RECOLLECTIONS

Vitamins, metabolic antagonists, and molecular evolution

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The years 1930 to 1996, which span my life as a biochemist, have seen many great advances in science. I divide my years into three periods. First, I worked on the identification and synthesis of vitamins. Second, I studied antibiotics and folic acid antagonists. Third, I worked on the genetic code and molecular evolution.

Identification and synthesis of vitamins

In 1933, it was possible to keep rats on a reasonably purified diet, but not chicks. They died in a few weeks, even if large amounts of yeast were added. It was a great challenge to find out what was needed to sustain life, growth, and reproduction in chickens. A good lead was the discovery at the University of Wisconsin that heating a diet for chicks consisting of a mixture of corn, wheat middlings, and casein destroyed a substance that was needed for growth and the prevention of dermatitis. I started to work with this finding at Davis, CA. Normality was restored by adding liver extract. Soon we found that two “factors” were involved: one was riboflavin and the second an unidentified “filtrate factor” that remained in an aqueous liver extract after treatment with fullers’ earth. In collaboration with S. Lepkovsky, studies with rats showed that another essential dietary substance adsorbed on fullers’ earth was also present; this turned out to be vitamin B₆. In 1939 we found that the filtrate factor was pantothenic acid, a growth factor for yeast cells in restricted culture media that had been studied and named by Roger Williams at Oregon State University. It consisted of beta alanine coupled to an unknown hydroxy acid, which was later identified by Merck scientists in the form of a lactone, “pantoyl lactone.” In the hands of Sidney Babcock, at Davis, pantothenic acid was synthesized by mixing beta alanine with the lactone and raising the pH by adding sodium hydroxide. This was far simpler than using esterification. As shown by Fritz Lipmann, Esmond Snell, and W.L. Williams, pantothenic acid is a constituent of coenzyme A and thus is involved in transacetylation reactions in tissues.

Chickens and turkeys are subject to a nutritionally induced deformity of the hock joint, called perosis. Manganese deficiency was shown at Cornell University to cause perosis, but when I tried in 1939 to prevent perosis with manganese in baby turkeys, the

deformity increased in severity with higher levels of manganese but was alleviated by yeast. I tried several pure substances and found in 1940 that choline prevented perosis. This finding led to the use of choline as a dietary ingredient in poultry feeds. I also studied vitamin B₆ deficiency in chicks and found that convulsions occurred in deficient chicks. This gave a clue to convulsions in infants on some formula diets that were low in vitamin B₆.

The era of vitamin discovery was an exciting time. Riboflavin, vitamin B₆, nicotinic acid, pantothenic acid, folic acid, and biotin were newcomers during the 1930s. The finding in 1937 that nicotinic acid cured pellagra in human beings was of great importance in public health. One of the symptoms of pellagra is dementia, and the introduction of nicotinic acid into their diet enabled the recovery of dementia patients to an extent that it was possible to close some mental hospitals in the southeastern USA. The program to enrich cereal grain products, including bread, was implemented and today the statement of ingredients on the wrapper of a loaf includes thiamine, riboflavin, and niacin (the colloquial name for

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Thomas H. Jukes was born in Hastings, England, in 1906. He studied biochemistry at the University of Toronto (B.S.A. 1930, Ph.D. 1933). He went to University of California, Berkeley as a National Research Council postdoctoral fellow in 1933, and to University of California, Davis, as a faculty member 1934–1942, where he studied vitamins. In 1942, he went to Lederle Laboratories as a nutritional researcher, and worked on cancer chemotherapy, also on antibiotics. He returned to University of California, Berkeley in 1963, where he studied the genetic code and molecular evolution, especially the neutral theory.

nicotinic acid). At least one other vitamin, folic acid, should be added to this list.

There has been a long delay in authorizing folic acid enrichment of foods; an important preventive measure against spina bifida in infants, which is a family tragedy. The FDA is still hesitating. The allegation is that folic acid may "cover up" neurologic symptoms in untreated or relapsed pernicious anemia and allow these symptoms to progress. This idea is based on comparing patients receiving folic acid with others who have been injected with vitamin B₁₂. But this is an incorrect evaluation. The comparison should be made between *untreated* patients and those treated with folic acid. In this case, there is no difference. C.J. Dickinson has pointed out that folic acid will neither cause nor accelerate the neurological deterioration in pernicious anemia. Also, pernicious anemia usually does not develop until after the menopause, and women with B₁₂ deficiency are often sterile. There seems to be no reason for not encouraging the enrichment of food with synthetic folic acid.

Folic acid antagonists and cancer chemotherapy: antibiotics in animal feeds

My resources and facilities in the Poultry Husbandry Department at Davis, CA, were indeed meager, and the second period of my scientific life started when I accepted an offer from Lederle Laboratories Division, American Cyanamid Company, in Pearl River, NY, as a nutritional researcher. Their interests included nutrition in anemia, synthesis of vitamins, and cancer chemotherapy. In 1946, I was given a new laboratory and a new department. One of our biochemists, aged 29, Alfred Franklin, added a crude folic acid antagonist, "x-methyl folic acid" to the diet of rats. X-methyl folic acid had been synthesized by using an intermediate containing an additional methyl group to the reaction mixture for preparing folic acid by M.E. Hultquist and J.M. Smith, Jr., two organic chemists with American Cyanamid. Franklin found that x-methyl folic acid reduced the white blood cell count of rats almost to zero, and more important, the reduction could be stopped or reversed by raising the level of folic acid. This was a promising lead for the treatment of leukemia, but it did not work for human patients. The finding was put in the background by another discovery: aminopterin, a compound identical with folic acid except for substitution of -OH by -NH₂ at the 4-position on the pteridine ring was synthesized by Seeger, Smith, and Hultquist. It was thought that aminopterin might be a replacement for folic acid. Instead, it was highly toxic, and its effect was not reversible by folic acid; therefore, it was not a folic acid antagonist. It contained a diaminopyrimidine group that made it an irreversible inhibitor of an essential enzyme, dihydrofolic (FH₂) reductase, as shown in Figure 1. Methotrexate, which is aminopterin with a methyl group added at the 10-position, is shown as a diaminopyrimidine in Figure 1. It was synthesized by J.M. Smith and co-workers in 1948 (Stokstad and Jukes, 1987).

The property of aminopterin as an inhibitor of dihydrofolic reductase led to the first successful chemotherapy of childhood leukemia by S. Farber and co-workers in 1948. Other clinical reports soon followed. R. Hertz and his colleagues treated malignant chorionic tumors successfully in women with remissions lasting over five years. The net effect was stopping the synthesis of DNA at the step from deoxyuridine to thymidine (Fig. 1). The effect was bypassed by administering tetrahydrofolic acid or its derivative leucovorin (Fig. 1). This was of importance in protecting patients against methotrexate toxicity. Methotrexate has found wide usefulness beyond cancer chemotherapy; it is used in treatment of psoriasis and

Fig. 1. Methotrexate blocks the synthesis of thymidine from uracil deoxyribotide.

rheumatoid arthritis. Aminopterin and methotrexate initiated the field of rational cancer chemotherapy. Resistance to methotrexate may develop as a result of amplification of the FH₂ reductase gene (Fig. 1). Diaminopyrimidines are used as antibacterial agents (trimethoprim) and as antimalarials (pyrimethamine). Their therapeutic effect is by inhibition of FH₂ reductase. Folic acid must be reduced first to dihydrofolic acid and then to tetrahydrofolic acid (FH₄) before participating in metabolism (Fig. 1). This was demonstrated in vivo by us and in vitro by F.M. Huennekens.

Our group at Lederle also identified and synthesized leucovorin (Fig. 1), thioctic acid (lipoic acid), and bioppterin, and showed that selenium prevented alimentary exudative diathesis in chicks.

In 1948, the isolation of the anti-pernicious-anemia factor was announced by Merck and Glaxo laboratories. The Merck group was aided by an observation by Mary Shorb that *Lactobacillus lactis Dornier* gave a growth response to the factor, which they named vitamin B₁₂. At Glaxo, Lester Smith relied on clinical tests with human subjects. Vitamin B₁₂ was red, and contained cobalt. This solved the riddle of cobalt deficiency "wasting disease" in ruminants, especially in Australia. Vitamin B₁₂ is not made by green plants and its only known source is microbial growth, which takes place among other locations, in the paunch of ruminants. The absence of vitamin B₁₂ from vegetable sources became crucial in World War II when fishmeal disappeared from the market because of submarine warfare. Soybean meal became the main protein for chicken feed, and there was an urgent need for an "animal protein factor" (APF), which was actually vitamin B₁₂. The search for APF led us to test the organism *Streptomyces aureofaciens*, which is the source of antibiotic aureomycin, and also makes vitamin B₁₂. We found that chicks receiving a supplement of *S. aureofaciens* fermentation grew 24% more rapidly than those receiving liver extract. In the words of Jeffrey Fisher, in a book with the unappetizing title, *The Plague Makers* (1994) "in a very short time this discovery became the biggest boon ever to livestock and poultry production, transforming it from the small business it still was in the early 1950s into the multibillion dollar industry it is today . . . before the use of antibiotics . . . bloody diarrhea caused death in young pigs and chickens. They died by the thousands, suffocated by air-sac diseases. Baby calves frequently perished from a condition called scours. These problems were alleviated by antibiot-

ics." Fisher states that 20 million pounds of antibiotics a year, twice as much as is used for humans, are added to animal feeds.

Questions have been raised that this use of antibiotics promotes the occurrence of transferable resistance to pathogenic bacteria from non-pathogens. If this resistance is widespread, it should have abolished the efficacy of antibiotics in animal feeds. Resistant *Salmonella* can be a serious public health problem; however, salmonellosis is not usually treated with antibiotics. The "growth promoting" effect of antibiotics is actually the neutralization of a growth-depressing effect caused by unidentified intestinal microflora. This is shown by the observation that germ-free chicks grow more rapidly than non-germ-free controls, and germ-free chicks do not show a growth response to antibiotics.

Molecular evolution, neutral theory, genetic code

It was time in 1962 to enter the third period of my scientific life. The discovery of the structure of DNA by Watson and Crick in 1953 had revolutionized genetics. But what was the biochemistry that enabled the genetic message in the base sequence of DNA to imprint itself upon living organisms? The answer resided in the genetic code. In 1962, Nirenberg and co-workers and Ochoa and colleagues found that in Nirenberg's cell-free *E. coli* system for protein synthesis, synthetic polyribonucleotides containing various proportions of U, C, and A, gave rise to polypeptides with varying preparations of serine, proline, leucine, threonine, asparagine, and histidine. More precise experiments, especially those by Khorana and co-workers and also by Nirenberg and Leder, established the three-letter code words for all the amino acids that participate in biological protein synthesis. The identity of these "magic twenty" amino acids, together with other basic facts, were set forth in a classical review by Crick in 1958, *On Protein Synthesis*.

Clearly, biochemistry was being revolutionized. The staid pattern of purifying enzymes and calculating their constants had become obsolete. I remember a seminar by Don Helinski of C. Yanofsky's lab in 1962 in which he showed that a glycine-coding locus in tryptophan synthase could mutate to arginine and then back to glycine: GGA-AGA-GGA. I decided that a new day had dawned in biology, and I wished to participate in it. I resolved that I would leave Lederle and return to Berkeley after 21 years to work under a grant, "Chemistry of Living Systems," funded by NASA. No more vitamins, no more antibiotics. The funding, in the wake of Sputnik, was sufficient that I was able to offer research appointments to several young molecular biologists. Two of these, Mituru Takanami and Hiroshi Yoshikawa, eventually became presidents of the Japanese Molecular Biology Society after returning to Japan.

The Watson-Crick model of DNA said that its sequence of bases was repeated in each generation. But how far back was this true? Clearly, the continuity had to reach back to the first DNA molecule. A new brand of science, molecular evolution, was propelled into existence. My first start in Berkeley was to write a textbook, *Molecules and Evolution*, published in 1966. In it, I noted that the sequence of amino acids in a protein such as cytochrome *c* that occurs in many different species varied without any change in its function. The variations increased in number as the divergence of species widened. I said that "the changes produced in proteins by mutations will in some cases destroy their functions but in other cases the change allows the protein molecule to continue to serve its purpose." Two years later, in 1968, M. Kimura proposed that "many of the mutations involved (in evolution) must be neutral ones," thus giving birth to the neutral theory of molecular evolu-

tion. Simultaneously, Jack King and I were preparing a long manuscript for *Science*, "Non-Darwinian evolution," (1969) which also proposed the neutral theory. These proposals were preceded by the work of others, especially Sueoka, who found that changes in amino acid content of bacterial proteins took place in concert with changes in A + T or G + C content of DNA in a manner that coincided with the base composition of codons. My interest in the genetic code continued. Was it "frozen" and "permanent"? In 1981, Sanger's group at Cambridge showed that the genetic code in mitochondria had two changes from the universal code: AUA for methionine instead of isoleucine; UGA for tryptophan instead of chain termination. Evidently the genetic code was not frozen.

Stop codons are recognized by release factors (RF). These block the function of peptide linkage by the final amino acid in the growing polypeptide chain, which is released from the ribosome. In 1985, I received a manuscript from Syozo Osawa in Nagoya describing the finding that UGA codes for tryptophan in the small bacterial species *Mycoplasma capricolum*, whose DNA is inordinately high in A + T. I suggested that mutational pressure may have led to the replacement of UGA stop codons by UAA, so that UGA codons, if they reappeared, were available for capture by tryptophan. The RF for UGA would have disappeared to make this possible. A mutation of tryptophan anticodon CCA, which pairs only with codon UGG, to UCA, which pairs with codon UGA and UGG, would enable UGA to become a tryptophan codon without any disruption of protein synthesis (Jukes, 1985). This proposal for "stop codon capture" became extended to changes in the mitochondrial codes for many species of organisms and for nuclear codes in other species such as ciliated protozoa, in which stop codons UAA and UAG have been "captured" by glutamine. In this process, the only termination codon for ciliates is UGA, and codons UAA and UAG disappear. Anticodons UUA and CUA are formed from a duplicate of Gln anticodon UUG. The net result is that ciliates have four glutamine codons: CAA, CAG, UAA, and UAG (Jukes et al., 1987).

Addendum

The problem of creationism

Creationism is an impediment to the teaching of science in elementary and secondary schools. Creationists demand that equal time be given to "creation science" in classrooms and in school science textbooks. This is their misnomer for a story in which the age of the Earth (and the universe) is limited to 10,000 years, and the Genesis flood of Noah was an actual event.

Creationists intimidate publishers into deleting mention of evolution from school science textbooks. I have spent a lot of time in combatting the creationist attacks on science, including participation in a court trial.

The fight against creationists in the USA pales into insignificance beside the struggle in Australia, where the leading anti-creationist, Professor Ian Plimer, wrote to me on 26 November 1995, that he had spent "30 hours per week and a total of \$300,000 of my own money" on anti-creationism during three years.

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