presence of an inhibitor or toxic factor relatively more effective at lower concentrations, or of a stimulating factor relatively more effective at higher concentrations. In the case of strongly colored samples, optical compensation can be achieved in the reading of the standard curve by insertion of a noninoculated sample at the proper dilution, or insertion of suitable color filters.

Microbiological assays should stress accuracy over precision. Standardization of an assay method should include comparisons with at least one other organism having a different nutritional pattern and specificity towards the compound being assayed. Such a comparison was made for cyanocobalamin (vitamin $B_{12}$) content of human blood and serum, using four microorganisms differing in their cobamide requirements and metabolism (2).

A good basal growth medium is part of a microbiological assay; it must contain appropriate concentration of all stimulating factors likely to be encountered. Many media prove good enough for purified materials, but are unsatisfactory for biological fluids such as blood and serum, and for cells and tissues.

Before the introduction of the microbiological assay for vitamin $B_{12}$ in human serum, deficiency could be diagnosed only by symptoms of hematological stimulation upon administration of liver extract or vitamin $B_{12}$ preparations. A similar problem was experienced with folic acid. Because folic acid is metabolically related to vitamin $B_{12}$, microbiological assays had to be devised for different diagnosis. This has been solved not only for the vitamin $B_{12}$-folic acid pair, but also for thiamine, pantothenic acid, nicotinic acid, biotin, folinic acid, vitamin $B_6$ (pyridoxamine and pyridoxal), and unconjugated pteridines (3). These additions to the diagnostic arsenal have proved their value for assaying the titer of the respective vitamins in blood, serum, urine, and cerebrospinal fluid, and in tissues.

During the last 12 years our work has centered on investigations of the role played by vitamins in the etiology and symptomatology of metabolic diseases. At the time of the inception of our efforts in this field, the determination of vitamins had been limited to chemical methods. Since then, we have developed numerous microbiological assay methods for the estimation of the various members of the vitamin $B$ group.
Nucleogenic Vitamins

In the field of vitamin B\textsubscript{12}, we have contributed a series of studies comprising a comparison of various assay organisms, elucidation of the distribution of vitamin B\textsubscript{12} between body fluids and tissues, and investigation of subjects of clinical significance.

Moreover, our work has resulted in the establishment of a \textit{Lactobacillus casei} test for the determination of folic acid on a routine basis, which has received general recognition here and abroad. We have also developed assays for the important folinic acid (citrovorum factor) and for unconjugated pteridines. The development and improvement of these assays were followed by, and in most instances interwoven with, their application to physiological and pathological situations.

The diagnosis and therapeutic management of pernicious anemia and the complex of related anemias, including those following upon gastric surgery, require accurate routine methods for the determination of vitamin B\textsubscript{12} and folic acid in blood. We had developed and used an 18-hour folic acid microbioassay, using a thermophilic \textit{B. coagulans}, which showed specificity; however, the specificity was lost by mutation after 3 years. The organism was replaced by the equally accurate and specific test with \textit{Lactobacillus casei} (3), which shows complete correlation with the clinical diagnosis. It yields the most dependable estimate of the folic acid activity in the patient, compares favorably with other microbioassay methods, and is superior to the histidine load test (formiminoglutamic acid determination) whose failure under certain conditions we have demonstrated. The method, in conjunction with our \textit{Ochromonas malhamensis} assay for vitamin B\textsubscript{12}, permits clear distinction between macrocytic anemias of different etiology.

The distribution of B\textsubscript{12} between blood cells and plasma was studied. Plasma levels closely reflect the vitamin B\textsubscript{12} supply of the tissues, even though the blood cells from pernicious anemia patients often contain more B\textsubscript{12} than do normal cells (2, 4, 5).

Vitamin Surveys in Normal Blood Serum and CSF

Studies of the nucleogenic vitamins and of several other members of the vitamin B group for which we have developed microbioassays, serve to establish a base line for their physiologic levels in normal
young subjects. The extension of this work to 8 vitamins in the blood and serum of 6 mammalian species forms a useful foundation for animal experiments; differences between species serve for guidance in the selection of laboratory animals for specific investigations (6).

In various neurological conditions, the titer of folic acid and vitamin B₁₂ in the cerebrospinal fluid was found to be substantially elevated, often to a high multiple of the normal values. Such high titers are found with particular frequency in cases of multiple sclerosis, but the great variability in the symptomatology of this disease during its ups and downs does not yet permit a meaningful correlation (7a, 7b).

In conjunction with this study, we compared the level of the various vitamins in cerebrospinal fluid with that in blood. The ratio between these two body fluids varied from 1:1 to 1:250 (8). Some vitamins can pass the blood liquor barrier without obstacle while others are retained in the blood to the same extent as the blood proteins, possibly, as in the case of vitamin B₉, because of conjugation with the proteins. This ratio may be a parameter of the potential importance of vitamin therapy in neurological disease.

Vitamins in Mother and Newborn

We have studied with our microbi assay methods of the vitamin picture during pregnancy and parturition. Pregnant women were selected from the prenatal clinic with a view to excluding those receiving multivitamin therapy. The titer of the maternal blood was compared with that of the fetus, as represented by the cord blood. It was invariably found that, of the vitamins studied, the fetus attracts all at the expense of the mother. In some cases, the values for fetal blood are above the normal blood value; in other cases the fetal blood stays within the normal range, but the mother's blood is actually depleted; e.g., in the case of folic acid and of thiamine. This corresponds with the observation in countries where beriberi is endemic and where pregnant women are found to be especially susceptible to thiamine deficiency. In general, the ratio of the vitamin titers between fetus and mother range between 3:1 and 7:1 (9a, 9b).

In the course of these studies a unique exception was found in the case of diabetic mothers, where the thiamine ratio between fetus and mother is reversed. These mothers, suffering from diabetes or developing it in conjunction with pregnancy, have babies who suffer from
infantile gigantism, weighing up to 12 pounds, with edematus appearance, enlargement of the internal organs (especially the heart), and accelerated respiration. These symptoms are reminiscent of beriberi in its wet form.

Liver Disease and Complications of Diabetes

In studies of vitamin B₁₂ level of the serum in diabetics, no correlation was found with the incidence of retinopathy. In the course of this work, it was found that patients with hepatic disease retain the greater portion of a B₁₂-load test. This load test was further developed and correlated with other liver function tests (10a, 10b).

In some recent subsequent studies, Deysine et al. (11) observed the dramatic lowering of various vitamins in the serum of animals and patients after exposure to a number of general and local anesthetics and related hepatotoxins. Specific relationships exist between the chemical type of the anesthetic and the affected vitamin. Folic acid and thiamine are most frequently involved. These observations are of potential significance for pre- and postoperative vitamin therapy in surgical shock.

Certain types of malabsorbers find relief of their hematological symptoms by parenteral, but never by peroral administration of folic acid. These patients experience dramatic remissions of their symptoms upon peroral administration of the synthetic alpha-isomer of pteroyl diglutamic acid.

While the parenteral administration of folic acid in such patients yields a normal curve for the blood folic acid titer, peroral dosage with folic acid does not at all increase the low level of folic acid. In contrast, the alpha-isomer of the conjugate is well absorbed, regardless of the path of administration, as shown by the rise of the folic acid activity in the serum.

Endocrine Conditions and Iatrogenic Effects

An antagonism between vitamin B₁₂ and the thyroid hormones was discovered. Hyperthyroid patients have subnormal B₁₂ levels in blood and urine, while the opposite holds for myxedematous patients. The hyperthyroid state enhances the B₁₂ turnover and demand (13, 13). This relationship was confirmed on the cellular level, where thyroid hormones antagonize the growth of microorganisms.

The establishment of quantitative methods for the determination
of vitamins in body fluids and tissues by microbiological assay techniques should stimulate the search for the significance of vitamins in disease, not only in nutritional deficiency, but in the much wider field of metabolic disturbances. Functional vitamin deficiencies are produced by malabsorption, by inhibition of the vitamin function through products of the body, and particularly through drugs and other toxic substances. Vitamin deficiencies may be relative deficiencies, as when an individual's metabolism is deranged so as to require enhanced quantities of a given vitamin to cure or to counteract certain symptoms; e.g., in Darier's disease (keratosis follicularis) (14).

**Hypervitaminoses**

There is moreover, the field of hypervitaminoses, which has been explored with respect to the fat-soluble vitamins, but hardly touched as regards the water-soluble vitamins. The production of "combined system disease" by folic acid therapy of pernicious anemia is in the group of hypervitaminoses, but many more instances await recognition. The indiscriminate use of polyvitamin preparations by poorly informed clinicians is bound to mask such states and to delay their discovery. Also, the use of flushing doses of vitamins in diagnostic tests may cause acute hypervitaminoses and may also mobilize hitherto unknown growth factors from the tissues.

**Conclusions**

Like other constituents of blood or serum, the deviation of a vitamin titer from the normal range may mean a number of things: reduced intake or absorption, increased utilization, increased demand, increased excretion will all cause a decrease of titer; their opposites, an increase.

A vitamin is often the etiological center of a disease, as vitamin B₁₂ and folic acid in macrocytic anemias. Here, because of the obvious implications for diagnosis and therapy, the determination of the "nucleogenic" vitamins, B₁₂ and folic acid, is imperative in the routine of clinical hematology.

Where the connection between a vitamin and a disease is less transparent a wide field remains open for the discovery of meaningful correlations between vitamin content of body fluids and tissues to physiologic or pathologic events. To single out the case of a physi-
ological state of general importance, the vitamin equilibrium between mother and newborn offers now possibilities for the management of pregnancy, especially in regions where preventive "shot-in-the-dark" polyvitamin therapy is financially impossible. In the field of pathology, the significance of vitamin B$_{12}$ in toxemia of pregnancy deserves further study.

The so-called normal range of blood and serum vitamin levels is always derived from observations on healthy young subjects. Why not a comparison with healthy old subjects, whose percentage in the population is steadily increasing? Much may be learned about the cause of the decrease of physiological function and of the increased susceptibility to organic disease in old age, if the role of vitamins as parameter of these alterations were investigated with a view to preventive therapy.

A glance at vitamins in clinical medicine opens a wide panorama with challenging aspects in hepatic conditions, in oxalosis and calculus disease, in obscure but widely spread neurological diseases, and in many others. Astute clinical observations, combined with knowledge of the function and mechanism of vitamin action, will bring vitamin analysis into the picture as a useful tool.

Special mention must be accorded to iatrogenic effects, where the usefulness of novel synthetic drugs is impaired by untoward side effects of obscure etiology. Some of these side effects may find their explanation in the inhibitory action of the drug upon a vitamin, as in the case of Primidone vs. folic acid (15).

The mode of the toxic action of Thalidomide has not yet been determined. Because of our previous experience with the use of protozoa in the detection of metabolic lesions induced by drugs, as exemplified already in the case of Primidone, Frank and Baker have studied the antimetabolic nature of thalidomide, using Ochromonas danica, Ochromonas malhamensis, and Euglena gracilis; they found inhibition of growth which was overcome by nicotinic acid or amide, by nicotinamide adenine dinucleotide (NAD, formerly DPN), or by vitamin K. This points to interference by thalidomide with the pathway of cellular oxidative phosphorylation. While thalidomide is remarkably nontoxic to the nonpregnant animal, the blocking of normal morphogenesis in the fetus must be ascribed to this metabolic interference. In general, such relationships appear to be fortuitous until the structural chemical kinship of drug and vitamin is recognized.
References