

The Principles of Humane Experimental Technique

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CHAPTER 5

REPLACEMENT

With respect to the "analogical" ... resemblances between organic beings ...

The Uses of Microorganisms

Nutritional Research and Assays

The study of nutrition is a substantial one numerically (Table 18), and experiments upon animals for this purpose take the form of inducing, by feeding controlled diets, deficiency symptoms of various forms. The mildest of such symptoms is a general decline in weight and well-being; often quite specific and almost certainly distressing pathological states are produced, such as polyneuritis or rickets. This sort of procedure is necessary both in research and assay when animals are used. It is therefore of interest that replacement has made great strides here, chiefly through the use of microorganisms.

Apart from bulk requirements, and the proper proportions of the major types of large organic molecules (carbohydrates, fats, proteins), nutrition study centers on specific factors needed in the diet. Among these we may distinguish between amino acids and vitamins. A variety of specific amino acids are necessary to higher animals, but even those acting in specific processes are needed by man in gram amounts daily, as compared with milligrams or micrograms of B vitamins. Finally, among the vitamins themselves, we may distinguish between those with highly specific functions--the fat-soluble A, D, E, and K--and those associated with general reactions common to most living cells--the water-soluble C (ascorbic acid) and the vitamins of the B group (Prosser, 1950; see also Snell, 1953; Novelli, 1953; Reed, 1953).

The last distinction is important in practice. Whereas the vitamins of the B complex are essential to most organisms (including microorganisms), vitamins A, D, E, and K are "strictly vertebrate requirements, well known only for birds and mammals" (Prosser, l.c.). Thus, in both study and assay of the B vitamins, microorganisms have

been able to play a leading role, while in the case of the fat-soluble vitamins progress in humanity has so far had to wait for physical or chemical methods of assay. Fortunately, a variety of physical and chemical tests are by now available for the A vitamins, as well as for ascorbic acid (Melnick and Oser, 1947; cf. Barnholdt, 1956). But D still seems to defy replacement, "there being as yet no suitable test for this potent substance, at the low concentrations found in, for example, butter and margarine, other than one involving prevention or cure of rickets in animals" (Bacharach, 1955a). Attempts to develop chemical tests continue (cf. Anon., *Nature*, 1954). A serious obstacle is the difficulty of discriminating between the two important forms of this vitamin complex, which have different significance for different higher animal species (Prosser, 1950; Melnick and Oser, 1947). An unusually complex microbioassay has been proposed by Kodicek (1950) for the two important D vitamins. "The method is not an easy one to carry out, but it seems worthy of further investigation" (Sykes, 1957). Prosser (1950) suggests another interesting way out of the difficulty--the study of shell- or carapace-forming molluscs and crustacea. If deficiency is found to impair shell formation in snails, for instance, and this can be repaired by specific D substances in specific ways, a very cheap assay would be at hand. This would be a case of comparative substitution, but it is doubtful if exoskeletal defect could be anything like as distressing as rickets, provided the snails were kept in suitable conditions. Replacement here by one means or another would be very welcome. Vitamin D assay is a heavy burden on the rat (Tables 7 and 10).

We may now turn to the positive side of the subject--the use of microorganisms for the study, and above all for the assay, of nutritional factors. This was already well established in the forties, and its history has been discussed by Snell (1948--himself among the earliest contributors) and Dunn (1949). An excellent survey of the present position has been given by Sykes (1957). In some fields, chemical methods are replacing microbiological ones, but since both are absolutely humane we need not consider this secondary change.

The opening paragraphs of Snell's review (1948) tell a story of the greatest interest. The modern use of microorganisms for vitamin assay results from "an essential academic study--the nutritive requirements of microorganisms." At first, microbiologists had been mainly concerned to develop nutrient media from crude and ill-defined materials, since their priority was the task of culturing the multitude of microorganisms found in nature. Only in the thirties did systematic study begin of the precise nutritive requirements of these cultures, and "... this period of intensive investigation thus coincided with that during which rapid advances in knowledge of animal nutrition were being made... The resulting cross-fertilization between the two fields has immeasurably speeded" advance in both. It was soon found that many

animal vitamins and microbiological growth factors were the same. Some were discovered primarily in work on animals (e.g. thiamine, riboflavin, pyridoxine, choline), others in work on microorganisms (e.g. inositol, biotin, pantothenic acid, pyridoxal, pyridoxamine, p-aminobenzoic acid). "Both animals and microorganisms are inextricably involved in the early history of nicotinic acid and folic acid." Thus for each B vitamin necessary for animal growth there are known a number of different microorganisms which also require that vitamin for growth.

"In many cases (e.g. biotin, pantothenic acid, folic acid, inositol), the growth responses of such organisms were adapted to provide a quantitative or semi-quantitative measure of the amount of the growth factor present even before the responsible factor was identified, and this measure was used to guide the course of concentration from natural materials in the initial isolation of these substances. It is from such procedures... that our present microbiological assay methods have developed."

They could have developed even more rapidly--a microbioassay was proposed in 1919, but the suggestion was not fully realized till 1939 (Sykes, 1957). Nevertheless, the story remains one of the most dramatic instances of explosive progress in science and technology. Why this was so is quite clear from the passage just summarized. It was the result of ample communication between two specialized disciplines, each of which could make use of fundamental knowledge acquired by the other. This general point will be reconsidered later. As a more special inference, we may notice the importance in replacement, of fundamental study of the replacing techniques--in this case the microorganisms themselves.

Each microorganism species has its own spectrum of growth factor requirements, determined by the two factors of synthetic repertoire and optimal growth conditions. Required substances are obviously those needed but not synthesized. The researcher and assayer thus have at their disposal a lavish supply of models of high discrimination. Those used for nutritional assay include bacteria, yeasts, molds, and ciliate protozoa. Among these the most prominent are the members of the bacterial genus *Lactobacillus*. These organisms, which produce lactic acid during growth, offer a practical advantage. Assays can be based not only on estimates of growth, but more simply on the titration of the acid produced. But sometimes the array of *Lactobacillus* strains does not provide a convenient model, and many other groups of bacteria and other groups of microorganisms are used.

Besides the B vitamins, specific amino acids are generally needed by organisms, and are not synthesized by all microorganisms. Hence, these substances, too, can be assayed on the latter. The development of these assays followed hard on the heels of vitamin advance (Dunn, 1949). Still more recently, a ciliate species has been found to

use unhydrolyzed proteins, and several workers have determined nutritive values of proteins by microbioassay (see Allison, 1955).

In the development of particular assays, we observe a blend, common enough in science, of deduction and empiricism. In 1949, McIlwain made a kinetic analysis of acid formation during a procedure for assay of nicotinic acid on *Lactobacillus arabinosus*. This assay had been one of the three methods generally recommended as sound by the Association of Vitamin Chemists two years previously. He discovered the hitherto unknown and effectively fortuitous reason why variation could occur in the quantity of bacterial growth without detriment to the assay, and also why accurate temperature control was not required. At the same time he was able to suggest further improvement on a logical basis. At certain stages of development, the subject takes on a slightly mysterious aspect. *Ochromonas malhamensis* is very like higher animals in the specificity of its requirements for vitamin B12 (the most recent of the B complex to be worked out at the assay level).

"But in practice the activity measured by microorganisms is not necessarily a reliable prediction of the gross vitamin B12 activity of feeding stuffs for higher animals. In fact, the results of a comparison of chick and microbiological assays of a variety of feeding-stuffs indicate that in general the less specific microorganism, *Lactobacillus leishmannii*, approximates more closely to the findings by chick assay. The agreement is possibly fortuitous, but is nevertheless useful at the present stage of development of methods for assessing vitamin B12 activity" (Shrimpton, reported in Anon., *Nature*, 1955; a perfect instance of the correlation principle).

This last paradox calls attention to the fact that microbioassay is not without its practical difficulties. If we may use the phrase, it takes some time to get the bugs out. First, the general nutritional and cultural requirements of the test organism have to be fairly well known. The mold *Neurospora* is of special interest here. Wild-type strains of *Neurospora crassa* are extremely modest in their requirements. They need only one vitamin (biotin), a source of carbon and energy, and some inorganic salts (Beadle and Tatum, 1941). These authors were able to produce mutant strains defective in one or another synthetic capacity, and therefore specifically requiring one of a great variety of vitamins (Beadle and Tatum, 1945). They did not rely upon nature to provide them with models--they *made* their own!

Next the precise determination of the control medium, to which vitamin is to be added, is of critical importance. Trouble may arise if this basic medium contains all factors (the vitamin apart) essential for growth, but lacks a few factors which markedly stimulate growth. This difficulty may be overcome by suitable procedure, but is only finally eliminated by complete knowledge of the organism's requirements for *optimal* growth (Snell, 1948). The second major snag is liable to arise in the

extraction of the vitamin from the tissues to be tested. Here the problem is to obtain the vitamin in a form in which the test organism can use it, free of substances which may interfere in various ways with the assay. Sometimes different extraction methods have to be used for different tissues with the same test organism (*ibid.*).

When all these obstacles have been surmounted (especially the disturbance, by interfering substances, of specificity and parallelism with higher animal assays), there remains a final difficulty. "It is quite apparent that most of the extraction and hydrolytic procedures have not exact counterparts within the animal organism itself. The question may then be asked--Is the microbiologist measuring total rather than available vitamin content?" (Melnick and Oser, 1947). Although this question was asked a long time ago, it has been far from completely answered. Measurement of the total amount of vitamin contained in a given foodstuff does not necessarily reflect the amount which a human would derive from it under normal conditions of food preparation and feeding.

This is a drawback due to lack of fidelity in the microbioassay models. But the use of higher animals is not necessarily indicated, for here as in other respects their fidelity may leave much to be desired. Higher vertebrate species differ markedly in their nutritional spectra (*cf.*, *e.g.*, Bird, 1947; Morris, 1947; Coward, 1957), and also in a variety of metabolic ways related to vitamin availability and utilization. The question of availability can, therefore, only be answered decisively at present by studies on man himself (or the domesticated animal species concerned). But the more the biochemist learns about microorganisms, the more possible it may become to simulate in microbioassay the conditions under which vitamins are actually utilized by man.

The main specific microbioassays at present in use have been listed by Sykes (1957), who discusses the potentialities of this replacing technique in general. It only remains to note a recent suggestion that new recruits for nutritional microbioassay may come from the bacterial populations of the soil (Lochhead and Burton, 1956).

Before leaving the subject of nutrition, we may notice that other replacing techniques are becoming available for this purpose. Optimal nutrition is that which is optimal not only for growth of the individual but for reproduction and (in the case of the female) viability of the progeny. Study of this aspect initially took the form of depriving female animals and observing the effects on their young. The development of chemical analogues of the vitamins, which compete with the latter and hence inhibit their effects, has made possible a different approach. Specific antimetabolites are injected into hen's eggs (without previous manipulation of the mother's diet), and from their effects the function of the inhibited vitamin in development can be inferred. Nicotinic acid, B6, and folacin have been studied in this way. Apart from its use in the study of its own species, the hen's eggs treated in this way may provide a useful model

of low fidelity but high discrimination for studies of vitamin function in other animals and man (Cravens, 1952). Tissue cultures of mammalian organs are also coming to be used for purposes of nutritional study (Pomerat and Leake, 1954; Fell, 1954).

Other Uses of Microorganisms

For routine assay of the antibiotics (apart from tests for toxicity, pyrogens, and histamines), microorganisms are naturally used *in vitro*, except where they have been replaced by chemical tests (Grove and Randall, 1955). The uses of microorganisms in some other contexts have yet to be explored. Several groups of microorganisms metabolize steroids (Sebek and Michaels, 1957), so it is conceivable that they could be employed in some endocrinological contexts. The nutritional example has shown that microorganisms have more in common metabolically with higher animals than might have been supposed; there may be other similarities to be exploited.

But we can close this chapter on a pleasing and topical note. The youngest of the biological sciences is that of radiobiology--the study of the effects of radiations on living tissues. In this product of the atomic age, absolutely humane replacing techniques are already playing a part. Besides use of tissue cultures (e.g. Dixon, 1952; Buchsbaum, 1951), lessons about protection from radiation are already being learned from microorganisms (Hollaender and Stapleton, 1953). As new fields of biology open in the future, it may become a matter of routine to apply the lessons of the past and turn as soon as possible to the techniques of replacement.