Some Microbiologic Prospects for Discerning Vitamin Interrelationships

S. H. HUTNER, PH.D.,* HELENE A. NATHAN, M.A.,† HERMAN BAKER, PH.D.,‡
HARRY SOBOTKA, PH.D.,§ AND S. AARONSON, PH.D.||

VITAMIN interrelationships underlie much of a problem once posed in this JOURNAL: "Today the nutrients essential for man appear to have been delineated. Yet how they interact and how their reduction or lack precisely affect the organism so as to give rise to specific clinical and pathologic changes which comprise human deficiency disease states are questions which have yet to be solved."[1]

Many of the answers must come from microbiologic enterprises. As we will show here, use of particle-ingesting (phagotrophic) protozoa opens to attack some problems which would be intimidatingly expensive to pursue solely with higher animals, not to mention with human patients.

PHAGOTROPHIC PROTOZOA

"True" protozoa are animals; that is, they eat. An omnivorous protozoon necessarily has digestive enzymes equivalent to a higher animal's, perhaps with more versatility in digesting microbial bodies. Several such protozoa can be grown in chemically defined media. Which most resembles man? To appreciate the practical import of this question, contrast the cost of a few racks of test tubes and roomfuls of rats or chicks for the equivalent experiment.

Two kinds of phagotrophs in pure culture are represented by the ciliate *Tetrahymena pyriformis* and yellow-brown photosynthetic chrysomonad flagellates such as *Ochromonas malhamensis* and *Poteriochromonas stipitata*. Other protozoa are in pure culture, e.g., small amebae and *Paramecium*, but this discussion is restricted to *Tetrahymena* and *Ochromonas*.

(1) *Tetrahymena: Possibilities in Its Amino Acid, Biotin, Vitamin B_6, and Folic Acid Requirements*

T. pyriformis, unlike man, requires neither fat-soluble factors (aside from the sterol requirement of a few strains) nor vitamin B_{12}, but it does require exogenous purine (primarily guanine) and thioctic (lipoic) acid. Its vitamin B_6 requirement cannot so far be bypassed by the amino acids (including D-alanine) effective for such lactic acid bacteria as *Streptococcus faecalis*.[2,3] We are trying to see whether feeding heat-killed *Str. faecalis* grown on a vitamin B_6-free medium to *Tetrahymena* supplemented with likely vitamin B_6-bypassing compounds, will bypass *Tetrahymena's* requirement. It would not be surprising if effective sparing, let alone bypassing, should demand a much longer roster of nitrogenous compounds than hitherto assembled: Vitamin B_6 enters

From the Haskins Laboratories, New York, and the Department of Chemistry, Mount Sinai Hospital, New York, N. Y.

* Staff Member, Haskins Laboratories; †Research Associate, Haskins Laboratories; ‡Department of Chemistry, Mount Sinai Hospital; §Head, Department of Chemistry, Mount Sinai Hospital; ||Research Associate, Haskins Laboratories and Assistant Professor of Biology, Queens College, New York, New York.

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into every known reaction having to do with amino acid or with moving nitrogen in and out of heterocycles. This makes the ease in bypassing the vitamin B_6-requirement of *Str. faecalis* hard to interpret. The present inability to bypass the vitamin B_6 requirement of *Tetrahymena* implies that when *Tetrahymena* serves as an assay organism for vitamin B_6, it is responding mainly to this nutrient, not to products of its catalytic activity. This implies further that the vitamin B_6 requirement of man may be spared by products of vitamin B_6 activity, and that such sparing should be taken into account in surveying foods for vitamin B_6 or in assaying vitamin B_6 in body fluids.

We are now studying the practicability of such assays with *Tetrahymena*. The problem of the vitamin B_6 requirement of *Tetrahymena* can be attacked by simultaneously feeding *Tetrahymena* an assortment of organisms whose vitamin B_6 requirement can be bypassed. The nonphagotrophic flagellate *Crithidia fasciculata* has a vitamin B_6 requirement that we have spared but not bypassed with a mixture of amino acids.[2] Which organism's vitamin B_6 requirement is most akin to man's is a problem to be solved in the future.

We may suppose that to bypass the vitamin B_6 requirement of both *Tetrahymena* and *Crithidia* one has to provide D-amino acids, as when vitamin B_6 is bypassed in vitamin B_6-requiring bacteria. Such a finding would heighten the likelihood that D-amino acids are essential metabolites for higher animals; it would make more attractive vitamin B_6-bypassing experiments with higher animals. To induce sharp vitamin B_6 deficiencies, it would probably be necessary to add a vitamin B_6-antagonist to the diet. We have not found a satisfactory antagonist for vitamin B_6 in protozoa; isonicotinic hydrazide holds some promise.

One can lead a phagotroph to a particle, but will it ingest? How could one tell whether negative results in bypassing experiments with phagotrophs reflect a failure of ingestion of particulate food, e.g., bacterial bodies or, if it is ingested, a failure to digest? One way might be to see whether *Tetrahymena* can satisfy one or more of its amino acid requirements from the particulate food in question. *T. pyriformis* has much the same pattern of amino acid requirements as man; it has been proposed—with convincing assay data—as a replacement for higher animals in measuring the biological value of proteins.[4]

Another tough problem is posed by the folic acid requirement of *Tetrahymena*. Here folic acid is spared, not bypassed, by thymidine plus the other metabolites effective for such folic-assay bacteria as *Str. faecalis* and *Lactobacillus casei*.[5] If the requirement for folic acid in man follows the *Tetrahymena* pattern, as seems likely, then *Tetrahymena* is the best organism for measuring the folic acid activity of foods and body fluids. In some experiments we supplied thymidine and other folic products as folic-sparers together with bodies of *Str. faecalis* grown on a folic-free medium, i.e., on a medium in which folic acid was wholly replaced with purines, pyrimidines, and amino acids. This bacterial supplement seemed to have little if any effect on *Tetrahymena* beyond that given by thymidine. The next move might be to supplement the *Str. faecalis* bodies with bodies of *C. fasciculata* grown on a folic-free medium.

To bypass the folic acid requirement of *Crithidia*, the new pteridine growth factor, biopterin, had to be supplied.[2] This brings up a new problem: can biopterin by bypassed? Bypassing folic acid for *Crithidia* thus resembles the Quaker Oats box whose label shows a Quaker holding a box whose label shows a Quaker, etc. Since no other biopterin-requiring organism is known, *Crithidia* cannot be given a crude (i.e., an unfractionated natural product) rendered biopterin-free by the device of bypassing the biopterin requirement. Another course is to inactivate the biopterin in a crude by illuminating the preparation: biopterin, like folic acid, is light-sensitive. Retention of *Crithidia*-factor activity beyond the inactivation of an added control amount of biopterin would point either to new bypassing compounds or to unfamiliar forms of *Crithidia* factor. Obviously the same procedure could be followed directly in bypassing the folic acid requirement of *Tetrahymena* or, for that matter, higher animals. Here too we meet the Quaker Oats or Chinese box situation: to spare folic acid in many bacteria, vitamin B_{12} is needed. Can this

vitamin B_{12} requirement be bypassed in turn?

(2) *Ochromonas malhamensis*

Since *Ochromonas* has a vitamin B_{12} requirement seemingly identical with man's, the *Ochromonas* method is now the official British method of measuring vitamin B_{12}; rats and chicks here became technologically unemployed. *Ochromonas*'s requirement for the vitamin has no more been bypassed than has man's. The implication is that vitamin B_{12} helps synthesize unknown compounds. In preliminary experiments, *Ochromonas* was fed *Escherichia coli* 113-3 grown on methionine rather than vitamin B_{12} and, separately, on *L. leichmannii* grown on thymidine instead of vitamin B_{12}. *Ochromonas* still needed vitamin B_{12}. This may mean that: (1) the bacteria were practically free of vitamin B_{12}—their vitamin B_{12} requirement may have been bypassed; (2) *Ochromonas* uses vitamin B_{12} for syntheses absent in bacteria.

Ochromonas has no clear-cut requirements for amino acids. To tell whether particulate food is being effectively utilized, one might exploit instead its biotin and thiamine requirements: If *Ochromonas* can get both vitamins from particulate food, presumably the particles are being effectively utilized. Assays for thiamine and biotin by means of *Ochromonas* are likely to yield realistic parallels for higher-animal activity.[6] *Tetrahymena* also requires biotin and thiamine, which provides a unique cross-check—one kind of animal against another. *Tetrahymena* and *Ochromonas* are on different evolutionary lines; they are far more different from each other than the rat is from the chick.

Pernicious anemia exemplifies an interrelationship, a reciprocal mobilization, between two vitamins, B_{12} and folic acid. Microbiologic data, marshalled in a review in preparation, lead one to think that folic acid is necessary for the synthesis of at least three different parts of the vitamin B_{12} molecule; further, that folic acid joins with vitamin B_{12} in the synthesis of (1) itself; (2) methionine; (3) purines; and (4) thymidine. Folic acid (or folic-catalyzed products) seem necessary in turn for its own synthesis. The academic biochemist must be thankful for the existence of pernicious anemia, and hence to clinicians, for calling attention to vitamin B_{12}. The yet obscure folic acid-vitamin B_{12} link in pernicious anemia stands as a sign post to a web of reaction pathways controlled importantly by multiple feedbacks. Awareness of catalysts which, amid their other activities, apparently catalyze their own synthesis, raises the question of where the controls on growth, conventionally ascribed to the nucleic acids, enter. Tracing vitamin interrelationships inevitably leads to deep waters: What limits the autonomy of "self-duplicating" intracellular systems? What is the significance of "priming" or "sparking" reactions—whence the original primers? And, more broadly, how is metabolism and growth coordinated at the molecular level?

PROSPECTS AND CONCLUSIONS

T. H. Jukes says, "nothing is cheaper than an idea." How easy it is to draft grandiose research projects! We plead in extenuation that we have confined our speculations to experiments actually in progress or plainly being designed. Nonetheless some experiments are so complicated that pilot experiments with microorganisms seem the prudent course before experimenting along these lines with higher animals. Some work with protozoa, such as on "temperature factors," designed to find out why the requirement for vitamin B_{12} in animals is increased sharply in hyperthyroidism, is probably at the present limits of practicability with higher animals.[7,8] We must hope that the protozoa used do have a metabolism like ours—that, to put it not all together facetiously, they are humanoid. Our present candidate is *O. malhamensis*. The limiting factor in reaching a decision may turn out to be the slowness of parallel experiments with higher animals.[7] An eminent chemist, now dead, once asserted to one of us that the limiting factor in hematology had been the development of chemical methods for fractionating blood constituents, not the development of analytical methods. The rejoinder was that knowledge of vitamin B_{12} had not advanced rapidly when the analytical method consisted of a hospital ward of patients with pernicious anemia; once microbiological methods were aligned with clinical findings, then large-scale

chemical fractionation of vitamin B_{12}—at least on this side of the Atlantic—could proceed rapidly, through intermediate stages of rat and chick assays. This and many other examples attest that it is of the utmost importance to reduce the lags in the sequence microbe→higher laboratory animal→man. The choice of microbial research problems is shaped to some degree by a desire to complete the circle: man→microbe. Indeed it is important to reduce the lag in traversing this research circle in both directions.

The need to make microbiological assays as simple as possible for clinical use is often impressed upon us. Toward this end we have listed many pointers.[9]

Electrical engineers speak of the retina, the brain, or of the organism itself, as a "black box:" sensory signals enter, and appropriate behavior follows—how is the interpreting or transducing done in these black boxes? The pursuit of vitamin interrelationships, especially among vitamins having broad synthetic functions, such as those just discussed, will help pry the lid off the metabolic black box, and so, eventually, off the organismal black box—which is to say that we will have the insights into the reaction chains underlying nutritional requirements that clinicians, such as the one quoted at the opening of this discussion, are seeking.

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