The objective of this two-part symposium, begun in 1995 and continued in 1996, was to describe some of the discoveries made during the past 150 years that changed the direction of thinking in nutrition. These discoveries all illustrate the strength of the scientific method as a process for gaining reliable knowledge of the natural world.

Philosopher of science Karl Popper proposed that the scientific method begins, not with the accumulation of facts, but with recognition of an unsolved problem. This leads to conjecture about a solution, i.e., formulation of a hypothesis. The essence of the process is to subject hypotheses to critical examination and experimental tests that have the potential to refute them. It is basically a process for detecting error; its strength lies in its self-correcting nature. If a hypothesis fails to withstand a test with the potential to refute it, it must be discarded or modified. It is equally important, nonetheless, to defend hypotheses vigorously to ensure that they are not rejected without being tested thoroughly. Although the ability of a hypothesis to withstand such tests does not establish unequivocally that it is valid, as assumptions that are false are eliminated by repeated testing, we achieve an increasingly better approximation of reality.

The process is illustrated by two articles on early studies of protein that were included in the symposium. During the 1820s, protein was accepted as an essential nutrient on the basis of feeding studies with dogs. Subsequently the German school of Liebig and Voit postulated on theoretical grounds that protein was the source of energy for muscular work. This hypothesis was challenged by Fick and Wilsenius in 1866 in an elegant nitrogen balance study performed during a mountain climbing expedition. Their results, interpreted by Frankland, proved conclusively that the hypothesis was erroneous (Paper 2). Nonetheless, the assumption that a high protein intake was uniquely important in stimulating vigor of mind and body was accepted for another 40 years until it was challenged in 1904 by Chittenden, who demonstrated that healthy young men remained vigorous with a protein intake about half that recommended by Voit (Paper 5).

Thomas Kuhn, another philosopher of science, has concluded that major advances in science do not occur gradually, but suddenly, and constitute “scientific revolutions.” He uses the term “paradigm” to describe the theoretical assumptions, laws and techniques that dominate scientific experimentation by a particular community of scientists during a given period. Eventually, however, observations that are at variance with the current paradigm are encountered. The paradigm is recognized as being inadequate, and a new and radically different hypothesis is proposed as the result of unusual insight, usually coupled with new methods. This leads to a new paradigm, and a period during which it is consolidated follows. An example given by Kuhn of a scientific revolution is the discovery by Copernicus, at a time when essentially all astronomers believed that the earth was the center of our solar system, that in fact the sun was the center of the solar system and the planets, including Earth, revolved around it. The history of the various sciences, Kuhn proposes, is characterized by a series of paradigms interspersed with periods of “normal science” during which problems falling within the limits of the prevailing paradigm are explored. This view is complementary to that of Popper, who emphasized the need for constant hypothesis testing and modification to ferret out error. Scientific revolutions have occurred in biology and medicine, of which nutrition is a part, since the time of Hippocrates.

Some examples of scientific revolutions in biology and medicine are the discoveries of Harvey, Lavoisier and Darwin, each of which made existing paradigms obsolete. Harvey, in 1628, discovered that blood pumped by the heart through the arteries passed to the veins and circulated back to the heart. This was the demise of the hypothesis, postulated by Galen in the 2nd century, that the blood oscillated back and forth within the arterial system. Lavoisier’s discovery, in 1777, that combustion was a chemical process in which oxygen combined with other elements with the release of energy made untenable the century-old hypothesis that combustion represented loss of “phlogiston.” Charles Darwin in his classic study The Origin of Species, published in 1859, assembled evidence that new species had evolved continuously over millions of years. His theory of evolution demonstrated that biblical creationism, the belief that species arose intact through supernatural intervention, and which was almost universally accepted in countries that had adopted Western religions, was incompatible with scientific observations.

All of the symposium presentations that follow discuss experiments that influenced nutritional thinking. Some describe experiments that challenged accepted concepts and resulted in their displacement with new ones; others are reports of
discoveries that arose from exploration of specific aspects of the new concepts. Several fit Kuhn's concept of scientific revolutions that bring about a rapid change in the paradigm of a field.

A major paradigm shift in nutrition was the discovery of the essentiality of organic and inorganic micronutrients. Despite a number of observations during the 19th century that diets composed of purified food constituents did not support growth or even life, this shift did not occur suddenly as the result of a single discovery; it occurred over a period of more than 60 years. The lag was attributable in large measure to resistance to the new paradigm by many scientists who were influenced by the prestige of Liebig and who accepted, almost as dogma, his concept that energy sources, protein and a few minerals were the sole principles of a nutritionally adequate diet. Only after the inadequacy of Liebig's hypothesis had been demonstrated in many experiments that should have changed nutritional thinking, but did not, was the new paradigm generally accepted. Four of the papers describe experiments that contributed to the shift in paradigm.

Gerrit Grijns, in the 1890s, extended the work of Eijkman in Java (Indonesia) showing that chickens fed a diet of white rice developed polyneuritis, a disease resembling beriberi. The disease was prevented by including rice polishings or beans or water extracts of them in the diet. He concluded that chickens needed an organic complex provided in adequate quantities by rice polishings and beans but not by polished rice. His observations had little immediate effect on orthodox nutritional views, even though they ultimately contributed to the basis for the new paradigm (Paper 4).

Liebig's concept that the nutritional value of foods and feeds could be predicted from their proximate composition (nitrogen, ether extract, ash, and carbohydrate by difference) was tested directly by Hart and colleagues in 1907. They found that calves from cows fed an all-wheat ration survived only a short time even though the wheat ration was balanced for major nutrients to match an all-corn ration that proved to be fully adequate. This was a clear demonstration of the inadequacy of Liebig's concept (Paper 6).

Subsequently, McCollum found that rats fed a simplified diet of casein, carbohydrate and minerals stopped growing unless supplied with a fat-soluble factor present in butter but not in olive oil. Rats fed a polished rice diet were found to need a water-soluble factor B, as Grijns had shown, as well as the fat-soluble factor A (Paper 7). During this period, Holst and Froelich in Norway induced a scurvy-like disease in guinea pigs by feeding them diets resembling those of Grijns. This disease was prevented by providing the guinea pigs with lemon juice or cabbage.

Also, between 1909 and 1914, Osborne and Mendel at Yale, following an earlier observation by Hopkins in Cambridge that tryptophan was essential for the survival of mice, discovered that some plant proteins did not support growth of rats unless the rats were supplemented with other amino acids (Paper 8). Hopkins, and Funk in London, both postulated in 1912 that diseases such as scurvy, beriberi and rickets were dietary deficiency diseases. Only between 1910 and 1915, after these and other demonstrations of the inadequacy of Liebig's concept, was the new paradigm of the essentiality of minor constituents of foods widely accepted.

Acceptance of the new paradigm was followed by a period of unparalleled discovery in nutritional science from about 1915 to the 1950s, during which some 40 essential nutrients were identified and characterized and their functions explored. Several of the articles included in the symposium discuss representative experiments of this expansion of knowledge within the new paradigm.

Iron was known early in the 19th century to be a component of hemoglobin, but the belief that only organically bound iron was available to the body was an obstacle to understanding the role of minerals in nutrition. The demonstration by Stockman in 1893 that inorganic iron was used efficiently for hemoglobin synthesis corrected this erroneous assumption (Paper 3). Thirty-five years later, Hart and associates discovered that copper was essential for the utilization of iron in hemoglobin formation. It is now known that copper promotes uptake of iron by transferrin and increases the utilization of iron by erythroblasts for hemopoiesis (Paper 10).

After the discovery that yellow carotenoid pigments and colorless oils both had vitamin A activity, a conflict between competing hypotheses about the nature of vitamin A precursors was resolved by Thomas Moore, who in 1930 showed that the yellow β-carotene was converted to colorless vitamin A in the animal body (Paper 11). Thiamin was shown by Lohmann and Schuster in 1937 to be a component of the coenzyme thiaminpyrophosphate, and its role in pyruvate metabolism in the animal body was elaborated by Peters (Paper 12). Observations by Goldberger that protein as well as protein-free extracts of yeast could cure pellagra posed a problem that was resolved when Krehl and colleagues discovered in 1945 that the amino acid tryptophan was a precursor of niacin in the body (Paper 15). That complex interactions and antagonisms can occur among trace minerals was discovered by Dick and associates, who observed that copper deficiency occurs in animals with a normally adequate intake of copper if their intake of molybdenum and/or sulfur is high (Paper 16). In 1972, selenium was shown by Rotruck and co-workers to be essential for the action of glutathione peroxidase (Paper 20).

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Also during the 1970s, through the work of Kodicke in Cambridge and Deluca in Wisconsin, the prevailing view that vitamin D acted directly to promote intestinal absorption of calcium and regulation of bone metabolism was shown to be in error. They discovered that vitamin D, through the combined actions of the liver and kidney, was converted to a hormone that mediated the actions attributed to vitamin D. This represented a new concept: the action of a vitamin depending on its conversion to a hormone (Paper 19).

Another major paradigm shift in nutrition resulted from discoveries about the ability of the body to synthesize and degrade nutrients and tissue constituents. The shift occurred in phases, two of which are discussed in the symposium.

Claude Bernard, the great French physiologist, conjectured about the source of glucose in the blood of dogs consuming a diet that contained neither sugar nor starch. By a series of carefully conducted experiments during the 1850s, he discovered liver glycogen and the process of gluconeogenesis by which glucose and glycogen could be synthesized in the liver from non-glucose precursors, enabling this organ to supply glucose to the blood (Paper 1).

The use of isotopically labeled compounds by Schoenheimer in the 1930s to follow the metabolic fate of fatty acids and amino acids administered orally revealed for the first time that these nutrients were incorporated rapidly into depot fat and body proteins, respectively, and that their metabolites continued to be excreted over many days. Through his work, the concept of distinct exogenous (dietary) and endogenous (tissue) metabolism was replaced with the concept of the "dynamic state of metabolism," the continuous breakdown of tissues with the constituents of both food and tissues entering a
common pool from which new tissue components were synthesized (Paper 14).

The demonstration by Becker and colleagues that sucrose and fructose are toxic to young pigs and calves represents an extension of this paradigm, one of many, illustrating that metabolic pathways for some nutrients may not be functional at birth and undergo development during the early stages of growth (Paper 18).

With the successive discoveries of essential nutrients between 1915 and 1950 and the virtual disappearance of dietary deficiency diseases, emphasis in nutrition was on ensuring that diets would provide adequate quantities of all essential nutrients to prevent impairment of growth and development. Although it was recognized that requirements declined with increasing age, little attention was given to the long-term effects of total food intake. One of the first challenges to the paradigm that if essential nutrient intake was adequate throughout life, other dietary factors would be of little consequence, came from Clive McCay. He argued that short-term trials with the emphasis on rapid growth did not provide an adequate test of the most desirable nutritional state throughout life. He found that, although rats allowed to freely eat a nutritionally adequate diet grew most rapidly, those allowed only restricted amounts of food could survive much longer (Paper 13). Competing hypotheses about the basis for these effects remain unresolved, but they have opened new directions in nutritional thinking, especially in relation to appropriate body weight and energy intake for adults.

Emphasis on the paradigm of nutritional essentiality also distracted attention from investigations of the nonessential components of foods and from the ancient paradigm that foods contain nutrient, medicines and poisons. The finding that broccoli in the diet increased resistance of guinea pigs to X-irradiation and that this effect was not related to its contribution of known nutrients shifted attention back to the nonnutrient components of foods (Paper 17). There is now evidence that substances in cruciferous plants and some other foods may increase resistance to cancers. These observations have led to acceptance of a scientifically based form of the paradigm that foods can affect health by their contributions of chemicals other than essential nutrients through their influence on susceptibility to certain diseases.

The work reviewed here illustrates how much has been learned through the use of animal models. It also illustrates that caution must be exercised in extrapolating findings in one species to another. For example, rats were an excellent choice for studies of vitamin A and thiamin deficiencies, but failure to produce the equivalent of either pellagra or scurvy in this species led a leader in the field to conclude that these diseases in humans were not, after all, due to dietary deficiencies. These experiments also illustrate the need for caution in assuming that observations made at one stage of life apply throughout life.

The history of nutrition illustrates that new paradigms and concepts do not necessarily make earlier ones obsolete; several may exist together and overlap, with all being valid frameworks for investigation. What is the outlook for new paradigms and concepts in nutritional science? Application of techniques from genetics and molecular biology to nutritional problems has led in recent years to advances in understanding the roles of nutrients and their metabolites in the regulation of gene expression with respect to metabolic adaptations, the action of hormones, and responses of the immune system. Undoubtedly other new paradigms and concepts, unanticipated now, will follow.

We believe that these proceedings illustrate, on the one hand, the tremendous advances resulting from the scientific approach to nutrition and, on the other, the importance of continually maintaining a critical approach to even well-accepted hypotheses and concepts.

**Paper 1: The Liver Forms, Stores and Secretes Glucose (Claude Bernard, 1860)**

Presented by Patricia B. Swan, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 95, April 11, 1995, in Atlanta, GA.

In 1834, the 21-year-old Claude Bernard left the hills of the Rhône Valley and went to Paris to seek his fortune as a playwright. A professor of literature at the Sorbonne read one of his plays, Arthur of Brittany, and counseled Bernard to enroll in medical school instead. Heeding this advice, Bernard entered the College de France in the fall (Bernard 1979). There he became intrigued by lectures in physiology given by François Magendie. Most chemists and physiologists of the time believed that only plants synthesized lipids, carbohydrates and proteins, whereas animals merely degraded them. The macromolecules within the body were therefore assumed to come largely preformed from the diet (Holmes 1974). A few skeptics questioned these ideas, because there sometimes seemed to be more fat in an animal's body than could have come from its diet. Bernard was captivated by Magendie's demonstrations of the intricacies of animal physiology, and from 1841 to 1844 he served as his laboratory assistant, gaining knowledge of techniques in animal experimentation (Holmes 1974).

**Studies on Glucose**

Soon Bernard began his own experiments, studying digestion and certain functions of the nervous system. He extended the digestion studies to examine the fate of sugars within the body and demonstrated that cane sugar (sucrose) was converted to grape sugar (glucose) in the gastrointestinal tract (Grmek 1968). Cane sugar, injected directly into a vein, was excreted unchanged in the urine; injected grape sugar, however, disappeared. Thus, glucose seemed to be the major form of sugar used within the animal body, and when Magendie, assisted by Bernard, fed starch to a dog, glucose was found in the dog's blood. Thus, glucose was a normal constituent of blood, at least after starch consumption, not just a sign of the diabetic condition as had been thought previously (Grmek 1968).

Early in 1848, Bernard began a systematic study to learn where glucose is used within the body. Following Lavoisier's idea, he conducted experiments with dogs that he thought would show glucose was burned in the lungs; however, these experiments yielded contradictory or uninterpretable results (Grmek 1968, Holmes 1974). In the early experiments, he had only insensitive methods for detecting and quantifying glucose and needed to use large quantities. He was assuming that these large quantities would be used almost instantly. Moreover, he used animals of various physiological conditions, and he sometimes fed the glucose and sometimes injected it. Improvement of a method for the detection of glucose based on its ability to reduce copper in an alkaline potassium tartrate solu-
tion significantly improved his results. Gradually Bernard improved his experimental techniques, and the early work set the stage for later, more successful, experiments.

The Source of Blood Glucose

In July 1848, Bernard conducted an experiment with a female that had been nursing a litter of pups. He did not feed her for one day and, as expected, found no glucose in her gastrointestinal tract, but to his surprise, he did find glucose in her blood (Grmek 1967). “What was the source of this glucose?” After this experiment, he altered the direction of his research to find the answer to this question (Grmek 1968, Holmes 1974).

In August, Bernard used a dog that had been fed only meat (no carbohydrates) for eight days and found a large amount of glucose in the portal vein, smaller amounts in the heart and the neck, but none in the chyle, stomach, intestine or urine. He exclaimed that the source of this glucose was “incomprehensible” (Grmek 1968).

A few days later, using a dog that had been fed only lard and tripe, he found no glucose in the mesentery (before the portal vein), but “enormous” quantities of glucose in the liver (Grmek 1968). Within the next four days, Bernard measured the glucose content of the liver of many different species, finding significant amounts of glucose in most. He concluded that liver of healthy animals contains glucose independent of a source of glucose in the diet (Bernard 1850, Bernard and Barreswil 1848).

Subsequent experiments provided evidence that the liver was the source of glucose in the body. By placing a tie between the liver and the portal vein, Bernard was able to show that the source of glucose previously found in the portal vein was the back flow of blood from the liver, not an alternative source prior to the liver (Bernard 1849 and 1850). For this work he received the Prize for Physiology in 1851 (Olmsted 1938) and the doctorate of science (Bernard 1853). It was also the beginning of Bernard’s important concept of the body’s ability to regulate its internal environment (Bernard 1878).

Search for the Source of Glucose in the Liver

In 1855 Magendie died, and Bernard was named to the Chair in Physiology at the Collège de France (Olmsted 1938). In this role he continued to pursue a variety of studies related to digestion, diabetes, toxins and the nervous system. During this time, he typically measured glucose in duplicate in several tissues of the animals he was studying. On one occasion he made the first measurement on a liver on one day, but did not make the duplicate measurement until the following day, after the liver had been allowed to stand at room temperature overnight. To his surprise, the content of glucose had increased markedly (Bernard 1855, Grmek 1967).

Bernard next decided to perfuse an isolated liver with cold water until the perfusate was free of glucose. He then allowed the liver to stand at room temperature for some hours. Upon resuming perfusion, he again found significant amounts of glucose in the perfusate. It appeared, therefore, that something within the liver was giving rise to glucose and it clearly was not making glucose from other elements in the blood. He took this as proof that there was a source of glucose within the liver (Bernard 1855).

Bernard then began the tedious job of isolating the glucogenic material present in the liver. He eventually recognized its chemical similarity to starch in plants and reported that it was present in opalescent extracts of the liver and formed a white precipitate when alcohol was added. It gave a red-wine color with iodine and was hydrolyzed by diastase, from saliva or plants, to produce glucose (Bernard 1857a, 1857b, and 1857c).

A Productive Decade

Within 10 years, Claude Bernard had made three major discoveries: 1) Glucose is a normal constituent of liver. 2) Liver is the source of blood glucose. 3) Liver forms glucose and stores it as glycogen, which, upon degradation, yields glucose.

Bernard’s experiments and the theories he derived from them were major contributions to the science of nutritional physiology. His exceptional skill in the surgery required for these studies, and the understanding that he developed regarding the use of intact animals in experimentation, earned him recognition as the “father of experimental medicine.” His major textbook (Bernard 1865) became a classic in the field, and he later received many honors, including membership in L’Académie Française (Bernard 1979, Olmsted 1938).

Literature Cited


Paper 2: Protein Cannot Be the Sole Source of Muscular Energy (Fick, Wislicenus and Frankland, 1866)

Presented by Kenneth J. Carpenter, University of California, Berkeley, CA 94720-3104 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, D.C.

By 1865 it had been the general “textbook” view for over 20 years that the energy needed for muscular contraction came from the destruction of a portion of the muscle’s own substance, i.e., protein. This had been stated by the organic chemist Justus Liebig in his influential Animal Chemistry. On page
233, he added that the protein broke up during the release of energy and that the nitrogenous fraction was converted to urea and excreted by the kidney, so that the total amount of work performed (i.e., both internally, as in the heart muscles, and externally) was proportional to the nitrogen excreted in the urine (Liebig 1840).

Liebig’s second point was essentially disproved by the finding that prisoners receiving a constant daily ration of food excreted no more urinary nitrogen during 24 h in which they had worked a treadmill than on days when they had rested (Smith 1862). However, it was still possible that protein had been a sole muscle fuel and that more had broken down on rest days by some alternative mechanism. It certainly seemed that nitrogen intake was the main determinant of its output.

A Swiss physiologist, Adolf Fick, saw that the best conditions for a critical experiment would be to do a considerable amount of measurable work while eating a protein-free diet. Then if the heat energy obtained from the oxidation of protein to urea and carbon dioxide were known, and also the relation of heat energy to mechanical work, it should be possible to determine whether the amount of body protein metabolized was sufficient to have powered the work done.

Fortunately, Fick’s brother-in-law, the chemist Edward Frankland, was at work in England developing a method for measuring the heat of oxidation of organic materials. High pressure “bomb” calorimeters had not yet been developed, but he was able to ignite a mix of potassium chlorate and manganese dioxide with the test material in a little “diving bell” immersed in an insulated water tank. Using a series of controls to adjust his results for the rise in temperature of the bath, he was able to obtain an impressive set of results for a long series of food materials and for urea (Frankland 1866).

The most directly relevant results are set out in Table 1. He assumed that metabolized protein yielded one-third of its own weight of urea, and he therefore subtracted the residual gross energy of this quantity of urea when estimating the energy released from the metabolism of 1 g of protein in the body.

By this time it also seemed well established that the mechanical equivalent of heat was such that the energy needed to raise 1 kg a distance of 423 m was at least approximately equivalent to 1 kilocalorie (Joule 1843).

Now the human trial was needed. Fick and his university colleague Johannes Wislicenus passed a night at a hotel near the foot of a convenient mountain. At 0500 h next morning they set out, carrying urine collection equipment, and walked steadily up a steep path until, at 1320 h, they reached another hotel at the summit. They were in a cold mist throughout the climb and did not believe that they had had significant losses from sweating. From noon the previous day until 1900 h on their exercise day, their only food was cakes made from starch paste fried in fat; they also drank strongly sweetened tea and some beer and wine over the period.

Their results for their urinary nitrogen excretion, and the subsequent calculations, with slight “rounding off” of their values, are set out in Table 2. It is seen that even without making any allowance for the internal work of breathing and respiration, and even if the muscular system were 100% efficient, the quantity of protein metabolized was insufficient to have provided the energy needed for their climb; in fact it was 51% for one subject and 43% for the other.

The climbers concluded that “the burning of protein cannot be the only source of muscular power” (Fick and Wislicenus 1866). And Frankland, on page 684 of his review of these and other results, added: “Like every other part of the body the muscles are constantly being renewed; but this renewal is not perceptibly more rapid during great muscular activity than during comparative quiescence. After the supply of sufficient albuminized matter [protein] in the food to provide for the necessary renewal of the tissues, the best materials for the production, both of internal and external work, are non-nitrogenous material. . .” (Frankland 1866).

These conclusions were not immediately accepted, but they stimulated further long-term trials that were confirmatory, although Liebig himself never admitted in so many word that he had been wrong (Carpenter 1994).

### TABLE 1

<table>
<thead>
<tr>
<th>Name of substance dried at 100°C</th>
<th>Heat units (kcal)</th>
<th>Kg-m of force</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy developed by 1 of each substance:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>When burnt in oxygen:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef muscle purified by ether</td>
<td>5.10</td>
<td>2161</td>
</tr>
<tr>
<td>Purified albumen</td>
<td>5.00</td>
<td>2117</td>
</tr>
<tr>
<td>Urea</td>
<td>2.21</td>
<td>934</td>
</tr>
<tr>
<td>When consumed in the body:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef muscle purified by ether</td>
<td>4.37</td>
<td>1848</td>
</tr>
<tr>
<td>Purified albumen</td>
<td>4.26</td>
<td>1803</td>
</tr>
</tbody>
</table>

1 From Frankland (1866).

### TABLE 2

<table>
<thead>
<tr>
<th>The results from the climbing trial</th>
<th>Fick</th>
<th>Wislicenus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (+ objects carried), kg</td>
<td>66</td>
<td>76</td>
</tr>
<tr>
<td>Urinary N (0500–1900 h), g</td>
<td>5.74</td>
<td>5.55</td>
</tr>
<tr>
<td>Protein metabolized (N x 6.25), g</td>
<td>35.9</td>
<td>34.7</td>
</tr>
<tr>
<td>Caloric equivalent of protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>at 4.37 kcal/gf kcal</td>
<td>157</td>
<td>151.6</td>
</tr>
<tr>
<td>Work equivalent (at 423 kg-m/kcal), kg-m</td>
<td>66,400</td>
<td>64,100</td>
</tr>
<tr>
<td>Net work in ascending 1956 m against gravity, kg-m</td>
<td>129,000</td>
<td>149,000</td>
</tr>
</tbody>
</table>

1 Fick and Wislicenus (1866).
2 Frankland (1866).

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**Literature Cited**


Paper 3: Inorganic Iron Can Be Used to Build Hemoglobin (Stockman, 1893)

Presented by Richard A. Ahrens, Department of Nutrition and Food Science, College of Agriculture and Natural Resources, University of Maryland, College Park, MD 20742-7521 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.

The condition of anemia was originally named morbus virgineus by Johannes Lange (Lange 1554). Lange was a physician of Lemberg and Rector of Leipzig University. He considered this disease to be peculiar to virgins and to be due to a retention of menstrual blood. His therapy involved instructing virgins afflicted with this disease to marry as soon as possible. He cited no less an authority than Hippocrates, in his treatise De Morbis Virginum, as also recommending marriage to cure this disease.

J. Varandal renamed this disease “chlorosis” (Varandal 1615). The popular English term was the “green sickness,” referring to the greenish hue assumed by Caucasians when their blood is low in hemoglobin. Chlorosis soon became a central feature in medical textbooks describing the diseases of women. Because chlorosis was a sign of virginity, European artists often painted young women during this era with a greenish hue. In art, if not in fact, chlorosis was a widespread condition.

By the mid 19th century, the disease of chlorosis was accepted by many physicians as being associated with neurotic and hysterical manifestations (Bullough and Voght 1973). Chlorosis became a form of neurosis. This view of chlorosis was an impediment to the acceptance of dietary therapy for its treatment. It was a refinement of the view that anemia was due to virgins in women, but it continued to perpetuate a sex bias. Bullough and Voght (1973) pointed out that sex bias flourished during the latter half of the 19th century as a male “backlash” against women’s demands for more education, greater political equality and the elimination of male stereotypes about woman’s place. Medical practitioners were almost all men, and many of them were hostile to any change in the status quo in male-female relationships. Medical schools that had admitted a few women early in the 19th century began to reject female applicants purely on the basis of their sex. Nursing schools were established in growing numbers to provide an alternative for females. By the latter part of the 19th century, chlorosis became an extremely common diagnosis (Clark 1887). It is necessary to appreciate this historical context to understand some of the resistance to accepting a nutrient deficiency as the cause of this disease.

Pierre Blaud in France recommended the use of pills containing ferrous sulfate for the treatment of chlorosis (Blaud 1832). The average dose amounted to approximately 150 mg/d, and considerable success was achieved. Despite this success, however, there was considerable resistance to the acceptance of chlorosis as a simple dietary iron deficiency. One of the obstacles to be overcome was the just-discussed sex bias that tended to associate chlorosis with the neuroses of women. Another obstacle to be overcome, however, was the inability of investigators using the balance method to demonstrate that inorganic iron could be absorbed from the gastrointestinal tract. V. Kletzinsky conducted a series of experiments (Kletzinsky 1854). In all of his studies, the amount of iron recovered in the feces was almost exactly equal to the amount of inorganic iron ingested. A third obstacle to be overcome was the toxic effect of intravenous injections of ferrous sulfate in dogs.

During the 1880s, Gustav von Bunge wrote two influential papers in which he concluded that only organic sources of iron were of value in treating chlorosis (Bunge 1885 and 1889). Von Bunge’s interest in iron dated back to 1874 when he analyzed both blood and milk and recognized that blood was rich in iron and milk had very little. He developed a philosophy that people are always best served when they get essential nutrients from foods. That philosophy also applied to iron. To quote von Bunge, “Why should a patient buy his iron in the pharmacy and not on the market with the usual foodstuffs?” This is a philosophy with many adherents today. As von Bunge implemented what he believed, however, it soon became a personal crusade in which he claimed that “the iron which the doctors give to chlorotics to form hemoglobin is not absorbed at all.”

As Gustav von Bunge got into his crusade he was soon claiming that iron therapy was successful because of the power of suggestion. It was well known, after all, that most chlorotics were women and often exhibited nervous or psychic disturbances. He felt that this made them “highly suggestible.” The true villains, according to von Bunge, were those who advised young women to practice vegetarianism. He spent much of what remained of his life arguing against vegetarianism and was enthusiastic about the nutritional value of meat in the human diet. He died in 1920, just as Prohibition was beginning as the “noble experiment” in the United States. He was an implacable foe of alcohol consumption all his life and looked forward to the results of this experiment with U.S. citizens as the guinea pigs. He anticipated that a model U.S. society would result from Prohibition and that Europe would then soon follow this great example (McCay 1953). It is undeniably fortunate that he did not live to see the result of this particular experiment.

When he wasn’t blaming the power of suggestion for the beneficial effects of inorganic dietary iron on chlorosis, von Bunge had a second explanation. Bullough and Voght (1973) noted that such contradictions were common among researchers studying “women’s diseases” during the late 19th century. Von Bunge adopted Kletzinsky’s theory (1854) that susceptible patients became chlorotic through the production by gut bacteria of hydrogen sulfide, which then reacted with organic iron compounds in the ingesta to produce insoluble ferrous sulfide.
If inorganic salts of metals having insoluble sulfides were given as dietary supplements in large quantities, these should take up most of the hydrogen sulﬁde, leaving more of the organic iron compounds free for absorption.

Ralph Stockman of the Edinburgh University School of Medicine put Kletzinsky’s, and thereby von Bunge’s, theory to the test (Stockman 1891). He did tests on chlorotic patients to determine if inorganic iron worked directly or by the indirect mechanism of binding with hydrogen sulﬁde. His results are summarized in Figure 1. He gave subcutaneous daily injections of ferrous citrate providing 32 mg of iron to three chlorotic young women and found an increase from 44% to 52% of normal hemoglobin concentration in 10 d. After 24 d the women had blood hemoglobin concentrations that were 72% of normal. Stockman then tried giving another four subjects 550 mg/d of iron by mouth in the form of ferrous sulﬁde and enclosed in keratin capsules that released the iron salt in the small intestine. Iron in this form could not be expected to bind any additional hydrogen sulﬁde. Nevertheless he found an increase from 48% to 60% of normal hemoglobin concentration in 12 d. After 33 d the women had blood hemoglobin concentrations that were 84% of normal. He also gave 9.6 g/d of bismuth dioxide to chlorotic women having blood hemoglobin concentrations that were 55% of normal and found these hemoglobin levels to be only 54% of normal 9 d later. Manganese dioxide gave a similar result. These latter two salts were quite capable of removing hydrogen sulﬁde from the gut, but they had no value in treating chlorosis.

It would seem that the elegant refutation of Gustav von Bunge’s hypothesis by Ralph Stockman in 1893 should have made it apparent that inorganic iron had great value as a nutrient. In another paper two years later, Stockman (1895) showed that chlorosis in young women was explained by their low overall intake of food, particularly of meat, which resulted necessarily in low iron intake, at a time when the combined burdens of growth and menstrual blood loss increased their need. He showed, through the use of a more speciﬁc analytical procedure for iron in foods that avoided interference from chicken’s crop and that the silver skin contained an antidote mechanism of binding with hydrogen sulﬁde. His results are

TABLE 1

<table>
<thead>
<tr>
<th>Date</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1889</td>
<td>Atwater (chemistry of U.S. foods)</td>
</tr>
<tr>
<td>1896</td>
<td>Eijkman (polynutrients from polished rice)</td>
</tr>
<tr>
<td>1901</td>
<td>Grifﬁn’s (need for unidentified micromutrients)</td>
</tr>
<tr>
<td>1907</td>
<td>Holst and Frohlich (guinea pig scurvy)</td>
</tr>
<tr>
<td>1912</td>
<td>Funk (“vitamine” concept)</td>
</tr>
<tr>
<td>1914</td>
<td>McCollum (fat-soluble factors)</td>
</tr>
<tr>
<td>1915</td>
<td>Jansen and Donath (thiamin isolated)</td>
</tr>
<tr>
<td>1936</td>
<td>Williams and Cline (thiamin synthesized)</td>
</tr>
</tbody>
</table>

Recently we reported on Christiaan Eijkman’s work on polyneuritis in chickens performed during the 1890s in Indonesia (Carpenter and Sutherland 1995). The timeline shows how early it was when this work was being performed: at the same time as Atwater’s calorimetry work, and before the “vitamine” concept of Funk and McCollum’s studies of fat-soluble factors (Table 1). This was a time when the infectious theory of disease was dominant, and it was while Eijkman was looking for an infectious cause of beriberi, a serious disease in Indonesia at that time, that he recognized a similarity with polyneuritis seen in chickens (Eijkman 1990). He found that polyneuritis appeared in fowls when they were fed a diet of polished rice, but that by adding the silver skin, which had been removed during polish ing, the polyneuritis could be prevented or cured. From the results of many feeding experiments, Eijkman concluded that the polyneuritis was due to a nerve poison produced during the fermentation of starch in the chicken’s crop and that the silver skin contained an antidote to this poison.

Eijkman’s work was cut short by ill health, and in 1896 he had to return to Holland. The research was continued by another young Dutch military surgeon, Gerrit Grifﬁns. He had obtained his medical education in Holland and then studied physiology in the laboratory of Carl Ludwig in Germany (Grifﬁns 1901). In 1892 he was sent to Indonesia to assist in another of Eijkman’s studies, that of the physiological adaptation of Europeans to tropical conditions. But Grifﬁns was shortly recalled to military service, and when he was able to return to Batavia (modern-day Jakarta), Eijkman had already left for Holland. Grifﬁns was then appointed to carry on the investigations into the cause of polyneuritis in chickens. His official commission was to “investigate the physiologi-
TABLE 2

Grijs' key experiments

<table>
<thead>
<tr>
<th>The question asked</th>
<th>The answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Did a lack of minerals in a diet of white rice cause polyneuritis?</td>
<td>No, diseased chickens were not cured.</td>
</tr>
<tr>
<td>2. Was the removal of fat with the silver skin the cause of the disease?</td>
<td>No, adding oil to the diet did not prevent the disease.</td>
</tr>
<tr>
<td>3. Was a lack of protein causing the disease?</td>
<td>No, birds fed a supplement of high protein soybean still developed polyneuritis.</td>
</tr>
<tr>
<td>4. Could the protective substance be extracted from rice silver skin and mung bean?</td>
<td>No, the extracts did not prevent or cure the disease because they decomposed during extraction.</td>
</tr>
<tr>
<td>5. Was starch required to produce polyneuritis?</td>
<td>No, it appeared in chickens fed just autoclaved meat.</td>
</tr>
</tbody>
</table>

1 Based on the paper by Grijs (1901).

cal and pharmacological properties of the tannin contained in red rice” and to determine if the pigment found in red rice could be considered as a curative or preventive remedy for beriberi.

Grijs was aware that it was not just red rice that prevented polyneuritis but all unpolished rice, and he decided to continue to study the whole silver skin and not just to focus on the pigment. His first feeding experiments confirmed Eijkman’s conclusions that polyneuritis was not caused by a lack of fat, protein or mineral (Table 2). In his 1901 report, Grijs remarked: “In judging the suitability of a food, we have not finished when we have determined the quantity of albumen... fat, carbohydrates and salts, even when we have applied the corrections for digestibility. We can indeed calculate from this whether a balance of nitrogen will be possible with it and whether the work which must be performed better internally and externally, can be obtained from it, but not whether permanent health is possible.”

Grijs believed that a number of substances existed, whose actions were not explained, but which played an important part in the prevention of disease. He illustrated this idea with two examples: “how very difficult it is, in spite of all the chemical analyses of mother’s milk, to find a good substitute for it and how frequently we find that, when we think one has been found, we are again disappointed” and “the peculiar fact that scurvy, which usually develops from lack of fresh food, which sometimes occurs on long sea voyages, is usually cured when the patients can again obtain fresh meat and fresh greens.” He concluded that still-unknown substances may be responsible.

Grijs used two approaches for investigating these “unknown substances.” One was to prepare different fractions from the silver skin, and the other was by comparative assay (Grijs 1901). He first boiled rice bran in a large quantity of water for 24 h and then strained, filtered and evaporated the liquor to give a dried extract. He used fowls that were already consuming a polished rice diet and gave them the extract via a stomach tube. All the birds died with symptoms of polyneuritis. Increasing the dose of the extract further had no effect; neither did feeding the residue from the extracted bran. Grijs concluded that the “protective substances of the silver skin were for the most part lost through the methods of preparation used.”

Grijs was also looking for a food material that when given in small amounts with polished rice, would prevent an outbreak of polyneuritis. He tested the mung bean (which he had noticed was often included in chicken feed) and the soybean. The results of his feeding experiments showed that both the skin and kernel of the mung bean prevented polyneuritis; however, the soybean was less effective. Comparing the composition of these two legumes, he saw that soybean was the richer in protein, fat and minerals but less effective as an anti-neuritic substance (Table 3). This supported his belief that polyneuritis was not caused by a lack of these three nutrients. In later experiments he found that extracts of mung bean were just as liable as those from silver skin. He stated that “we therefore had the same experience with Phaseolus radiatus (mung bean) as with the seed coat of the rice... at every attempt to isolate the active constituents, they perished... in different conditions they apparently became decomposed” (Grijs 1901).

Eijkman had reported that the addition of some meat to sago, tapioca and arenga starch diets did not prevent polyneuritis. However, removing starch and feeding meat alone did cure the condition. From these results, Eijkman had concluded that starch was a significant harmful factor in the etiology of polyneuritis, but this explanation did not satisfy Grijs. He felt it important to determine whether polyneuritis could develop independently of starch consumption. He therefore fed four birds meat that had been extracted with water for 2 d, and all died with signs of polyneuritis. He then fed eight birds meat that had been autoclaved, and six of these also developed polyneuritis. Thus Grijs concluded that the development of polyneuritis was not connected with starch and was even wholly independent of the presence of carbohydrate. These experiments also confirmed that the nerve degeneration was not caused by a lack of protein (Table 2).

In a discussion of polyneuritis and beriberi, Grijs put forth two explanations for the symptoms that occurred: “either we presume a deficiency, a partial starvation, ... or ... there is a microorganism which exercises a degenerative influence on the nerves” (Grijs 1901). Concerning the possibility of a deficiency or partial starvation, Grijs stated that very little was known about the metabolism of the peripheral nervous system and that “if for the maintenance of the peripheral nervous system, a certain substance or group of substances is indispensable, which are immaterial for the metabolism of the muscles, then it may be assumed that very little of them is necessary. When therefore in certain foods the substances indispensable for the nervous system are lacking or are present in insufficient quantity, in the first place any reserve supply, which is present either in the nerve itself or in the blood or in some other organ, will be used up... (and) disturbances will develop.”

He explained that polyneuritis did not develop with total

<p>| Table 3 |</p>
<table>
<thead>
<tr>
<th>Composition of mung bean (P. radiatus java) and soybean (S. hispida tumida java)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mung bean</td>
</tr>
<tr>
<td>Albumin</td>
</tr>
<tr>
<td>Fat</td>
</tr>
<tr>
<td>Ash</td>
</tr>
</tbody>
</table>

1 Modified from table of analyses published by Grijs (1901).
starvation, because in this situation the muscles were drawn upon to provide the needed protein and that this process released the "protective substance," which therefore became available to the nerves so that degeneration was prevented. Grijns used the notion of individual differences to account for why some birds did not develop polyneuritis: "one person needs a much larger quantity of food than another to maintain his physical equilibrium, while doing the same work . . . . If therefore the total metabolism shows important differences, there is no reason why, separate tissues which together furnish the total metabolism, should not have individual differences. Therefore a food which contains just enough of the still unknown nerve nutritive substances for one fowl contains too little for another."

In regard to the concept of a microorganism causing nerve degeneration, Grijns believed that this depended on the nourishment of the tissue to resist infectious organisms. He concluded that, irrespective of the causal factor of polyneuritis, "there occur in various natural foods substances which cannot be absent without serious injury to the peripheral nervous system . . . . The distribution of these substances in the different food stuffs is very unequal . . . . Attempts to separate them have resulted in their disintegration . . . (showing) they are very complex" (Grijns 1901).

**Literature Cited**


**Paper 5: Dietary Protein Standards Can Be Halved (Chittenden, 1904)**

Presented by Vernon R. Young and Yong-Ming Yu, School of Science and Clinical Research Center, Massachusetts Institute of Technology, Cambridge, MA 02139 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.

The essentiality of a dietary substance, which was later named "protein" by the brilliant Swedish chemist Jac Berzelius (Korpé 1970), had been recognized by the middle of the 18th century by Beccari and by Haller (Munro 1969 and 1985). However, it was not until about a century later that definitive pronouncements were made about the dietary needs for proteins in human subjects. Thus, surveys of diets in the United Kingdom by Lyon Playfair, in Germany by Carl Voit, in the United States by Wilbur Atwater, as well as by others in other countries, revealed, in relation to protein intake and the total fuel value of the diet, that "all over the world people who can obtain such food as they desire use liberal rather than small quantities . . . ." (Benedict 1906). It was from these kinds of data that conclusions were drawn about the necessary intakes of protein, and Voit, who commanded considerable attention and scientific respect, concluded that—based on his assessment of his work in Munich—the protein intake of the average working man should be 118 g daily and that higher intakes would be necessary for heavy workers. Atwater, a pupil of Voit, supported this conclusion (Table 1). However, and in part through the expanded use of the nitrogen balance approach (developed initially by Boussingault for his studies in Alsace on the utilization of foodstuffs by milk cows), others began to question whether intakes lower than those shown in Table 1 would not only be adequate but possibly offer benefits for improvements in health. Arguably, the most significant of these others was Russell Henry Chittenden. Thus, Benedict (1906) says: "Of all the experiments heretofore made in which the low protein diet was used, none can compare with the exhaustive series of experiments recently completed by Professor Chittenden of New Haven." Indeed, they were impressive and after the "dust had really settled" they were destined to have an enduring and profound effect on the course of research in, and thinking about, human nutritional requirements.

In 1882, Chittenden was appointed Professor of Physiological Chemistry at the Sheffield Scientific School at Yale University, two years after he had completed his Ph.D. degree in physiological chemistry, the first such degree awarded by a university in the United States (Vickery, 1945).

Chittenden (1904) presented a detailed account of his series of experiments in a monograph entitled Physiological Economy of Nutrition: With Special Reference to the Minimal Protein Requirement of the Healthy Man. An Experimental Study. This is remarkable considering that his experiments began only in 1902 and continued well into 1904 and that this occurred well before the convenience afforded by computer-based data retrieval and summary techniques, not to mention desktop publishing. In this publication, he indicates that he had first questioned the premise that the dietetic standards adopted by mankind represented the real needs or requirements of the body (p. 3, Chittenden 1904): "We may even question whether simple observation of the kinds and amounts of foods consumed by different classes of people under different conditions of life have any very important bearing upon this question." He was the sort of mentor that any student would have been privileged to serve under: willing to challenge dogma and chart an entirely new experimental approach.

His experiments began with an opportunity to observe for several months the dietary habits of Horace Fletcher, an American of independent means. Chittenden noted that Fletcher’s nitrogen intake averaged 7.19 g, and in the words of Dr. Anderson, the director of the Yale Gymnasium, “Mr. Fletcher of Venice performs this work with greater ease and with fewer noticeable bad results than any man of his age and condition I have worked with” (p. 14, Chittenden 1904).

**Table 1**

<table>
<thead>
<tr>
<th>Authority</th>
<th>Protein</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranké</td>
<td>100</td>
<td>2324</td>
</tr>
<tr>
<td>Munk</td>
<td>105</td>
<td>3022</td>
</tr>
<tr>
<td>Voit (1881)</td>
<td>118</td>
<td>3055</td>
</tr>
<tr>
<td>Rubner</td>
<td>127</td>
<td>3092</td>
</tr>
<tr>
<td>Moschott</td>
<td>130</td>
<td>3160</td>
</tr>
<tr>
<td>Atwater (1894)</td>
<td>125</td>
<td>3315</td>
</tr>
</tbody>
</table>

1 From McCoy (1912), based on dietary intake studies.
TABLE 2

A typical day’s record of R. H. Chittenden’s diet and nitrogen balance after 18 mo on his self-imposed experiment

<table>
<thead>
<tr>
<th>Sunday, June 26, 1904</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet</strong></td>
<td></td>
</tr>
<tr>
<td>Breakfast: Coffee 122 g, cream 31 g, sugar 8 g.</td>
<td></td>
</tr>
<tr>
<td>Dinner: Roast lamb 50 g, baked potato 52 g, peas 64 g, biscuit 82 g, butter 12 g, lettuce salad 43 g, cream cheese 21 g, toasted crackers 23 g, blanc mange 164 g.</td>
<td></td>
</tr>
<tr>
<td>Supper: Ice tea 225 g, sugar 29 g, lettuce sandwich 51 g, strawberries 130 g, sugar 22 g, cream 40 g, sponge cake 31 g.</td>
<td></td>
</tr>
<tr>
<td>Body weight = 57.4 kg (initial weight in November 1902 = 65 kg. Age 47 y)</td>
<td></td>
</tr>
<tr>
<td>Protein intake = 0.64 g/kg</td>
<td></td>
</tr>
<tr>
<td>N balance = −0.07 g N</td>
<td></td>
</tr>
<tr>
<td>Energy intake = 1549 kcal</td>
<td></td>
</tr>
</tbody>
</table>

1 Data from Chittenden (1904).

Back then was no different than today, in that Chittenden needed financial support for the conduct of his investigations. He secured funds from the Carnegie Institution of Washington and the Bache Fund of the National Academy of Sciences, and he also received large donations from Fletcher and John H. Patterson of Dayton, Ohio.

The overall investigation consisted of three major experiments, each characterized by a long-term period of dietary protein restriction combined with nitrogen excretion measurements and supplemented in some studies with assessments of physical and mental well-being. Chittenden (1904) states “The writer, fully impressed with his responsibility in the conduct of an experiment of this kind, began with himself in November 1902.” He therefore served as one of the subjects in his study of five university professors and instructors, including his former student Lafayette Mendel, who by that time had become professor.

On the basis of his own experience (Table 2), including the disappearance of the rheumatic problem he had been having in his knee joint and the findings with the other four Yale professionals, Chittenden concluded that the minimum “protein” requirement was 93–103 mg N/kg body wt (about 0.6–0.64 g protein·kg⁻¹·d⁻¹), which anticipates, by 80 years, the mean requirement figure of 0.6 g protein·kg⁻¹·d⁻¹ proposed by FAO/WHO/UNU (1985)!

The next two series of studies confirmed and strengthened these initial findings; one of these was with 13 members of a detachment from the U.S. Army Hospital Corps, who were housed in Vanderbilt Square at Yale for 6 mo. The study included measures of physical and mental condition and of blood composition in addition to nitrogen excretion and balance over the 6-mo period. The conclusion was that 50 g of protein daily can establish nitrogen equilibrium and that there is, at this approximate intake, a maintenance of physical strength and vigor and an ability to respond to sensory stimuli. In a follow-up letter written to Chittenden by one of the participants, Private First Class J. Steltz, and on behalf of the other men, he stated: “The men are all in first-class condition. . . . We eat very little meat now as a rule, and would willingly go on another test.” The extensive findings in a third series involving eight Yale University athletes merely served to replicate all of these data and the interpretations that had been drawn from them.

Thus, Chittenden concluded that one-half of the 118 g of protein called for daily by the ordinary dietary standards is quite sufficient to meet all the real physiological needs of the body, certainly under ordinary conditions of life” (p. 475, Chittenden 1904).

Perhaps it ought to be noted that these experiments did not actually establish an average, minimum physiological requirement for dietary protein in these groups of subjects because 1) the low protein diets were freely chosen by the professional group, 2) the athletes were instructed to diminish the intake of protein but without imposition of a specific diet, and 3) the soldiers were given meals that contained lowered amounts of protein than provided by ordinary army rations and apparently with some reduction in the total “fuel value” of the food. The food given to each soldier was weighed, and at the close of every meal the uneaten food was determined and subtracted from the initial weight.

Although there has been some concern about the precision and accuracy of the nitrogen balance data generated from Chittenden’s experiments (McCay 1912, Carpenter 1994), including the reliability of the urine and fecal collections and determination of nitrogen intake, the results of these series of experiments were coherent and dramatic. They presented a strong case that the physiological needs for protein were much lower than values represented by free-choice intakes of dietary protein.

Chittenden’s conclusions were neither quickly nor universally accepted. For example, McCay (1912) referred to the onslaught to Chittenden’s findings and ideas, but he used his own data from dietary surveys of Bengalis, as well as the data of others, to reach a conclusion that “Voit stands today absolutely vindicated.” Although Cathcart (1911) was in “complete agreement with Professor Chittenden’s statement that life can be maintained and frequently maintained at a high level on relatively low protein intake,” he was not sure if it was desirable to keep a low intake as a general rule, and he expressed concern about the quality of protein. Later, he (Cathcart 1921) voiced reservations that were related to the lowered resistance to disease in persons consuming low protein diets. However, Chittenden (1911) had already argued that the problem with McCay’s studies was that the diets of the populations studied in India lacked unidentified trace nutrients. This was probably true, in retrospect, given the public health problems of iron, vitamin A and iodine deficiencies that are prevalent in southeast Asia today.

The protein standards for healthy individuals continued to be set by the opinions of individuals until national and international committees were convened to establish dietary recommendations. An early international committee was set up by the League of Nations, and in 1936 the recommendation was that “the protein intake for all adults should not fall below 1 gramme of protein per kilogramme of body weight . . .” (League of Nations 1936). No scientific justification was presented in support of the recommendation. In 1943 the U.S. Food and Nutrition Board of the National Academy of Sciences issued its first Recommended Dietary Allowances, and in this report 66 g of protein daily was recommended. These early figures proposed by expert groups were somewhat higher than those found to be sufficient by Chittenden, but they were far below the liberal standards that had been widely adopted during the middle 19th and on into the early part of the 20th century. It seems clear, however, that by the 1950s the metabolic approach used by Chittenden and others for establishing protein requirements had been well embraced, to the exclusion of the dietary intake approach followed by Voit, Atwater and others. For example, at the Princeton Conference in 1955, W. R. Aykroyd, the director of the nutrition division of the Food and Agriculture Organization, stated: “We need
not linger on Carl Voit and his recommendations on protein requirements, which were over influenced by what was observed among the population of Munich in 1880” (FAO 1957a). Indeed, the first FAO report specifically concerned with protein requirements (FAO 1957b) depended heavily upon the review by Sherman et al. (1920) of the literature on nitrogen balance and adult requirements, which included extensive reference to Chittenden’s work. Parenthetically, Sherman’s paper might be viewed as a forerunner of modern-day meta-analysis! In any event, this 1955 FAO committee suggested that the average minimum requirement of adults for reference protein was 0.35 g/kg body wt and proposed a daily safe practical allowance of 0.66 g/kg. Although the more recent recommendations (FAO/WHO/UNU 1985) differ from those given in the 1957 report of FAO, this latter assessment undoubtedly would have given Chittenden great satisfaction, and it served as a vindication of the data obtained and conclusions drawn from his visionary studies, commenced 50 years earlier in the former New Haven residence of Joseph E. Sheffield.

Although Chittenden explored and made important contributions in the areas of digestive physiology and the action of proteolytic enzymes, an activity that had been enhanced by his year-long sojourn with Kühne in Heidelberg, and in toxicology, including heavy metal poisoning and disorders created by alcohol, it was his studies of the protein requirements of humans that may well be regarded as his greatest contribution to the advancement of nutritional science. When Chittenden died on Boxing Day (December 26) 1943, he had been a member of the National Academy of Sciences for more than 53 years!

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FAO (1957b) Protein Requirements. FAO Nutritional Studies, no. 16. Food and Agriculture Organization of the United Nations, Rome, Italy.


**Paper 6: Liebig’s Concept of Nutritional Adequacy Challenged (Hart et al., 1911)**

Presented by Alfred E. Harper, University of Wisconsin, Madison, WI, as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.

Imagine that we have fallen back 90 years through time. It is 1906. We know that protein and a few minerals (sodium, potassium, calcium, phosphorus, iron) are essential nutrients, but we are unaware of the essentiality of trace elements, vitamins or fatty acids. Liebig’s concept from the 1850s that protein, a few minerals, and sources of energy (fat and carbohydrates) are the sole principles of a nutritionally adequate diet undoubtedly would have given Chittenden great satisfaction, and we are unaware of the essentiality of trace elements, vitamins or fatty acids. Liebig’s concept, the reports lie buried in the scientific literature (McCollum 1957, p. 201).

E. B. Hart has just been appointed Professor of Agricultural Chemistry at the University of Wisconsin to succeed S. M. Babcock. Babcock has observed that milk production by cows consuming rations composed of different feedstuffs differs considerably even when the rations are formulated to have the same gross composition (proximate analysis). He tells Hart that he is skeptical of Liebig’s claim that the “physiological value” of a ration can be predicted from knowledge of its gross chemical composition (Hart 1932) and encourages Hart to test Liebig’s hypothesis.

In 1907, in collaboration with G. C. Humphrey of the Animal Husbandry Department, Hart plans what will come to be known as the “Wisconsin single grain experiment”. He hires E. V. McCollum to conduct the chemical analyses and Harry Steenbock as a student assistant. The objective of the experiment is to compare the performance of four groups of heifers fed rations composed entirely of the corn, wheat or oat plant or a mixture of the three, all formulated to be closely similar in gross composition and energy content (Hart et al. 1911).

The animals, 16 Shorthorn heifers about 6 mo of age and weighing 300–400 pounds (lb), are to be carried to maturity and through two consecutive reproductive periods. The four rations, designed to provide 14 lb dry matter/d, consist of the following (lb/d): corn meal 5, corn gluten 2, corn stover 7; oat meal 7, oat straw 7; ground wheat 6.7, wheat gluten 0.3, wheat straw 7; and a mixed ration consisting of equal parts of the other three.

The average amounts consumed by the four groups during the course of the experiment (14.5 to 15.2 lb/d) did not differ appreciably. Values for crude digestibility of the different rations averaged 65 ± 3% for both dry matter and nitrogen and were not significantly different.

Weight gains of the groups after 1 and 3 y are shown in Table 1. Although the corn-fed group gained considerably more weight than the wheat-fed group, variability within groups was such that, as can be seen from the large SD, the results did not provide convincing evidence that the growth responses were different.

The first clear evidence of differences in the responses of the groups was from observations on the appearance of the
TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Year 1</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pounds</td>
<td>pounds</td>
</tr>
<tr>
<td>Corn</td>
<td>471 ± 64</td>
<td>726 ± 55</td>
</tr>
<tr>
<td>Oat</td>
<td>408 ± 55</td>
<td>824 ± 89</td>
</tr>
<tr>
<td>Mixture</td>
<td>410 ± 37</td>
<td>724 ± 149</td>
</tr>
<tr>
<td>Wheat</td>
<td>353 ± 67</td>
<td>665 ± 86</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. Adapted from Hart et al (1911).

animals after the first year. Cows consuming the corn ration had smooth coats, were full through the chest, and appeared healthy. Those consuming the wheat ration had rough coats, were of smaller girth, and appeared gaunt. The other two groups were intermediate between the corn- and wheat-fed groups.

There were major differences in reproductive performance (Table 2). Calves born to cows consuming the corn ration were strong and vigorous in both years and all lived. The cows consuming the wheat ration all delivered 3–4 wk prematurely. Their calves were weak both years, and none lived beyond 12 d. Again, results for the other two groups were intermediate. In 1909, the first year of calving, cows consuming the oat and mixture rations produced weak calves, with only two from the oat group and one from the mixture group living beyond a few days. In 1910, the performance of these two groups was better. The calves were carried to term, and all of the calves from the oat-fed group and two from the mixture-fed group lived but were weaker than those from the corn-fed group. Average weights of the calves, were 78, 72, 62 and 49 lb for groups fed corn, oat, mixture and wheat, respectively.

Milk production of each group was measured for 30 d each year following cessation of colostrum secretion. The difference between the amounts of milk produced by the wheat- and corn-fed groups was large (Table 1). Average values for the groups (lb/d ± SD, number of observations in parentheses) were as follows: corn-fed group, 26 ± 3.5 (8); oat-fed group, 22.9 ± 6.5 (6); mixture-fed group, 20.4 ± 1.5 (5); wheat-fed group, 14.1 ± 2.6 (4). (In calculating the average for the wheat-fed group I deleted two values, one for a cow that died of illness, and one abnormally low value). No differences were observed in milk composition (total solids, total protein, casein, ash or fat) or in the characteristics of the milk fat.

The wheat ration was known from chemical analyses to provide less calcium, magnesium and potassium than the other rations. Two cows in the wheat-fed group were therefore given a supplement of these minerals during 1 y of the study to raise their levels of intake to those provided in the diets of the other groups. The condition of the cows was not noticeably improved. The calf produced by one of them was small and weak and lived only a few hours.

After the experiment was completed, some animals were switched to other rations for an additional year (1910–1911). The vigor and health of one cow that was switched from wheat to corn improved rapidly. It produced a calf weighing 81 lb, compared with 47 and 48 lb for calves produced the previous 2 y. One cow that was switched from the corn diet to the wheat ration supplemented with extra calcium, magnesium and potassium deteriorated. Its calf was stillborn 18 d prematurely and weighed only 36 lb, compared with 93 and 85 lb for those born the previous 2 y.

The authors concluded that 1) the nutritive value of a ration could not be predicted reliably from measurements of its total digestible nutrients and energy content, 2) the differences in performance were unlikely to be due to differences in the protein component because animals fed the diet that provided a mixture of proteins did not grow as well as some of those fed the single grain diets, and 3) mineral inadequacies were unlikely because the mineral supplement did not improve the performance of cows consuming the wheat ration. They did not claim to have eliminated these possible explanations conclusively.

They stated “we have no adequate explanation of our results.” They did not attribute the inferior performance of the wheat-fed group to a deficit of some unidentified essential nutrient. They did, however, propose that the physiological value of a food or feed could be determined by measuring growth or other responses of animals fed diets in which a portion of a basic ration was replaced by the product to be tested.

Is it possible, with hindsight, to identify specific nutritional deficits that can account for the inferior performance of the cows fed the wheat ration? Levels of calcium and magnesium were low in the wheat ration. A level of 0.16% of magnesium is adequate for dairy cattle, but the level of 0.16% for calcium is marginal (Shepherd and Converse 1939) and 0.3% is now recommended (Scott 1986). Nonetheless, the reproductive performance of cows consuming the wheat ration, as Hart and colleagues noted, was not improved when they were given supplements of calcium and magnesium. Also, the fat content of the wheat ration was low. Dairy cattle require at least 2% of fat (Shepherd and Converse 1939). Low milk production, as was observed in the wheat-fed group, is an early sign of fatty acid deficiency in lactating dairy cows.

In addition, the carotenoid content of forage crops declines during storage, and vitamin A depletion occurs commonly in cows maintained during the winter on forage that has been stored for several months. A predominant sign of vitamin A deficiency in dairy cows is premature calving, with the calves often born dead or surviving for only a short time. Reproductive performance of cows receiving the corn ration, which would be expected to provide a high level of carotenoids, was excellent. That of the cows fed the wheat ration was poor.

McCollum (1964) attributed this to loss of most of the leaves of the wheat plant during threshing. However, reproductive performance of the oat-fed group was poor the first year but much better the second, suggesting that the carotenoid content of feedstuffs varies from year to year. It would thus seem

TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>1909</th>
<th>1910</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Condition and survival of calves</td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>Strong and vigorous both years; all lived</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6d premature 2 live</td>
<td>12.5 d premature 1 died</td>
</tr>
<tr>
<td>Oat</td>
<td>All weak 1 live</td>
<td>1 week, 1 fairly strong</td>
</tr>
<tr>
<td></td>
<td>2 died, 2 live</td>
<td>All lived 2.5 d premature</td>
</tr>
<tr>
<td>Mixture</td>
<td>1 fair, 1 weak</td>
<td>1 week, 1 fairly strong</td>
</tr>
<tr>
<td></td>
<td>12 d premature 1 died</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>3 weak, 1 stillborn</td>
<td>1 week, 2 cows died</td>
</tr>
<tr>
<td></td>
<td>None lived &gt;12 d</td>
<td>All lived 2.5 d premature</td>
</tr>
<tr>
<td></td>
<td>26 d premature 2 live</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 d premature</td>
<td></td>
</tr>
</tbody>
</table>

1 Adapted from Hart et al (1911).
highly probable that differences in reproductive performance of the groups were due to differences in vitamin A status, owing to differences in the carotenoid content of the rations, possibly complicated in the case of the wheat-fed group by an inadequate intake of fat and a marginal intake of calcium.

The results of this experiment provided the first clear evidence from research in the United States that the nutritive value of a diet depended on factors other than its content of protein, a few minerals, and energy sources. It sounded the death knell for Liebig and Voit's concept of a nutritionally adequate diet. It contributed, together with observations by European investigators on the inadequacies of purified or restricted diets for chickens, rats and guinea pigs (see introduction to this symposium), to a sharp change in the direction of thinking about the nutritional essentiality of constituents of foods. Maynard (1962) stated that the findings of Hart and his associates "stimulated much of the work which resulted in mental rations, the leaves of the corn and oat crops largely remained intact. However, the wheat leaves were so fragile that virtually all were lost. Thus, "We actually fed [some of] the cows wheat grain and straw." In the many other references to this noted experiment the fortuitous flaw has been overlooked.

The failure of the wheat diet, together with his concurrent and diligent searching of the available literature—especially the abstracts in Maly's yearbooks—persuaded McCollum as to the real possibility of some indispensable nutrients remaining unrecognized. This, plus his discussions with Dr. Babcock, convinced him that the heifer feeding project would not alone yield really significant new knowledge, but it stimulated him to think and move in a new and fertile direction. After some four months he suggested to Dean Henry and Dr. Hart that the "most promising approach to the study of the nutritional requirements of animals was through experiments with small animals fed simplified diets composed of purified nutrients. Small animals were desirable because they eat little, and we could do the extensive chemical work necessary for purifying the dietary ingredients. Small animals grow rapidly to maturity, reproduce at an early age, and have a short span of life... I recommended using rats...." (McCollum 1964). They opposed the use of rats in an agricultural experiment station, but Babcock encouraged him and McCollum proceeded on his own, without discontinuing any of his other pressing work. He procured "a dozen young albino rats from a pet-stock dealer in Chicago and paid for them myself" (McCollum 1964). Owing to his meager resources and limited knowledge in dealing with small experimental animals, he began to learn through trial and error. His extensive reading program had given him some acquaintance with the Pavlovian ideas on relationships between the taste of food and its digestibility. Thus he focused for a while on adding to the purified diets different flavors pleasant to people. No promising results were obtained. However, this and other experiences advanced his wisdom in the selection of research hypotheses and the planning of experiments.

Most fortunately, two years after he had gone to Wisconsin, he was voluntarily and unexpectedly joined by a young woman resident of Madison, Marguerite Davis, who had recently graduated in chemistry at the University of California at Berkeley, and asked if she might take care of the rats.

There soon began the experiments that were in time identified as McCollum's biological method for the analysis of food. The first definitive publication of research in which Miss Davis had a significant role was in establishing the "necessity of certain lipins in the diet" (McCollum and Davis 1913). This was an unexpected outcome in the early phases of their exten-
Two years later, McCollum and his graduate student Corcelia Kennedy suggested the provisional use of alphabetical terms for this lipin and other essential “organic complex(s),” using a prefix designating characteristic solubility. Thus for this essential factor(s) they proposed the term “fat-soluble A” (McCollum and Kennedy 1916). Soon this gave way to the term “vitamin A.”

McCollum and Davis also focused on discovering the nature of the nutritional deficiencies of the cereal grains. Particular attention was given to the nature of the dietary deficiencies of rice. In their article were 42 growth charts (McCollum and Davis 1915). Each chart portrayed the growth for each rat in an experimental lot, the composition of the ration used, the kind of test substance (accessory) added, if any, etc. Some of the results are summarized in Table 1. In their introduction they stated in part:

“In the present communication we present experimental data showing the specific properties of polished and of unpolished rice as a food, and show the supplementary relationship between these and certain purified and naturally occurring food-stuffs . . . . These studies, in addition to extending our knowledge concerning the dietary position of rice, have contributed to our understanding of the factors involved in normal nutrition, especially as regards the unknown accessory constituents of the diet which have received so much attention in recent years in connection with the ‘deficiency diseases’, scurvy and beri-beri.”

A substantial proportion of the research was on “the supplementary relationship between certain extracts of naturally occurring food-stuffs and polished rice” in which most of the extractants employed were water, ethanol and acetone (McCollum and Davis 1915).

This was in regard to answering the question of the presence of “water-soluble accessory” in polished and unpolished rice. Water extract of wheat embryo was far superior to an acetone extract in supplementing a diet based on polished rice. McCollum and Davis (1915) concluded that “such knowledge, when available for a wide variety of foodstuffs must, we believe, be of great value in the formulation of human diets which will promote health.” Thus this study contributed to the illumination of all their studies on cereal grains. While their work

![FIGURE 1](https://academic.oup.com/jn/article-abstract/127/5/1017S/4724174) Prepared from McCollum’s growth charts. This male rat received 3% cottonseed oil in its diet for the 7 wk of period 1 and then received 3% olive oil that had been shaken with soaps from saponified butter fat. The dotted line represents the normal growth rate of these rats (from McCollum and Davis 1914, chart 2, rat B).

**TABLE 1**

<table>
<thead>
<tr>
<th>Lot (no. of male and female rats)</th>
<th>308</th>
<th>316</th>
<th>317</th>
<th>383</th>
<th>395</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5 M, 1 F)</td>
<td>(6 M)</td>
<td>(6 M, 1 F)</td>
<td>(3 M, 2 F)</td>
<td>(4 F)</td>
<td></td>
</tr>
<tr>
<td>Approx. initial weight g</td>
<td>100</td>
<td>40</td>
<td>60</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polished rice</td>
<td>96</td>
<td>91</td>
<td>91</td>
<td>—</td>
<td>82</td>
</tr>
<tr>
<td>Unpolished rice</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rice polishings</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>88</td>
</tr>
<tr>
<td>Salt mix</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Egg albumen</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Casein</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Butter fat</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Approx. weight gain in 8 wk, g</td>
<td>0 or less</td>
<td>0–15</td>
<td>loss</td>
<td>65</td>
<td>50</td>
</tr>
</tbody>
</table>

1 Based on data in five different growth charts selected from 42 published by McCollum and Davis (1915).
on rice was in progress they learned more about the findings of E. B. Vedder and others on beriberi, and they “identified the nutritive value of the substance in their water extracts of wheat germ and egg yolk with the anti-beriberi factor” (McCollum 1957).

McCollum presented these findings in a lecture before the prestigious Harvey Society in New York in 1917 and concluded by stating: “These [supplements] have peculiar [not yet understood] dietary properties which the best chemical talent of today fails to recognize, but which are readily demonstrable by biologic tests” (McCollum 1917).

Five years later, in the second edition of his book The Newer Knowledge of Nutrition (McCollum 1922) he wrote:

“The biological method . . . was first developed with a view to discovering the nature of the deficiencies of individual natural food-stuffs. For this purpose the food under investigation is the principal component of the diet and is supplemented with small additions of one or more purified food substances (e.g., protein, inorganic salts, vitamins) [but even in 1922 no such isolated and chemically identified nutrient had been reported], in order to bring to light the nature of the additions which enhance its value. The method is applicable in another modification, however, which has yielded much valuable information concerning the relative values of many of our more important foods with respect to any one dietary constituent.”

The final and most comprehensive presentation on the biological method for the analysis of foods is in his history of nutrition (McCollum 1957).

### Literature Cited


McCollum, E. V. (1964) From Kansas Farm Boy to Scientist. The Autobiography of Elmer Verner McCollum. University of Kansas Press, Lawrence, KS.


### Paper 8: Some Amino Acids Are Indispensable for Growth (Osborne and Mendel, 1914–1916)

Presented by Kenneth J. Carpenter, Department of Nutritional Sciences, University of California, Berkeley, CA 94720-3104 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Atlanta, GA.

“Osborne and Mendel”—one of the most important and long-lasting collaborations in the history of nutritional science—and between two very different characters. Thomas Osborne was from a well-established New England family of lawyers and bankers and the fifth generation to go to Yale. He lived all his life in New Haven, worked in the Agricultural Experiment Station with no students and did not like to face an audience (Fruton 1995, Vickery 1932). Lafayette Mendel, 12 years the younger, was the son of Jewish immigrants running a clothing store in New Haven and a brilliant student at Yale. After two years of postdoctoral work in Germany, he came back on to the faculty, loved teaching and, unusually for his time, encouraged young women as well as men to do graduate work. His field was metabolism (Chittenden 1938).

In 1909, when they began to collaborate, Osborne was 50 years old and had already spent over 20 years isolating different vegetable proteins and demonstrating their chemical individuality and, in particular, how different they were in amino acid composition from animal proteins. Did that mean that they were necessarily nutritionally inferior? This was, obviously, of interest to Mendel, too, because he had been part of the big Yale study a few years earlier that used human subjects and ended with the recommendation that Atwater’s dietary standards for protein could be halved from 120 g to about 60 g for the average man, with no qualification as to the type of protein.

By 1909, several European workers had reported that enzyme digests of proteins could support nitrogen balance in adult rats and dogs, but that acid hydrolysates could not do so (Henriques and Hansen 1905). This was attributed to the destruction of tryptophan by acid digestion, and it was hypothesized that, although animals could manufacture linear amino acids, only the plant kingdom could synthesize cyclic ones such as tryptophan (Aderholden 1909).

Osborne and Mendel were skeptical of short-term nitrogen balance trials with rats, because of the problem of recovering all their urinary nitrogen and thus getting spurious positive balances. They thought that the only really satisfactory way to test the adequacy of dietary proteins was to test the amount of amino acids that were necessary for young animals to grow substantially in size, using purified diet components.

They soon found difficulties in maintaining growth in rats using starch, lard and mineral mixes even with casein as the protein source. They obtained improved growth by adding dried “protein-free milk,” i.e., separated milk from which the proteins had been precipitated by acidification and then boiling (Osborne and Mendel 1911). After the further inclusion of butter fat, growth continued to the mature weight of their animals (Osborne and Mendel 1914). With gliadin as their protein source in place of casein, rats hardly changed weight for 3 mo. But with added lysine they grew well.

At this time, with only crude gravimetric methods of analysis available, they believed that gliadin (isolated from wheat) had no lysine. Therefore, because rats would maintain weight

### TABLE 1

<table>
<thead>
<tr>
<th>Diet 1 (18% gliadin)</th>
<th>Diet 2 (17.64% gliadin, 0.36% lysine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. rats</td>
<td>4</td>
</tr>
<tr>
<td>Food eaten, g</td>
<td>99</td>
</tr>
<tr>
<td>Lysine, from gliadin, mg</td>
<td>160</td>
</tr>
<tr>
<td>Lysine, from supplement, mg</td>
<td>—</td>
</tr>
<tr>
<td>Total lysine intake, mg</td>
<td>160</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>+6.2</td>
</tr>
<tr>
<td>Est. protein gain, g</td>
<td>+0.99</td>
</tr>
<tr>
<td>Est. Lysine gain, mg</td>
<td>+79</td>
</tr>
</tbody>
</table>

1 Data from Osborne and Mendel (1916)

2 Assuming 0.92% lysine in gliadin, 8.0% in rat body proteins, and 16% protein in the weight gain by the rats.
with gliadin as the sole protein, lysine did not seem to be required for maintenance. However, by 1915, with an improved analytical procedure for lysine, they had found that gliadin did contain about 0.9% lysine, but they assumed that the rat’s muscle proteins, like those of animals’ muscles that had been analyzed, contained approximately 8% lysine, so that part of the lysine in the rats’ new tissues had to have come from the free amino acid supplement. They did not report any actual calculations to support this point, but Table 1 reproduces the relevant estimates that they could, and perhaps did, make.

Lysine could not apparently be synthesized by rats, even though it had no cyclic group. This was an important point because it had been thought it was the amino acids containing cyclic groups that animals would be found to be unable to synthesize. On the other hand, rats could apparently maintain themselves with dietary protein of very different composition from that of their own tissues.

This idea was confirmed by their parallel results with zein (from corn), which is completely lacking in lysine and also both tryptophan and glycine (Osborne and Mendel 1916). They showed most of their results with zein as growth curves Dementia, dermatitis, diarrhea and finally death associated from that of their own tissues. considered more likely than dietary deficiency to be the cause of South Florida, Tampa, FL 33606 as part of the minisymposium presented by Leslie M. Klevay, U.S. Department of Agriculture, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202 and Robert E. Olson, College of Medicine, University of South Florida, Tampa, FL 33606 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington DC.

Microbiology was transforming medicine at the end of the Victorian era. By the time Funk coined the term “vitamine” in 1912, the causative organism for tuberculosis had been known for 30 years. It is not surprising that infection was considered more likely than dietary deficiency to be the cause of pellagra. Both toxicity and heredity, two other causes of disease known at the turn of the century, had also been suggested.

Dementia, dermatitis, diarrhea and finally death associated with a diet of meat, maize and molasses described the pellagra syndrome. Unfortunately, the “meat” consumed by poor people was high in fat and low in protein. The dermatitis is photosensitive and confined to the areas of skin exposed to sunlight; Casal’s (1691–1759) necklace is the eponym attached to “the area of erythema and pigmentation around the neck in pellagra” (Terris 1964). The dementia was usually of the manic-depressive type and severe enough to justify admission to a mental institution.

Joseph Goldberger, who contributed extensively to our understanding of the causes of pellagra, was born in Austria in 1874 and immigrated with his parents to the United States in the 1880s. He grew up in New York City and entered the City College of New York as a high school graduate in 1890 to study engineering but changed his field to medicine two years later by enrolling at the Bellevue Hospital Medical College. He obtained his MD degree in 1895 and after internning for one year and practicing medicine in New York and Pennsylvania for three additional years, he joined the U.S. Public Health Service in 1899. He served as a quarantine officer in various ports including New Orleans, Tampico, Veracruz and Havana and studied yellow fever and typhus transmission by mosquitoes in those areas. In 1909, he solved the cause of Schambarg’s disease, a pigmented dermatitis, prevalent in crew members

## Table 2

<table>
<thead>
<tr>
<th>No. rats</th>
<th>Amino acid supplement to diet (g/100 g)</th>
<th>Mean weight change (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>None</td>
<td>−20 (±5)</td>
</tr>
<tr>
<td>6</td>
<td>0.54% Tryptophan</td>
<td>−7 (±6)</td>
</tr>
<tr>
<td>7</td>
<td>0.54% Tryptophan + 0.54% lysine</td>
<td>+22 (±5)</td>
</tr>
</tbody>
</table>

[1] Data from Osborne and Mendel (1914).

### Literature Cited


Presented by Leslie M. Klevay, U.S. Department of Agriculture, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202 and Robert E. Olson, College of Medicine, University of South Florida, Tampa, FL 33606 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington DC.

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on private yachts and in men living in private dwellings and boarding houses in the Philadelphia area. Goldberger and Schambor (1909) observed that these men slept on straw mattresses, and they finally identified a mite (Pediculoides ven-tricosus) as the vector for the disease. Thus, Goldberger had considerable experience in epidemiology and knowledge of infectious diseases when he was assigned by the Surgeon General in 1913 to undertake a study of the causation of pellagra.

Pellagra was not recognized as a problem in the United States until early in the 20th century. In 1912, Lavender of the U.S. Public Health Service estimated that more than 25,000 cases of pellagra had occurred in the United States in the previous five years and that the case fatality rate was 40%. The dominant thinking in the United States at the time Goldberger began his investigations was that pellagra was an infectious disease. As a result of studies in South Carolina, the Thompson-McFadden Pellagra Commission concluded in 1913 that “1) The supposition that the ingestion of good or spoiled maize is the essential cause of pellagra is not supported by our study; and 2) Pellagra is in all probability a specific infectious disease communicable from person to person by means at present unknown.” These conclusions were elaborated by Siler et al. (1914).

In less than three months after beginning his investigation, Goldberger (1914) published his first paper on pellagra. In a document of a little over three pages, Goldberger summarized the epidemiology of the disease as follows. Pellagra cannot be communicable. The cause is dietary. Prevention should result from a “reduction in cereals and vegetables and canned foods that enter to a large extent in the dietary of many of the people in the South and an increase in fresh animal food component such as fresh meats, eggs and milk.” In support of these views Goldberger pointed out that 1) in institutions where pellagra was prevalent, no case had ever occurred in nurses or attendants; 2) the disease was essentially rural; and 3) it was associated with poverty, which in turn was associated with a diet deficient in animal foods.

These conclusions, however, were reached by epidemiologic methods involving the association of variables and did not constitute proof of the etiology of the disease. Goldberger and his colleagues then proceeded to attempt 1) to cure pellagra by changing the diet of pellagrins to one rich in animal foods and 2) to demonstrate by direct studies the possible infectivity of secretions, scales and excreta from pellagrins. A year after publishing his first paper on pellagra, Goldberger and his co-workers demonstrated in back-to-back papers (Goldberger et al. 1915, Willets 1915) that pellagra could be prevented in institutionalized patients by a diet that included generous amounts of milk, eggs, meat, beans and peas and that pellagra could be successfully treated by the same regimen.

The second part of Goldberger’s plan was to demonstrate the nontransmissibility of pellagra by contact with nasopharyngeal secretions, blood and excreta from pellagrins (Goldberger 1916). In a heroic study on themselves conducted by Goldberger, Sydenstricker, Tanner, Wheeler, Willets, Goldberger’s wife and an additional 10 volunteers, defibrinated blood, nasopharyngeal secretions, feces, urine and dermatitic scales were administered enterally and parenterally in an attempt to cause pellagra. It must be noted that physicians in the public health service at that time had learned to accept such exposures as the risk of dealing with infectious diseases, and in fact Goldberger himself had contracted both yellow fever and typhus from his previous work. Various tissues, nasal secretions and excreta were obtained from 17 cases of pellagra of various grades of severity, including three fatal cases. Goldberger and Wheeler themselves were the first subjects, each receiving 5 mL of the defibrinated blood by intramuscular injection and also secretions. Three days later, Goldberger ate feces from an acutely ill patient together with the urine and dermatitis scales from two other patients. As a result, Goldberger developed diarrhea that lasted for about a week, but despite this both he and Wheeler joined three other volunteers for a similar round of tests with both injections of defibrinated blood from three patients and the oral consumption of scales and excreta. To maximize the chance of catching any infection from stools, they used fresh fecal material from the rectum of pellagrins by using an enema and then blended material from five subjects into pills that were consumed by the volunteers. The volunteers also took sodium bicarbonate before and after consuming these materials to reduce the acidity of the stomach to prevent a possible bacteriocidal action of gastric juice. Mrs. Goldberger received one injection of blood. Both Goldberger and Wheeler felt stiffness after the intramuscular injections, and several volunteers felt nauseous after ingestion of feces. Nonetheless, after five to seven months none showed any sign of pellagra. Because Goldberger’s group had failed to demonstrate any transmissibility of an infectious agent to themselves from pellagrins and had demonstrated both the preventative and curative action in pellagrins of diets rich in animal foods, they felt secure in their conclusion that the disease was dietary and not infectious. Nonetheless, they conducted another epidemiologic study of seven cotton mill villages in South Carolina beginning in 1916, which showed that the disease was not high in villages with poor sanitation but was high in villages with poor diets.

In 1920, the question remaining was: What was the agent in animal foods that prevented pellagra? Because the biological value of animal protein was in general better than the values for proteins in cereals and vegetables, Goldberger and Tanner (1922) proposed that an amino acid might be the pellagra preventative factor. Tanner actually conducted a trial of tryptophan in one pellagrous patient, which caused marked improvement of the dermatitis but little change in the diarrhea. He reported the finding in a progress report to Goldberger, but the result was not followed up (Tanner 1921, quoted by Hundley 1954). Goldberger also tested various foods in an attempt to cure black tongue, the pellagra analogue in dogs. Goldberger died prematurely at age 55 in 1929. He thus didn’t live to see the cure of black tongue with niacin as reported by Elvehjem et al. in 1937, although Goldberger and Sebrell (1930) did induce remission of this canine disease with liver extract. His idea that amino acids were critical in the pathogenesis of pellagra was confirmed by a finding by Krehletal. (1945), who proved that nicotinic acid could be formed from tryptophan. Subsequently Vilter et al. (1949) showed that tryptophan would cure pellagra in humans.

In summary, Goldberger was a well-trained physician, a brilliant epidemiologist and an imaginative clinical investigator. He studied a variety of infectious diseases and pellagra, which was not infectious, with a multidisciplinary approach that included epidemiology. He is still lauded as a exemplar of clinical epidemiology (Elmore and Feinstein 1994).

Literature Cited


Goldberger, J. (1914) The etiology of pellagra: the significance of certain epi-
Supplement 10: Copper as a Supplement to Iron for Hemoglobin Building in the Rat (Hart et al., 1928)

Presented by Leslie M. Klevay, U.S. Department of Agriculture, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 95, April 11, 1995, in Atlanta, GA.

Although Hopkins (1906) and Funk (1912) shrewdly predicted early in the 20th century that some diseases occurred because of dietary deficiency of accessory or essential food factors, the observations they cited were “unknown to medical men and chemists of that period” (McCollum 1957). Physiologists, biochemists and even the public (Allen 1931) caught up by 1928. Many important discoveries on the role of organic nutrients were made in the 1920s because Hopkins and Funk had opened their eyes (McCollum 1957).

The 1926 edition of Osler’s influential textbook (Osler and McCrae 1926) listed two classes of anemia. The secondary or symptomatic class included those due to blood loss, infection or intoxication. The primary or essential class included only chlorosis, pernicious anemia and sickle cell anemia. Of these three, only chlorosis had an effective treatment. Osler referred to the treatment of chlorosis by iron therapy as “one of the most brilliant instances—of which we have but three or four—of the specific action of a remedy” and stated “It is a minor matter how iron cures chlorosis.” Osler did not infer the existence of iron deficiency anemia (chlorosis was of unknown cause). Anemias from deficiency of ascorbic acid, cobalt, folate, niacin, pantothenic acid, pyridoxine, riboflavin, thiamine, vitamin E, etc., were unrecognized (Wintrobe 1967).

Hart, Steenbock, Waddell and Elvehjem certainly were well informed about nutritional opportunities and contemporary nutritional knowledge; they may have been somewhat ahead of physicians who read Osler. Their experimental design resembled that of Whipple et al. (1920), who used phlebotomy to make dogs anemic and then measured blood regeneration to assay dietary components for nutritional value. They produced anemic animals by dietary methods, however. In the preceding paper in their series, data were presented showing that iron salts of great purity were ineffective in correcting an anemia of rats confined to a diet of cow’s milk. However, an equal amount of iron fed as ash of lettuce, corn or beef liver (an acid extract of ash) was very potent in restoring normal hemoglobin.

The seventh paper (Hart et al. 1928) in their series established copper as an essential nutrient. Although the authors were modest, concluding only that the experiments “point to the need for a more intensive study of the rôle of small amounts of inorganic substances in the diet,” McCollum (1957) suggests that this experiment and other similar ones on trace elements “broadened immensely the outlook of physiologists, biochemists, and pathologists.”

Figure 1 contains charts on six rats of some 50 presented. Each chart showed both rat weight and hemoglobin plotted against time. Intakes of 0.3 g of Cohn’s liver preparation (approximately equivalent to 1.7 g of dried beef liver) produced a “marked growth response” in rat 597 without much improvement in hemoglobin. Cohn et al. (1927) fractionated livers and other organs, and the intermediate dose was similar to that of physicians who read Osler. Their experimental design resembled that of the Whipple et al. (1920) who used phlebotomy to make dogs anemic and then measured blood regeneration to assay dietary components for nutritional value. They produced
in the case of some molluscs and crustacea." McHargue, Warburg and Krebs had reported copper in human blood and in serum of "dog, cat, rat, guinea pig, frog, chicken and goose." In cattle, liver was found to be highest in copper among the organs, with lean meat being considerably lower.

"Recently the use of liver and liver extracts has come into prominence . . . in the treatment of pernicious anemia." "The fact that this preparation was found effective in the treatment of anemia in the rat exactly as it has been found effective in the treatment of pernicious anemia in man appeared to us rather significant." That the preparation contained copper and that copper alone was effective in rats suggested "more than a casual incidental connection." It was realized, however, that the "treatment of the two anemias need not necessarily be alike."

Thoughts from the 1990s

Instead of being the seventh paper in a series on iron, this could have been the first in a series on copper. Nowhere did the authors suggest that copper is an essential nutrient. Surprisingly little has been written on the definition of nutritional essentiality, although concepts from the trace element era have been collected and reviewed (Klevay 1987). Although rats grow reasonably well when deficient in copper, they cannot complete their life cycle without it.

The experiments of Hart et al. (1928) leading up to this discovery about copper were done without the use of statistics. Hill (1965) recalls some of his own work done in this era in which results were so clearcut that it had not occurred to him to use a test of significance. He discusses more recent situations when to "decline to draw conclusions without standard errors can . . . be silly." I'm told that replication by repetition and confirmation in more than one species prevented false conclusions in this earlier era. Thus, this experiment is all the more remarkable, although it is not quite fair to say that it was done with only a single rat.

Hart et al. (1928) cited seven references. The method for
measuring hemoglobin was not among them. Copper in the liver preparation was measured by the xanthate method without citation. Inspection of papers I, V, IV and VI in their series revealed no method of iron analysis. Hemoglobin was measured with either Feischl-Miescher or Newcomer hemoglobinometers, methods not likely to be as reliable as the cyanmethemoglobin method. Similarly, it is not clear which of the Cohn fractions was used. Two were active in treatment of pernicious anemia; another contained a “substance capable of reducing blood pressure.” One can infer (Schultze 1939) that cages were made of galvanized iron.

Nutritional scientists became preoccupied with the hematology of copper for more than a half century after this work. The reason for this preoccupation is not clear, but it may have been created by the vigor of Cartwright and Wintrobe. Perhaps just as the Buchners’ concepts of cell-free fermentation were resisted by many because of Pasteur’s experiments on spontaneous generation, many in nutrition began to think that hematology was everything in relation to copper.

A few were more flexible, however, and searched for other characteristics of copper deficiency. Keil and Nelson (1934) discovered what now is called glucose intolerance. Bennetts et al. (1942) first noticed cardiac catastrophes. The hematologists were not totally inactive in this latter field (Shields et al. 1962). Surely if the hypercholesterolemia of copper deficiency were not nearly invisible in plasma or serum (in contrast to hyperglycercydiridemia, which is mild in deficiency), copper and lipid metabolism would have been associated long before 1973 (Klevay). Now (Lei and Carr 1990) there is much more research being published on cardiovascular effects of copper deficiency than on hematology, although interest in the latter is reviving with a shift toward leukocytes (Percival 1995). It may be interesting to know what pathology might have been found by Hart et al. (1928) with even limited necropsy. Sometimes pathology is obvious (Allen and Klevay 1978).

Hart et al. (1928) commented that “Only when some important function . . . lends itself . . . to quantitative measurement are conditions suitable for making progress . . . !!” They had adequate methods for measuring copper, hemoglobin, etc., but the bioassay using rat 621 and others perhaps was most important, because this assay validated the other assays. No data were available on the “form in which Cu occurs in liver” or on “various materials” in which chemical form may have a “modifying effect upon the action of Cu.” These comments are valid in regard to much recent work on the biology of copper, as well.

**Literature Cited**


**Paper 11: The Conversion of Carotene to Vitamin A (Thomas Moore, 1930)**

Presented by James Allen Olson, Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011-3260 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.

In the early 1900s, Frederick Gowland Hopkins, Casimir Funk and others realized that minor organic components of foods played essential roles in growth and nutritional well-being. These minor essential constituents of the diet were termed “vitamines,” primarily because the first one discovered, thiamine, clearly possessed an amine function. That these mysterious vitamines were present not only in water-soluble extracts of living materials but also in fat-soluble extracts was soon noted by several investigators both in the United States and in Europe. McCollum and Davis (1913) at the University of Wisconsin and Osborne and Mendel (1913) at Yale University first identified foods containing this fat-soluble growth factor. Active foods included butter, egg yolk, whole milk powder, cod liver oil and many pigmented fruits and vegeta-
bles, whereas inactive foods included lard, olive oil and non-pigmented fruits and vegetables.

Between 1913 and 1919 two different types of foods clearly showed activity: 1) animal foods that were colorless or showed only a slight yellow color and 2) yellow-colored foods, largely of plant origin. The question then arose of the nutritional relationship between these two types of foods. There were several possibilities: 1) that the two different types of compounds were independently active on some physiologic system essential for growth; 2) that the pigmented compounds were active as such, or possibly were converted to active, so-called “leuko” forms; and 3) that only one of the types of compounds was active, but that the other type was contaminated with the active ingredient. In 1919, Harry Steenbock wrote: “It appears reasonably safe, at least as a working hypothesis, to assume that the fat-soluble vitamin is a yellow plant pigment or a closely related compound.”

The following year, Palmer and Kempster (1920) tested this hypothesis. They clearly showed that chicks grew well and laid eggs when fed a carotenoid-free diet supplemented with a colorless extract of pork liver. The field was suddenly thrust into turmoil. If the yellow plant pigments (carotenoids) were not the active growth-promoting compounds, why indeed, in the absence of animal products, did they stimulate growth and development?

A variety of factors confounded a clear analysis of the problem at that time. First of all, the chemical structures neither of vitamin A nor of carotenoids were known. Second, all lipid preparations, whether from animal or plant sources, contained many impurities. Third, some plant carotenoids stimulated growth, but other equally colored plant extracts did not. Four, other nutritional inadequacies often plagued the interpretation of results. For example, vitamin E was discovered to be an essential fat-soluble vitamin only in the early 1920s. Finally, the methodology for the measurement both of vitamin A from animal sources and of carotenoids from plant sources was rudimentary.

Following Palmer and Kempster’s work, the generally accepted view was that carotenoid preparations were contaminated with the colorless vitamin, which was found in pork liver and other animal tissues. Thus, carotenoids per se were thought to be inactive constituents of these extracts.

Thomas Moore, born in 1900, started his research career in the mid-1920s at the Dunn Nutrition Laboratory associated with Cambridge University in England. Following studies on the importance of light exposure in the formation of biologically active carotenoids in plants, he focused his attention on the nutritional relationship between carotene and vitamin A. Unconvinced by the “contaminant” hypothesis, he initiated studies on the conversion of carotene to vitamin A in the late 1920s. The two key objectives of his study were 1) to prove that purified carotene preparations did not contain vitamin A and 2) to show unambiguously that carotene was converted to vitamin A in vivo.

In the mid-1920s, it had become clear that carotene and vitamin A were quite different substances, based on their color, absorption spectra, solubility, and reactivity with antimony trichloride. In this latter reaction, vitamin A gave a vivid blue color at a wavelength maximum of 610–630 nm, whereas carotene gave a green-blue color of lesser intensity at a peak wavelength of 590 nm. The absorption maximum of β-carotene was approximately 450 nm, whereas liver oils containing vitamin A absorbed very weakly, if at all, at that wavelength but showed a maximum absorption at 328 nm. The main instrument used in these studies was the Lovibond tintometer. By its use, two types of units were defined: yellow units, which were high for β-carotene and very low for vitamin A, and blue units, based on the antimony trichloride reaction, which were very strong for vitamin A and weak for β-carotene. Thus, the ratio of yellow to blue units was 11 to 1 for carotene but 1 to 100 for vitamin A.

Moore’s full paper was published in the Biochemical Journal in 1930. Moore first determined whether or not carotenoid preparations contained vitamin A. He crystallized carotene 12 times from a concentrated extract of carrots. He then compared the growth-promoting ability of his crystalline carotene with that of a cod liver oil concentrate. Approximately equal amounts of carotene and of the oil concentrate stimulated the growth of vitamin A–deficient rats. The oil concentrate gave the expected strong blue color at 610–630 nm, characteristic of vitamin A. If the carotene preparation had contained vitamin A as a contaminant, then it also should have given a strong blue color. But it did not. Thus, Moore concluded that the carotene preparation did not contain vitamin A.

Moore then turned to studies on the conversion of carotene to vitamin A in vivo. He fed 22 rats a vitamin A and carotene–free diet for 28 to 77 d. Ten of the depleted rats were killed, and their livers were analyzed for yellow and blue units. Thereafter, the remaining rats were supplemented with various sources of carotene for 16 to 55 d. These rats were then killed, and their livers were analyzed. The data presented in Moore’s paper are summarized in Table 1. The 10 depleted rats showed a few yellow units but no blue units in their liver. Small doses of carotene stimulated the growth of animals but did not much change the presence of blue or yellow units in the liver. On the other hand, when large doses of carotene or red palm oil, which predominantly contains α- and β-carotene in roughly equivalent amounts, were administered, the blue units in the

### Table 1: Comparison of ingested and stored carotene and vitamin A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Daily dose of carotene</th>
<th>Total ingested units (yellow)</th>
<th>Blue (610–630 nm)</th>
<th>Yellow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depleted</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1–10</td>
</tr>
<tr>
<td>Carotene</td>
<td>10–50 μg</td>
<td>770–4400</td>
<td>0</td>
<td>7–16</td>
</tr>
<tr>
<td>Carotene</td>
<td>750 μg</td>
<td>24,000–39,000</td>
<td>2000–3700</td>
<td>40–110</td>
</tr>
<tr>
<td>Red palm oil</td>
<td>1.55 g</td>
<td>83,000–130,000</td>
<td>4500–5000</td>
<td>280–400</td>
</tr>
<tr>
<td>Fresh carrots</td>
<td>Ad libitum</td>
<td>ND</td>
<td>5300–16,000</td>
<td>250–600</td>
</tr>
</tbody>
</table>

1 Data from Moore 1930. ND = not determined.
paper. Liver pigments added to the diet of thiamin-deficient animals were shown to support an elevation of the blood pyruvate levels. This finding was later confirmed by others, including McCollum and Davis (1913), who demonstrated that the addition of liver pigments to the diet of thiamin-deficient animals increased the rate of oxygen consumption and pyruvate accumulation in vitro.

However, the explanation for the role of liver pigments in carbohydrate metabolism was somewhat puzzling. Platt and Lu (1935) noted that the addition of liver pigments to the diet of thiamin-deficient animals increased the rate of oxygen consumption and pyruvate accumulation in vitro. However, the lack of a significant difference between pyruvate content of normal and thiamin-deficient brains before incubation with added lactate was puzzling.

The explanation was provided by the work of R.H.S. Thompson and R.E. Johnson (1935), who correctly supposed that the normally large amount of pyruvate formed in the thiamin-deficient brain in vivo was detectable because the metabolism of pyruvate largely diffuses out into the bloodstream. Using pigeons and rats, they measured pyruvate indirectly with other keto compounds that can form bisulfite complexes, and they secured the fractional amount of pyruvate in the bisulfite-binding compounds by direct isolation of the pyruvate 2,4-dinitrophenylhydrazones and estimating it colorimetrically. Their findings are summarized in Table 1. It should be noted that Platt and Lu (1935) bridged from the experimental animals to humans by concordantly reporting the presence of pyruvate in the blood and cerebrospinal fluid of beriberi patients in the Orient, where the story began.

The significance of findings importantly focused by the investigators at Oxford, and even the broader value of basic research with its use of animals, was well reviewed by R.A. Peters (1936) in his lecture delivered at the National Hospital, Queen-Square. In summarizing “What has been learnt,” Peters states: “We may now take stock of the position. A purely in-vitro research with brain tissue of the bird was started in the first instance to improve the test for vitamin B-1 and later extended to elucidate the enzyme with which the vitamin cooperated. It has not only helped to settle these problems but it has proved the existence of pyruvate in normal metabolism. It has also shown that an in-vitro research upon brain tissue which takes advantage of the in-vitro labours of biochemists, can be applied to in-vivo events. This is an important step in this field. It is further encouraging that the work has led to the detection of pyruvate in the blood of beriberi patients, which may well prove diagnostic. Surely we

---

**Table 1**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Normal</th>
<th>Deficient</th>
<th>Cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigeon</td>
<td>3.96</td>
<td>11.31</td>
<td>5.29</td>
</tr>
<tr>
<td>Rat</td>
<td>4.22</td>
<td>9.39</td>
<td></td>
</tr>
</tbody>
</table>

1 Data from Thompson and Johnson (1935). Approximately 2.5 mg of each averaged number is not pyruvate.
could not have a better instance of the ultimately practical value of a purely academic research."

During the period when the metabolic connections of vitamin B1 to carbohydrate utilization at the level of pyruvate were being established, other work was leading to elucidation of the structure of the vitamin (Gubler 1991, McCormick 1998). The correct empirical formula of vitamin B1 determined by A. Windaus in 1932 recognized the inclusion of sulfur, and by 1936–1937 R. R. Williams and his coworkers determined the full structure and accomplished synthesis of what we today call thiamin. In 1925 Clive McCay took a postdoctoral position at Yale University, who was on leave to work with Mendel. Maynard, the head of the animal husbandry department at Cornell, retired in 1962 (Loosli 1974).

With the recognition that decarboxylation of pyruvate was a biochemical step somehow involving vitamin B1, the earlier work of E. Auhagen (1932), who separated the alkaline labile coenzyme called "co-carboxylase" from the yeast pyruvate "carboxylase," was given new importance. The structure of this functional coenzyme form of vitamin B1 was elucidated by instructor in chemistry at Texas A&M for a year. He was awarded a M.S. in biochemistry by Iowa State College in 1920, and had served as an instructor in chemistry at Texas A&M for a year. He was impressed with McCay, hired him. Thus, he began his 35-year career at Cornell, retiring in 1962 (Loosli 1974).

It can now be appreciated that thiamin pyrophosphate functions within α-keto acid decarboxylase subunits of three general types of multi-enzyme α-keto acid dehydrogenases, namely those for pyruvate, α-ketoglutarate and branched-chain α-keto acids, operating in our bodies. A second important role for the coenzyme of thiamin in the direct metabolism of carbohydrates is within transketolase, where thiamin pyrophosphate mediates interconversions with pentose phosphates (Horecker et al. 1953). A better index of thiamin status in humans evolved with development of erythrocyte transketolase assays. However, the classic work of R. A. Peters and his associates remains a seminal example of the prelude to thiamin coenzyme functions, whereas the efforts of R. R. Williams working on thiamin structure and of Lohmann and Schuster ascertaining the coenzyme nature of its pyrophosphate lead us toward the modern era of biochemical understanding.

### Table 2

<table>
<thead>
<tr>
<th>Isolation of cocarboxylase (100 kg yeast → 750 mg HCl salt)</th>
<th>C_{12}H_{21}O_{8}N_{4}P_{2}SCl</th>
<th>pyrophosphate ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Preparation of alcoholic heat extract.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Barium precipitation and elution.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Precipitation from an acid solution with ethanol and</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reprecipitation with methanol.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Absorption on Frankonit KL and elution with hot dilute</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pyridine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Fractional precipitation with methanol-ether.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Precipitation of poorly soluble picrolonates.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Precipitation of poorly soluble Ba and Ag salts.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Precipitation with phosphotungstic acid and crystallization as the hydrochloride salt.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Outlined from Lohmann and Schuster (1937).

<table>
<thead>
<tr>
<th>Chemical research</th>
<th>Biological research</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical analyses</td>
<td>Reconstitution of pyruvate carboxylase activity from beer and baker’s yeasts.</td>
</tr>
<tr>
<td>Acid hydrolases</td>
<td></td>
</tr>
<tr>
<td>Sulfite splitting</td>
<td></td>
</tr>
<tr>
<td>UV absorption</td>
<td></td>
</tr>
</tbody>
</table>

### Literature Cited


### Paper 13: To Live Longer, Eat Less! (McCay, 1934–1939)

Presented by Patricia B. Swan, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996 in Washington, DC.

In 1925 Clive McCay took a postdoctoral position at Yale University, with Lafayette B. Mendel, an experience that would lead directly to his classical studies of the effects of nutrition on the longevity of rats. He had been born (1898) in Indiana, received an A.B. degree in physics and chemistry from the University of Illinois in 1920, and had served as an instructor in chemistry at Texas A&M for a year. He was awarded a M.S. in biochemistry by Iowa State College in 1923 and a Ph.D. with C.L.A. Schmidt at the University of California, Berkeley in 1925. With his strong background in the chemical aspects of biochemistry, McCay was ready to turn his attention to questions more biological in nature (Loosli 1974).

At Yale, McCay learned of the earlier experiments in Mendel's laboratory (Osborne et al. 1917) demonstrating that female rats whose food intake was restricted were able to reproduce at more advanced ages than usual. McCay asked Mendel why they had not carried their experiments longer to determine the extent to which the life span of these rats had been increased. Mendel replied that McCay, as a young man, was in a better position to undertake such a long-term experiment (Loosli 1974).

While at Yale, McCay became acquainted with L. A. Maynard, the head of the animal husbandry department at Cornell University, who was on leave to work with Mendel. Maynard, impressed with McCay, hired him. Thus, he began his 35-year career at Cornell, retiring in 1962 (Loosli 1974).

### Nutrition and Longevity

Shortly after he went to Cornell, McCay began his first study of the effects of food restriction on the life span of rats,
Table 2 provides data regarding the weight and density of the femur at the time of death.

**Confirmation of the Relationship of Diet to Longevity**

In a second experiment, McCay and his group fed all rats identical amounts of a nutrient-dense diet, feeding that amount required to just maintain the weight of the restricted rats. This allowed them to determine whether the unlimited consumption of a nutrient-dense diet had shortened the life of the control group from the first experiment, and they concluded it had not. The growth of the rats was controlled by varying the amount of a mixture of sucrose, cooked starch and lard (38:57:5) that was fed, with the control rats being allowed to eat as much as they chose. In addition, the control group was divided, with half the rats being fed cod liver oil and half (as well as all rats with restricted intakes) being fed irradiated yeast and carotene as sources of fat-soluble vitamins. Supplementation with these different sources of fat-soluble nutrients

**TABLE 3**

Effects of various lengths of food restriction on the life span of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ave. age</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Unrestricted</td>
<td>670</td>
<td>308–896</td>
</tr>
<tr>
<td>Males (n = 17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (n = 16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II Restricted until d 500</td>
<td>865</td>
<td>805–1018</td>
</tr>
<tr>
<td>Males (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (n = 5)</td>
<td>811</td>
<td>555–1183</td>
</tr>
<tr>
<td>Restricted until d 500</td>
<td>806</td>
<td>366–1103</td>
</tr>
<tr>
<td>Males (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (n = 5)</td>
<td>990</td>
<td>793–1078</td>
</tr>
<tr>
<td>Restricted until d 700</td>
<td>874</td>
<td>772–1025</td>
</tr>
<tr>
<td>Males (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (n = 6)</td>
<td>912</td>
<td>406–1320</td>
</tr>
<tr>
<td>Restricted until d 1000</td>
<td>882</td>
<td>336–1127</td>
</tr>
<tr>
<td>Males (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (n = 4)</td>
<td>1033</td>
<td>815–1320</td>
</tr>
</tbody>
</table>

1 Data taken from McCay et al. (1939).
2 Data for controls, fed either carotene + irradiated yeast or cod liver oil, were combined.
Restricted rats did not attain the full size of the control rats. and amino acids and led to the concept of metabolic turnover.

Individuals whose food intake had been restricted for 1000 days led to the understanding of the metabolism of fatty acids and growth with few exceptions. Those exceptions were certain at Columbia University in the period from 1933 to 1941 and included: “The bones of the males respond to the realimentation more promptly than those of the female” (McCay 1952). Restricted rats were allowed to eat normally, they resumed growth upon the breeding period and duration of life of rats. Science 45: 294–295.

Table 4 presents the weight and density of the femur of rats in the second experiment. Again, both measures tended to decrease with increasing length of time of food restriction. Bone growth (length) was also measured, and the authors concluded: “The bones of the males respond to the realimentation more promptly than those of the female. The bones of the male retain the power to grow larger than those of the female” (McCay et al. 1939).

### Conclusions

McCay summarized the conclusions he drew from these experiments in a chapter he wrote (McCay 1939) and later revised (McCay 1952) as a contribution to a book on aging. “This indicated that the life span was flexible and that the possibility of its extension was unknown as well as that the retarded animals tended to outlive those that matured normally” (McCay 1952). “The second experiment gave essentially the same results as the first and indicated clearly that the retarded growth was the essential feature” (McCay 1952). He went on to say: “If one were to draw conclusions from these data for guidance of human beings he might summarize by stating: ‘Eat what you should, after that eat what you will but not too much.’”

McCay’s scientific work was interrupted by his military service during World War II, but after the war he continued his studies of the effects of nutrition on aging and the life span of rats and other species. He is best remembered by his students for the course he taught for several years on the history of nutrition. A collection of some of these lectures was published posthumously (McCay 1972). McCay died of a heart attack in 1967.

### Literature Cited


### Paper 14: The Dynamic State of Body Constituents (Schoenheimer, 1939)

Presented by Robert E. Olson, Department of Pediatrics, College of Medicine, University of South Florida, Tampa, FL 33606 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 95, April 11, 1995, in Atlanta, GA.

The discoveries of Rudolf Schoenheimer and his colleagues at Columbia University in the period from 1933 to 1941 revolutionized our understanding of the metabolism of fatty acids and amino acids and led to the concept of metabolic turnover.

Rudolf Schoenheimer was born in Berlin in 1898 and received his medical degree from the University of Berlin in 1922. Following a year of clinical training he did postdoctoral work in Leipzig with Karl Thomas from 1923 to 1926 and then in Freiburg with Ludwig Aschoff from 1926 to 1933, where he began work on the metabolism of cholesterol. In 1933, following the political upheaval in Germany, he emigrated to the United States and took up an appointment in the Department of Biochemistry at Columbia University, where he came in contact with Harold Urey, who had discovered deuterium in 1932, and David Rittenburg, one of Urey’s students who had also joined the biochemistry faculty at Columbia. Together they planned experiments with various isotopic compounds, including heavy water, and substrates labeled with deuterium and 15N; these experiments were to revolutionize concepts of fat and protein metabolism.

At the time that Schoenheimer initiated his isotopic studies of intermediary metabolism it was generally believed that the major components of the body, including body fat and protein, were chemically stable. It was assumed that there was very little exchange of nutrient molecules between the diet and the body.
Studies of Protein Metabolism

Schoenheimer and his associates next turned to the study of protein metabolism. They prepared doubly labeled $L(±)$leucine containing 3.60 atoms % excess deuterium in its hydrogen atoms and 6.54 atoms % excess $^{15}$N in its nitrogen atom. This concentration of both isotopes was high enough to allow admixture with several hundred parts of ordinary leucine and still permit detection by mass spectrometry. Adult rats were fed a stock diet containing the marked leucine and observed for 3 d, during which time there was no change in weight. At the end of the 3-d period the excreta and body tissues were analyzed. About 30% of the $^{15}$N administered was found in the excreta (2.2% of the isotope in the feces and 27% in the urine). The body protein retained the remaining 65% of the activity, indicating that the isotopic $N$ had been incorporated mainly into tissue protein. This finding was totally inconsistent with Folin’s view of exogenous protein metabolism.

It was found that different organs were not equally effective in the fixation of dietary nitrogen. As shown in Table 1, the proteins of the internal organs, serum and intestinal tract were the most active. The proteins of muscle showed less activity, but, because they constitute by far the largest part of the animal, the low concentration of $^{15}$N actually represented a high absolute amount of isotope. In fact, 66% of the administered isotope was recovered in muscle and only 33% in the combined internal organs.

Table 2 shows that not only was there an active incorporation of leucine into various organs and tissues, but the $^{14}$N also appeared in all other tissue amino acids studied except lysine. Particularly prominent in this exchange were glutamic and aspartic acids. This transamination observed by Schoenheimer in these studies was explained enzymatically by Braunitz and Kretzmann in 1937. The $^{15}$N in arginine was more enriched in liver than in kidney, mostly because of the ornithine-urea cycle in liver, discovered by Krebs and Hensel in 1932.

When the deuterium and $^{15}$N contents of the administered doubly labeled leucine were both measured, it was found that the $D/^{15}$N ratio in leucine was increased from 100:182 in the administered compound to 100:108 in the carcass, indicating the relative importance of the different sources of nitrogen.

Studies of Fat Metabolism

In Schoenheimer’s first study of the turnover of body constituents, he used deuterated water to track the biosynthesis of body fat. A proton from water is taken up in fatty acid biosynthesis in the reductive steps catalyzed by NADPH. Figure 1 shows the enrichment of fatty acids in the depot fat of mice over a period of 19 d. The isotope content of total fatty acids of the mice reached a maximum at d 6 with a $\frac{1}{2}$ time of 2.5 d. The total enrichment indicated that 14% of all fatty acids (mostly in adipose tissue) was replaced in this experiment. To demonstrate the destruction of fatty acids in mice fed a carbohydrate-rich diet, mice of the same weight were fed for 5 d a diet consisting of 20% fat enriched with deuterium and 80% whole wheat bread. Animals were killed at intervals. The destruction of the labeled fat proceeded at the same rate as the synthesis in the first experiment (Fig. 1). Schoenheimer also demonstrated that $[^{3}H]$-palmitic acid was not only deposited in the fat of rats but also converted into other fatty acids, including stearic and oleic acids but not linoleic acid, which had been shown earlier to be essential.
that a chemical reaction had occurred by which more than one third of the labeled nitrogen in the original leucine was replaced by ordinary nitrogen. This was further validation of transamination as a major reaction of amino acids.

Schoenheimer and coworkers then gave $[^{15}N]$ammonium citrate to immature rats fed a low protein diet. On this unnatural diet the animals lost weight, but despite the rapid disappearance of body proteins the rats synthesized new amino acids from dietary ammonia. The amide N in glutamine and asparagine was highly enriched, as was the $\alpha$-amino group of glutamate. Glutamine synthetase, glutamate dehydrogenase and carbamoyl phosphate synthetase are now known to be important enzymes for ammonia fixation into amino acids. Most of the isotope in arginine was in the amidine group and was removed by arginase, leaving a very small amount in ornithine in accord with the ornithine-urea cycle.

In these experiments, $[^{15}N]$ammonia was also incorporated into creatine, as expected from the work of Borsook and Dubnoff, who in 1941 demonstrating arginase to be a precursor of creatine. What Schoenheimer then showed was that the formation of creatine from $[^{15}N]$creatine was a spontaneous reaction without isotope dilution over a 29-d period. The slope of the curve indicated that about 2% of total body creatine was being converted to urinary creatinine (and replaced by low level of the essential amino acid tryptophan in its mixed dietary source of niacin, but the same group now tested rats 40% of their 15% casein diet with corn grits for 3 d. When niacin was added, growth was restored so that under these conditions the rats removed by arginase, leaving a very small amount in ornithine in accord with the ornithine-urea cycle.

In summary, Schoenheimer introduced a new and dynamic paradigm for fat and protein metabolism, replacing the static view of Folin and others. In fact, the idea of the major body constituents as a dynamic biochemical system has been extended more recently to include all aspects of metabolism, including systems involved in transport, hemopoiesis, endocrine and cytokine activity and even the genome.

### Literature Cited


### Paper 15: Tryptophan’s Role as a Vitamin Precursor (Krehl et al., 1945)

Presented by LaVell M. Henderson, Professor Emeritus, Department of Biochemistry, University of Minnesota, St. Paul, MN 55108 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 95, April 11, 1995, in Atlanta, GA.

When canine black tongue was recognized as the counterpart of pellagra, about 1925, experimental nutritionists adopted the dog for their studies of the causation of pellagra. Two hundred years after Casal’s description of this syndrome, it was established as a deficiency of a new B-vitamin, niacin, by the experiments of Elvehjem et al. (1938).

L. J. Teply used the microbiological method of Snell and Wright to assay a variety of foods for their niacin activity. Contrary to expectations, he found that corn contained enough niacin that it should prevent black tongue and pellagra. Krehl and co-workers confirmed this, but they found that the niacin requirement of dogs was three times greater when corn diets rather than sucrose-casein diets were consumed. Rats fed sucrose-casein diets had been found not to require a dietary source of niacin, but the same group now tested rats with corn diets. They reported (Krehl et al. 1945a) that replacement of 40% of their 15% casein diet with corn grits reduced growth from 25 to 4 g/wk. When niacin was added, growth was restored so that under these conditions the rats did require niacin. Corn is unusual in having a particularly low level of the essential amino acid tryptophan in its mixed proteins. Two months later, Krehl et al. (1945b) reported that tryptophan added at 40 mg/100 g diet replaced niacin in reversing the growth suppression caused by corn grits (Table 1).

### TABLE 2

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>7.95</td>
<td>1.90</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.85</td>
<td>0.89</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.16</td>
<td>0.70</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.89</td>
<td>0.25</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.50</td>
<td>0.20</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Amide N</td>
<td>0.78</td>
<td>0.51</td>
</tr>
</tbody>
</table>

1 Data from Schoenheimer (1942). Calculated for 100 atom per cent $^{15}N$ in liver administered.

### TABLE 1

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Basal</th>
<th>Basal + niacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% Casein</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>9% Casein + 40% CG</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>9% Casein + 40% CG + Trp</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>9% Casein</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>9% Casein + 0.2% Cys</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>9% Casein + 0.2% Cys + 0.078 Thr</td>
<td>3</td>
<td>19</td>
</tr>
</tbody>
</table>

Downloaded from https://academic.oup.com/jn/article-abstract/127/5/1017S/4724174 by guest on 28 December 2018
The most likely explanation for the interchangeability of niacin, required at 1 mg% of the diet, and tryptophan, required at 40 times that molar concentration, was the conversion of tryptophan to niacin. The fact that 2% acid-hydrolyzed casein (tryptophan destroyed) could replace 40% corn grits in causing the deficiency suggested that an increase in the levels of other amino acids was inducing the deficiency. This effect was termed "amino acid imbalance." The amino acids most effective in producing the deficiency were threonine and lysine (Hankes et al. 1948), but additional sulfur amino acids were needed to maximize the effect. Therefore, in subsequent studies 0.2% L-cystine was added to the basal diet (Table 1). The explanation offered for the effect of other amino acids on the growth of rats fed the 9% casein + 0.2% L-cystine diets is illustrated as follows:

\[ \text{Trp} + \text{AA (Thr or Lys)} \rightarrow \text{Protein} \]
\[ \downarrow \]
\[ \text{Niacin} \]

When an essential amino acid, such as threonine, is supplied at just below the optimum level, moderately good growth occurs and the marginal tryptophan level meets the needs for both protein and niacin synthesis. When the threonine in the diet is elevated, protein synthesis increases, tryptophan is drawn into this function at the expense of the alternate pathway to niacin, and the vitamin deficiency results. That this explanation for the effect of threonine is valid is supported by the results of similar imbalance studies that showed that lysine, valine, leucine and isoleucine also produce growth inhibition that is reversed by tryptophan or niacin (Koeppe and Henderson 1955).

Many isotopic labeling studies with rats have confirmed the conversion of tryptophan to niacin. The first evidence regarding the reactions involved came from experiments with mutants of the mold Neurospora crassa (Fig. 1). The first four intermediates in this scheme were verified in animal systems, and quinolinic acid was added when it was identified as an excretory product of tryptophan in mammals and a rather ineffective niacin substitute in rats and a niacin-less mutant of N. crassa (Henderson 1949).

Quinolinic and picolinic acids are formed by the incubation of 3-hydroxyanthranilate with mammalian liver (Fig. 2). Neither is degraded to carbon dioxide even in vivo. That they arise from intermediates in the degradation of tryptophan to glutamate was established by the report of Ghosh et al. (1962). It is evident that picolinate and quinolinate arise by formation of cyclic Schiff's bases from intermediates in the degradation of tryptophan, and they can be considered side reaction products of the degradative pathway.

The limited activity of exogenous quinolinic acid as a dietary replacement for niacin cast doubt on its intermediary role in the formation of niacin. This limited activity probably results from the failure of the salts of quinolinic acid to penetrate the cells in which it normally arises. It is a strong acid and because it is obliged to enter cells in the undissociated form, a proper pH for its penetration is much below physiological pH values. The role of quinolinate in the formation of niacin was clarified.
by the observations of Nishizuka and Hayashi (1963), who showed that it is converted to nicotinic acid mononucleotide in the presence of 5-phosphoribosyl-1-pyrophosphate by a soluble enzyme system from rat liver (Fig. 2). The wide variation among animal species with regard to the effectiveness of tryptophan as a source of niacin seems to reflect from the degree to which 2-acroleyl-3-aminofumarate is decarboxylated (Fig. 2). In cat liver, 86% is decarboxylated and only 9% is converted to quinolinate (Suhadolnik et al. 1957). At the other extreme, the rat liver decarboxylates only 12%, leaving 80% to form quinolinate. Livers from eight other species, including dogs and humans, fall between these two extremes. Cats cannot substitute tryptophan for niacin, whereas rats do not require niacin at the usual levels of tryptophan intake. Humans and dogs have a limited capacity to substitute tryptophan for niacin, so the clinical deficiency appears even with moderate intakes of tryptophan.

The prevalence of pellagra in poor, corn-eating populations in southern Europe, but not in natives of the western hemisphere, has been explained by the fact that the natives of the Americas used hot lime or other alkaline solutions in the preparation of their tortillas from corn meal. The observations of Krehl et al. (1945b), 50 years ago, have provided an explanation for the variation in niacin requirements among species and a partial explanation for the association between corn consumption and pellagra, based upon the very limited tryptophan content of corn. A full understanding of this association will depend upon the identification of the postulated substances in corn that form the vitamin on heating with alkali.


**Presented by Boyd L. O’Dell, Department of Biochemistry, University of Missouri, Columbia, MO 65211 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.**

The precise origin of the concept of antagonistic interaction of two or more essential trace elements is not entirely clear, but this essay reviews some of the first documented evidence for such a physiological antagonism. It not only describes the origin of the two-way interaction concept but extends the concept to that of a three-way interaction with copper, molybdenum and sulfur acting on each other individually and collectively.

In 1938 Ferguson and co-workers reported that the forage grown in the “teart” area of southern England contained higher concentrations of molybdenum than that of non-teart herbage. The teart area is an area of approximately 20,000 acres on which grazing cattle and sheep developed severe scouring and exhibited poor performance. Horses were not affected. The authors observed that addition of soluble molybdate to normal forage, in amounts equivalent to that consumed in the teart forage, produced the same signs of diarrhea in dairy cows. Although this paper did not show interaction of molybdenum with another specific nutrient, it did show the detrimental effect of trace quantities of molybdenum in the diet of ruminants (Ferguson et al. 1938).

Evidence of an antagonistic copper-molybdenum interaction came from Australia. The original observation in Australia was made during the investigation of an entirely different problem, enzootic jaundice, a disease in sheep that results from chronic copper toxicity. While seeking an explanation for a chronic copper toxicity that occurred in specific areas of the country, Dick and Bull (1945) observed incidentally that molybdate supplementation of cows and sheep decreased the liver storage of copper. Similar observations were made in New Zealand by Cunningham (1950), who studied the interaction of copper and molybdenum in relation to “peat scour.” In the United States, Comar et al. (1949) showed that molybdenum supplementation lowered copper storage in livers of cattle, and the effect occurred with intakes that gave rise to copper deficiency in areas of Florida. This series of studies established the concept of Cu-Mo antagonism and suggested that excess dietary molybdenum might induce copper deficiency. It also suggested that a low molybdenum concentration in forage of normal copper content might predispose sheep to copper toxicity.

Although these experiments demonstrated Cu-Mo antagonism, it soon became apparent that another dietary factor influenced the interaction. The concept of a three-way interaction among Cu-Mo-S emerged from a series of papers (Dick 1952, 1953a, 1953b and 1954) that constitute the basis of this present article. The first of the series (Dick 1952) showed that an unknown dietary constituent besides molybdenum had an effect on copper storage in livers of sheep. The salient data are shown in Table 1. Clearly the amount of copper stored increased as the proportion of oat to alfalfa (lucerne) hay in the diet increased with supplemental copper and molybdenum each remaining constant at 10 mg/d. With equal proportions of the two forages, total liver copper increased 35 mg over the 6-mo period. This value compares with an earlier observed increase of approximately 135 mg that occurred under similar conditions without added molybdenum. When oat hay was the sole source of forage, the increase was 113 mg even in the presence of additional molybdenum. It is also notable that the molybdenum concentration in the blood was 10-fold higher in sheep that consumed oat compared with alfalfa hay. This experiment showed clearly that a naturally occurring component of alfalfa that is largely absent in oat hay affected the physiological interaction of copper and molybdenum.

The next paper in the series (Dick 1953a) described experiments that identified inorganic sulfate as the component of alfalfa that lowered the blood molybdenum. Dick (1953a) found that an aqueous extract of alfalfa hay was rich in sulfate and that the extract had the same molybdenum-lowering

**Literature Cited**


TABLE 1
Liver copper and blood molybdenum concentrations of sheep fed different sources of forage supplemented with 10 mg each of copper and molybdenum per day for 6 mo

<table>
<thead>
<tr>
<th>Dietary forage (chaffed hay)</th>
<th>Increase in liver Cu²</th>
<th>Blood Mo conc.³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucerne</td>
<td>9</td>
<td>48</td>
</tr>
<tr>
<td>Lucerne: oat 1</td>
<td>19</td>
<td>96</td>
</tr>
<tr>
<td>Lucerne: oat 3</td>
<td>35</td>
<td>180</td>
</tr>
<tr>
<td>Lucerne: oat 3</td>
<td>70</td>
<td>376</td>
</tr>
<tr>
<td>Oat</td>
<td>113</td>
<td>447</td>
</tr>
</tbody>
</table>

1 Adapted from Dick (1952); n = 6 animals per group.
2 The increase in total liver copper is the difference between the estimated initial content and that at slaughter; the initial value was approximately 27 mg.
3 The values are the means of weekly blood samples taken over the period.

effect as the hay itself. The results summarized in Figure 1 show that the administration of potassium sulfate lowered blood molybdenum in a sheep fed oat hay supplemented with 10 mg of molybdenum per day. The lower blood molybdenum resulted from increased excretion of molybdenum via the kidney, an effect that was independent of urine volume. This and related experiments established the Mo-S interaction.

Subsequently, Dick (1953b) showed that sulfate is also the factor in alfalfa that, in combination with molybdenum, impaired liver copper storage. Pertinent data from two experiments are presented in Table 2. All sheep consumed approximately 10 mg of copper and some were supplemented with 10 mg of molybdenum per day. When the animals consumed oat hay, the addition of molybdenum had no effect on liver copper storage, but when they consumed alfalfa it markedly decreased copper storage. When the oat hay diet was supplemented with 2.2 or 4.4 g of sulfate per day as well as with molybdenum, copper storage was reduced to the same degree as in sheep fed alfalfa supplemented with molybdenum. The lower level of sulfate (2.2 g) was just as effective as the higher level in decreasing liver copper in sheep consuming oat or alfalfa hay. These experiments clearly established the existence of a three-way interaction among copper, molybdenum and sulfate as regards liver copper storage.

In spite of the decrease in liver copper storage observed when the diet was supplemented with both molybdenum and sulfate, the concentration of blood copper was increased, as shown by the data summarized in Table 3. The blood copper concentrations rose with increasing levels of both molybdenum and sulfate, and there appeared to be an interaction. Even though the blood copper concentration increased, the copper was not available for biological function. Sheep fed high levels of molybdenum and sulfate and a nominally adequate level of copper developed signs of copper deficiency even with blood copper concentrations that were normal or greater than normal. There was loss of crimp in the wool and decreased liver copper concentration. Clearly, under these conditions of high molybdenum and sulfate intake, blood copper was not a valid measure of copper status because there was evidence of copper deficiency in spite of normal or elevated blood copper concentrations.

An explanation of the Cu-Mo-S interaction must account for the fact that S renders both copper and molybdenum biologically unavailable and that molybdenum lowers copper availability only in the presence of dietary S. Suttle (1974) pointed out that the formation of cupric tetrathiomolybdate, a highly insoluble complex, could occur in the sulfide-rich environment of the rumen. This would account for low copper absorption but not for the high blood copper concentration. Normally all of the copper in blood is solubilized by trichloroacetic acid (TCA), but this was not true in cases of high molybdenum intake. To explain this phenomenon, Dick et al. (1975) prepared di-, tri- and tetrathiomolybdates and observed that their addition to blood in vitro resulted in a TCA-insoluble fraction that contained most of the copper. When tetrathiomolybdate was administrated to sheep intravenously, there was an immediate rise in the TCA-insoluble copper in the blood. Thus, formation of copper thiomolybdates, particularly CuMoS₄, in the rumen accounts for the poor absorption of copper when the intake of molybdenum is high. The absorption of thiomolybdate and subsequent formation of CuMoS₄ in the blood accounts for the high concentration of blood copper that is not bioavailable.

The experiments described here revealed a three-way interaction of copper, molybdenum and sulfate in ruminant animals, in which bacterial fermentation plays a major role in digestion. The interaction in monogastric animals is much less dramatic, because there is less sulfide available to form the thiomolybdate complexes. Ruminal microflora normally play a major role in this three-way interaction, but the interaction can be demonstrated in monogastric animals by administration of thiomolybdates. Interestingly, tetrathiomolybdate has been used to treat Wilson’s disease in human patients (Brewer et al. 1994). This genetic disease results in accumulation of cop-
Change in total liver copper in sheep fed forages supplemented with copper, molybdenum and sulfate for periods of 81–114 d

<table>
<thead>
<tr>
<th>Dry forage</th>
<th>Cu intake</th>
<th>Mo intake</th>
<th>Sulfate intake</th>
<th>Δ Liver Cu²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/d</td>
<td>g/d</td>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Oat hay</td>
<td>10.0</td>
<td>0.3</td>
<td>—</td>
<td>+54</td>
</tr>
<tr>
<td>Oat hay</td>
<td>10.0</td>
<td>10.3</td>
<td>—</td>
<td>+40</td>
</tr>
<tr>
<td>Lucerne</td>
<td>9.8</td>
<td>0.5</td>
<td>—</td>
<td>+20</td>
</tr>
<tr>
<td>Lucerne</td>
<td>9.8</td>
<td>10.5</td>
<td>—</td>
<td>−12</td>
</tr>
<tr>
<td>Oat hay</td>
<td>9.9</td>
<td>0.5</td>
<td>2.2 &amp; 4.4³</td>
<td>+15</td>
</tr>
<tr>
<td>Oat hay</td>
<td>9.9</td>
<td>10.7</td>
<td>2.2 &amp; 4.4</td>
<td>−20</td>
</tr>
<tr>
<td>Lucerne</td>
<td>10.1</td>
<td>0.7</td>
<td>2.2 &amp; 4.4</td>
<td>+39</td>
</tr>
<tr>
<td>Lucerne</td>
<td>10.1</td>
<td>11.1</td>
<td>2.2 &amp; 4.4</td>
<td>−18</td>
</tr>
</tbody>
</table>

1 Adapted from Tables 3 and 5 of Dick (1953b).
2 Calculated change in total liver copper during periods of 82 and 114 d; the mean d = 0 values, determined by biopsy, were 52 and 84 mg, respectively.
3 The effects of adding 2.2 and 4.4 g of sulfate, as the potassium salt, were not different, and the data were combined.

per in tissues, and thiomolybdate counteracts copper toxicity by complexation of the cupric ion and prevention of its absorption. What lesson derives from this work? In the first place, one cannot easily predict the outcome of good science regardless of its origin. In this case a project that was designed to determine the toxicology of a disease in sheep led to the elucidation of a complex interaction related to copper deficiency and eventually to a compound used in the treatment of a human disease.

**Literature Cited**


**TABLE 3**

Changes in blood copper concentrations in sheep fed a forage diet supplemented with graded levels of sulfate and given variable daily doses of molybdenum for 3 d

<table>
<thead>
<tr>
<th>Dietary sulfate intake</th>
<th>Increase in blood copper with indicated Mo dose²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 mg Mo</td>
</tr>
<tr>
<td>g/d</td>
<td>%</td>
</tr>
<tr>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>1.8</td>
<td>1.1</td>
</tr>
<tr>
<td>3.1</td>
<td>9.7</td>
</tr>
<tr>
<td>5.7</td>
<td>15.6</td>
</tr>
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1 Data from Table 1 of Dick (1954).
2 Change in the blood copper concentration of individual sheep over a 4-d period; the mean initial concentration was 81 mcg/100 mL. The daily intake of copper was 10 mg.


**Paper 17: Reduced Radiation Damage from Ingestion of Cabbage Family Plants**

Presented by Mindy S. Kurzer, Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.

By 1950, it had been noted that experiments on the effects of radiation in guinea pigs showed great variability in the results. Mme. M. Lourau and O. Lartigue (Lourau and Lartigue 1950) observed that although each series of experiments was fairly homogeneous, when one compared experiments, the lethal radiation dose varied by a factor of two or more. The cause of this variability in response was unknown. They stated that it was necessary to make a systematic study of the factors responsible for these differences in radiosensitivity.

Lourau and Lartigue were the first to show that diet composition was one of these factors. In the experiment reported in 1950, they studied 114 male guinea pigs. Test diets consisted of oat and bran supplemented with either cabbage at 50 g/d or beets at 75 g/d. The animals received a single whole-body radiation dose. Table 1 summarizes the results. For a given radiation dose, mortality was far greater in the animals fed beets: the first death was at 100 roentgen (R) with 100% mortality at 200 R. In the animals fed cabbage, the first death was at 250 R, with 100% mortality at 500 R.

In addition to their finding that guinea pigs fed beets died at lower radiation doses than those fed cabbage, Lourau and Lartigue commented that only certain lesions differed between the two groups. Although bone marrow changes were identical...
The first experiment on the effect of diet on radiation mortality in guinea pigs

Table 1 presents the results of the first experiment. The data shows the mortality percentages at different radiation doses for guinea pigs fed cabbage and beets. The authors proposed two explanations for the dietary effect on radiosensitivity: 1) cabbage may contain substances that protect against radiation damage, such as vitamins P and C; or 2) beets may contain substances that become toxic after irradiation. They preferred the second explanation, that beets were toxic to irradiated guinea pigs. The control diet supplemented with beets, cabbage or broccoli significantly reduced mortality from irradiation. Thus, they proved that Lourau and Lartigue were incorrect in their conclusion that beets contained a toxic substance and that Duplan was correct as to the protective effects of cabbage.

A few years later, M. Jean-Francois Duplan showed that in fact, Lourau and Lartigue’s first explanation was correct, that cabbage did offer protection against radiation damage (Duplan 1953). Duplan studied 70 male guinea pigs fed oat and bran diets. The test diets were supplemented with either cabbage or carrots in this study, and the animals received a single radiation dose.

The results of Duplan are shown in Table 2. For a given radiation dose, the animals consuming cabbage had much lower mortality than those consuming carrots. He saw no differences in lesions, but he did note that the animals consuming carrots lost much more weight than those consuming cabbage.

Duplan’s results suggested that Lourau and Lartigue may have been incorrect in concluding that beets contained a toxic substance. Rather than concluding that carrots contained a toxic substance, Duplan concluded that cabbage lowered the radiosensitivity of guinea pigs. He speculated that the radioprotective substances may have been antioxidant goitrogens present in the cabbage.

Spector and Calloway (1959) continued this investigation of the dietary factors that protect against radiation damage. They very importantly noted that Lourau and Lartigue did not have a control group in their experiment. The addition of a control group would have allowed them to distinguish between their two explanations (toxic substances in beets vs. protective substances in cabbage).

Spector and Calloway used an oat and bran control diet and the control diet supplemented with beets, cabbage or broccoli. They irradiated their four groups of guinea pigs with a radiation dose of 400 R. Their results are shown in Table 3. Although beet consumption did not affect mortality, consumption of either cabbage or broccoli significantly reduced mortality from irradiation. Thus, they proved that Lourau and Lartigue were incorrect in their conclusion that beets contained a toxic substance and that Duplan was correct as to the protective effects of cabbage.

Calloway and colleagues went on to investigate the substance conferring the protective effects (Calloway et al. 1963). Because the basal diet was devoid of vitamin A, and animals consuming this diet were known to become vitamin A deficient, they suggested that sources of vitamin A might be able to decrease the radiosensitivity of guinea pigs. Their results are shown in Table 3. In this experiment, they confirmed previous findings that both cabbage and broccoli lowered mortality in irradiated animals. They found that a number of other β-carotene-containing foods also exerted some beneficial effects. Mortality after 20 d was significantly lowered by consumption of the β-carotene-containing vegetables that they tested. Beets, apples and white potatoes had no effect. Supplementation with all essential vitamins reduced mortality somewhat, but not to the same degree as supplementation with...
vegetables. Supplementation with pure vitamin A or β-carotene alone had little effect on mortality.

Calloway and co-workers found that when an adequate purified diet was fed, the animals were somewhat resistant to radiation damage, suggesting that part of the beneficial effects of broccoli may have been due to improved nutritional status. On the other hand, the beneficial effects of broccoli could not be duplicated by feeding a mixture of 48 chemically pure ingredients patterned on the known composition of broccoli.

The experiments of the three groups considered together suggested that a nonnutrient substance present in cabbage and broccoli protected the guinea pigs from radiation damage. Experiments performed by Calloway to isolate the substance from alfalfa found it to be present in the water-soluble fraction, although the protective substance itself was not isolated.

These early experiments laid the foundation for subsequent work showing the beneficial effects of fruit and vegetable consumption in humans. Plants are now known to contain, in addition to vitamins and minerals, many nonnutrient compounds that have important biological effects such as antioxidant, anticarcinogenic, antimicrobial, anti-inflammatory, antiviral and immunomodulatory effects. These nonnutrient compounds include lignans, indoles, coumarins and flavonoids, which in 1949 were reported to protect mice from the effects of radiation (Shimoi et al. 1994). Perhaps these were the elusive substances responsible for the radioprotective effects first observed over 45 years ago.

**Literature Cited**


**Paper 18: Toxicity of Sucrose and Fructose for Neonatal Pigs (Becker et al. 1954)**

Presented by David H. Baker, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 96, April 16, 1996, in Washington, DC.

McRoberts and Hogan (1944) fed a liquid synthetic milk diet (30 g sucrose and 30 g casein per 100 g dry solids) to newborn pigs and reported poor performance and diarrhea, despite addition of “unrecognized factors” (vitamins) to the diet in the form of brewer’s yeast or beef liver extract. Subsequently, Bustad et al. (1948) tried a similar synthetic milk formulation for newborn pigs, and even when lactose was substituted for sucrose, the pigs had severe diarrhea and failed to survive longer than 22 d. In 1949, S. R. Johnson presented an abstract at the FASEB meeting in Detroit wherein it was reported that 2-d-old pigs would thrive on a synthetic milk diet containing lactose or glucose but would experience severe diarrhea and death when the diet contained sucrose as the carbohydrate source (Johnson 1949). Unfortunately, this abstract contained few details, and the work in question was apparently never published as a full-length paper.

Physiologists interested in reproduction had demonstrated in the late 1940s that fetal blood of sheep contained a high concentration of fructose (Bacon and Bell 1949, Barklay et al. 1949, Hitchcock 1949), and Goodwin (1957) later showed that fetal blood of pigs was also rich in fructose, although the level in the blood of pigs 24 h after birth was very low. Alexander et al. (1955) identified glucose as the precursor of fetal fructose, with the site of conversion being the placenta. Newton and Sampson (1951), though not mentioning it in their paper, must have assumed that fructose was an important energy source for fetal pigs, and perhaps newborn pigs as well. They obtained pigs at birth and deprived them of food for periods of 24 to 48 h, or until blood glucose had fallen to 20 mg/100 mL or less. These hypoglycemic comatose pigs were then given intravenous injections of various sugar solutions. Glucose injection produced a dramatic resuscitative response within a few minutes, and galactose gave a positive response also, although less immediate and less dramatic. Fructose injection was without benefit, and none of the six pigs given fructose were resuscitated. Taken together, the work of Johnson (1949) and Newton and Sampson (1951) suggested that baby pigs could not effectively utilize either sucrose or fructose.

**The Key Feeding Experiments**

In the early 1950s there was little interest in early weaning of pigs, and it was common practice to wean pigs at 8 wk of age. Also, this period was marked by keen interest in the newly discovered role of antibiotics and vitamin B-12 in pig nutrition. Today, there is great interest in artificial rearing of newborn pigs, so that effective carbohydrate sources in synthetic milk diets are very important.

D. E. Becker at the University of Illinois published three papers in 1954 that involved extensive testing of carbohydrate sources for pigs ranging in age from 1 d to 16 wk (Becker and Terrill 1954, Becker et al. 1954a and 1954b). With pigs during the period 1 to 10 d of age, liquid diets containing casein and various sugars were fed with ad libitum access, with the sugar component representing 56.6 g per 100 g of dry ingredients (Becker et al. 1954b). Of the seven pigs fed each diet, six fed the sucrose diet died, five fed the fructose diet died, and only one fed the dextrose diet died. Among surviving pigs, those fed dextrose gained weight, whereas those fed sucrose or fructose lost weight. Pigs fed either sucrose or fructose also exhibited severe diarrhea.

With pigs fed various carbohydrate sources (56.6 g/100 g dry solids) during the period 7 to 35 d of age (Becker et al. 1954a), three of the eight pigs fed sucrose died, whereas mortality was minimal in pigs fed lactose, dextrose, dextrin or cornstarch. Although the surviving pigs fed sucrose gained body weight as effectively as those fed the other carbohydrate sources, the three pigs that died had severe diarrhea. This experiment suggested that at least some pigs by 7 d of age can effectively hydrolyze sucrose in the gut and can also utilize both glucose and fructose for energy. Subsequently, Becker and Terrill (1954) demonstrated that 12-wk-old pigs could effectively utilize sucrose (50% of dry diet), but depressed growth and moderate diarrhea resulted when the semipurified soybean meal diet contained 50% lactose.

**Enzymatic Development in Pigs**

The work of Bailey et al. (1956) and Walker (1959) with small intestinal and pancreatic extracts from pigs at various
ages showed that intestinal sucrase activity was extremely low in newborn pigs and increased 10-fold at 1 wk, 60-fold at 2 wk and 200-fold at 5 wk of age. Intestinal lactase activity, on the other hand, was high at birth but declined thereafter.

Aherne et al. (1969a and 1969b) repeated some of Becker's earlier studies and found that 2- and 4-d-old pigs could not utilize either sucrose or fructose, whereas 7-d-old pigs survived when fed these sugars, although weight gains were somewhat lower in pigs fed sucrose or fructose than in those fed glucose or lactose. An intestinal loop in situ procedure together with assessment of blood sugar concentration established that fructose was absorbed from the gut intact, with little, if any, conversion to glucose in the gut mucosa. This result was confirmed by stomach tubing experiments wherein 3-, 6- and 9-d-old pigs showed marked elevations in blood fructose but not glucose when fructose was intubated. Urinary fructose excretion accounted for a sizable portion of the fructose administered, particularly in 3- and 6-d-old pigs. Intestinal fructokinase activity was very low in pigs of all ages, and hepatic fructokinase activity was also low in 3-d-old pigs but tended to increase with age or fructose feeding or both.

It seems clear that the toxicity of sucrose for neonatal pigs is caused by a low activity of intestinal sucrase. Also, pigs appear to absorb fructose intact, and during the first week of life they are poorly equipped to phosphorylate fructose in the liver (Cori et al. 1951) to facilitate its metabolism to triose phosphate. Biochem. Biophys. Acta 7: 304–317.


Black 1924), the isolation and identification of the structures of vitamins D (Askew et al. 1931) and D (Windaus et al. 1936) and a general understanding of the physiologic function of vitamin D in calcium absorption in the small intestine and the mineralization of the skeleton (Nicolaysen and Eeg-Larsen 1953). Kodicek and his group were pioneers in vitamin D metabolism research, using first bioassay and then very weakly labeled vitamin D (1956). After a decade of investigation, this group concluded that vitamin D functions directly without

Paper 19: Discovery of the Vitamin D Endocrine System

Presented by Hector F. DeLuca, Department of Biochemistry, University of Wisconsin-Madison as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 95, April 11, 1995, in Atlanta, GA.

In the early part of this century, during the discovery of the vitamins by McCollum and coworkers (McCollum and Davis 1913, McCollum et al. 1916) and by Osborne and Mendel (1917), came the idea that rickets (a disease rampant in northern Europe and northern United States) might be a dietary deficiency. The truth of this idea was readily demonstrated by Sir Edward Mellanby (1919), who raised the question whether the antirachitic activity might be due to the fat-soluble vitamin A, discovered by McCollum et al. (1916). However, McCollum and coworkers clearly demonstrated in 1922 that a separate fat-soluble substance was responsible for healing rickets (McCollum et al. 1922).

In the subsequent two decades came the discovery of the irradiation process for producing vitamin D (Steenbock and Black 1924), the isolation and identification of the structures of vitamins D (Askey et al. 1931) and D (Winders et al. 1936) and a general understanding of the physiologic function of vitamin D in calcium absorption in the small intestine and the mineralization of the skeleton (Nicolaysen and Eeg-Larsen 1953). Kodicek and his group were pioneers in vitamin D metabolism research, using first bioassay and then very weakly labeled vitamin D (1956). After a decade of investigation, this group concluded that vitamin D functions directly without

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further metabolism (Kodicek 1956). As illustrated by a paper stating that vitamin D itself is responsible for its biological activity in intestine (Haussler and Norman 1967), this idea persisted throughout most of the 1960s.

The underlying key to success in this area must be attributed to the chemical synthesis of radiolabeled vitamin D of high enough specific radioactivity to permit experiments with truly physiologic amounts of vitamin D (Neville and DeLuca 1966). Biologically active polar metabolites of vitamin D were discovered (Lund and DeLuca 1966, Norman et al. 1964), and further research demonstrated that the more polar metabolite not only had higher biological activity but also acted more rapidly (Morei et al. 1967). This provided the impetus for the isolation and chemical identification of 25-hydroxyvitamin D$_3$ (25-OH-D$_3$) in 1968 (Blunt et al. 1968) and its proof of structure by a long procedure demonstrating that the configuration of the hydroxyl in the 1-position and was thus called a tritium-deficient metabolite. This loss of tritium was not confirmed elsewhere, but the existence of the intestinal polar metabolite called “4B” was also reported (Haussler et al. 1968).

Continuing isolation and the identification of metabolites in the DeLuca group resulted in the finding of at least three polar metabolites, one of which proved to be 25,26-dihydroxyvitamin D$_3$ (Suda et al. 1970b). Another was believed to be 21,25-dihydroxyvitamin D$_3$ (Suda et al. 1970a), and a third was present in such small amounts in blood that its identification was not possible. It soon became clear that the functional metabolite in a target tissue could only be isolated with certainty from that target tissue. Therefore, from the intestines of 1600 vitamin D--deficient chickens given H-labeled vitamin D$_3$ came the isolation of what was termed “peak 5” by the DeLuca group. After several chromatographic steps, 8 $\mu$g of what still seemed to be slightly impure metabolite was obtained (Holick et al. 1971). From the fact that this metabolite was known to have a tertiary hydroxyl came the idea that this metabolite could be specifically modified and rechromatographed. Thus, the trimethylsilyl (TMS) derivative of the metabolite was made and treated so as to remove the silyl groups from the secondary alcohol functions but not from the tertiary alcohol on the 25-position (Holick et al. 1971). This was followed by chromatographic purification, providing 2 $\mu$g of pure 25 TMS metabolite. Mass spectrometry and specific chemical reactions were used to identify the structure as 1,25-dihydroxyvitamin D$_3$ [1,25-(OH)$_2$D$_3$] (Holick et al. 1971).

At the same time, Fraser and Kodicek (1970) made the important discovery that their “peak P” metabolite (“peak 5” metabolite from the DeLuca laboratory and “peak 4B” from the Norman group) is produced exclusively in the kidney. This important discovery was readily confirmed (Gray et al. 1971). The Kodicek group generated large amounts of the metabolite in vitro, but it was of insufficient purity to allow identification. Proof of the 1,25-(OH)$_2$D$_3$ structure came finally through direct chemical synthesis by a long procedure demonstrating that the configuration of the hydroxyl on the 1-position is indeed 1α (Semmler et al. 1972). Thus, the active metabolite of vitamin D proved to be 1α,25-(OH)$_2$D$_3$. Furthermore, this metabolite proved to be equally active in nephrectomized or sham-operated rats (Boyle et al. 1971, Holick et al. 1972), whereas its precursor (25-OH-D$_3$) had little biological activity in the nephrectomized rat. This provided clear evidence that the two-step process is required for vitamin D activation for function. Another important proof resulted when Fraser et al. (1973) demonstrated that vitamin D dependency rickets type I (an autosomal recessive genetic disorder causing severe rickets despite normal intakes of vitamin D) could be healed with the provision of physiologic amounts of synthetic 1,25-(OH)$_2$D$_3$, leaving no doubt of the two-step activation process in humans.

While the identification of peak 5 proceeded, Boyle et al. (1971) found that the production of peak 5 metabolite in vivo is strongly dependent upon the dietary calcium levels. Low calcium diets resulted in massive conversion to 1,25-(OH)$_2$D$_3$, whereas high calcium diets and vitamin D adequacy resulted in the production of another metabolite that proved to be 24,25-(OH)$_2$D$_3$. Pursuit of this led to the demonstration that the parathyroid gland mediated this regulation (Garabedian et al. 1972). Thus, in response to low blood calcium, parathyroid hormone is secreted that in turn stimulates 1α-hydroxylase in the kidney to produce the vitamin D hormone. The vitamin D hormone together with parathyroid hormone then provides for the mobilization of calcium from bone and renal reabsorption of calcium, and 1,25-(OH)$_2$D$_3$ by itself provides for the absorption of calcium and phosphorus (DeLuca 1974). Thus, the basic tenets of the vitamin D endocrine system were discovered from 1968 to 1973, as summarized in the Federation Proceedings lecture delivered in 1974 (DeLuca 1974).

**Literature Cited**

Paper 20: Glutathione Peroxidase: A Role for Selenium (Rotruck 1972)

Presented by Richard A. Ahrens, Department of Nutrition and Food Science, College of Agriculture and Natural Resources, University of Maryland, College Park, MD 20742 as part of the minisymposium “Experiments That Changed Nutritional Thinking” given at Experimental Biology 95, April 11, 1995, in Atlanta, GA.

The first practical biological interest in selenium occurred in the 1930s, when selenium was associated with alkali disease. This disease was called more graphically the “blind staggerers” in certain northern range areas and resulted from selenium poisoning from forage grown on those shale soils. The standard treatment for such poisoning was to provide very small amounts of certain arsenic compounds in the feed or water of livestock.

However, K. Schwarz and C. M. Foltz (1957) reported that traces of selenium prevented liver necrosis in certain vitamin E–deficient rats. Because vitamin E was known to be an antioxidant nutrient, a role for selenium in repairing or preventing oxidative damage was soon being sought. The first paper describing the enzyme glutathione peroxidase was also published in the same year by G. C. Mills (1957). Glutathione peroxidase was reported to be an erythrocyte enzyme that protects hemoglobin from oxidative breakdown. Nobody in 1957 linked these two discoveries!

In 1968 John T. Rotruck was assigned to the research laboratory of Professor W. G. Hoekstra to complete his Ph.D. studies at the University of Wisconsin. As these things often happen, John Rotruck was initially assigned to work with another faculty member who promptly went off on a sabbatical leave. Hoekstra worked largely with the metabolic effects of zinc. He was no particular expert in selenium or glutathione peroxidase, but he was familiar with the whole field of trace mineral research. Although attempts to understand selenium’s mode of action had focused on its potential role as an antioxidant, these theories had not been uniformly accepted. Indeed, John Rotruck recalls that some faculty members at the University of Wisconsin did not believe that selenium could be part of an enzyme. In 1968 and 1969, he was engaged largely in taking classes prior to the heavy research engagement that was to characterize his last two years in the Ph.D. program in biochemistry.

Being an enthusiastic graduate student, John Rotruck read the literature on glutathione biochemistry because of the similarities between selenium and sulfur chemistry and biochemistry. Glutathione is the common name of γ-glutamylcysteinylglycine. It is therefore a tripeptide composed of glutamic acid, cysteine and glycine. The sulfhydryl group of cysteine is reactive and tends to form disulfide bonds with other glutathione molecules when oxidized. Glutathione functions as a reducing agent to maintain other molecules in the reduced form and can convert hydrogen peroxide in the body to water. When it is oxidized to its double form linked by disulfide bonds, it can be reduced to the sulfhydryl form. The enzyme that does that is called glutathione reductase, and it uses NADPH as a source of hydrogen to achieve this reduction.

In August 1969, John Rotruck, perusing the Biochemistry Journal, came across a paper by R. E. Pinto and W. Bartley (1969). The paper was on the effect of age and sex on glutathione peroxidase and glutathione reductase activities in rat liver homogenates. This paper presented a pathway of glutathione metabolism that connected glutathione with the enzyme glutathione peroxidase. The reduced form of glutathione converts hydrogen peroxide to water most efficiently when it is catalyzed by the enzyme glutathione peroxidase. A figure in this paper illustrated the proposed relationship of glutathione, glutathione reductase, glutathione peroxidase and NADPH. It was also noted that glucose metabolism is the main source of NADPH. With this figure in front of him, John began to develop that same afternoon a hypothesis that selenium played a role in glutathione peroxidase. He generated a one-page research proposal that was presented to Hoekstra, complete with misspelled words and typographical errors, that same afternoon. Hoekstra said that, as far as he knew, this was the first research proposal of an involvement of selenium with glutathione peroxidase, and he reacted with enthusiasm. John, however, had another year of heavy course work ahead of him before he began to fully implement his proposal in the summer of 1970. Over the next several months the actual experimentation progressed rapidly, culminating in studies demonstrating incorporation of $^{75}$Se into glutathione peroxidase. This portion of John’s Ph.D. dissertation took about 12 months to complete.

The first publication (Rotruck et al. 1971) to come from this research reported on a glucose-dependent protection by dietary selenium against hemolysis of rat erythrocytes in vitro. That was also the year that John Rotruck left the University of Wisconsin and joined Procter and Gamble, where he had a long career from which he retired in 1995. An abstract from that research was first reported at the 1972 FASEB meetings (Rotruck et al. 1972a), and a subsequent publication on the prevention of oxidative damage to rat erythrocytes by dietary selenium appeared later that year in the Journal of Nutrition (Rotruck et al. 1972b). For this portion of the research, John actually used the methodology from the original paper by Mills (1957) on glutathione peroxidase to establish that glutathione peroxidase was virtually nonexistent in selenium-deficient rats.

These papers from Madison did generate some controversy, because L. Flohe’ (1971), working in Germany, used a spectrophotometric method to study bovine erythrocyte glutathione peroxidase and reported that it contained “no non-protein prosthetic group.” He responded negatively to the 1972 reports by Rotruck, but he did decide to recheck his earlier conclusions. In the next year, the last part of John Rotruck’s dissertation research was published in Science and demonstrated the uptake of $^{75}$Se by glutathione peroxidase and the fact that selenium was tightly bound to the enzyme (Rotruck et al. 1973). The controversy ended when Flohe’ et al. (1973) published a letter reporting that glutathione peroxidase was, indeed, a selenoenzyme. The 1973 paper by Rotruck and co-workers was identified as a Nutrition Classic in Nutrition Reviews in 1980 and as a “Citation Classic” in Current Contents in 1988. A RDA for selenium was established in 1989, and in 1992 John Rotruck and Bill Hoekstra received the Klaus Schwarz Commemorative Medal for this work.

John Rotruck recalls that it was the research environment at his time in the Biochemistry Department at the University of Wisconsin that made such discoveries possible. Just across the hall from him, Hector DeLuca’s students were explaining how vitamin D worked. Down the hall from them was John Suttie’s laboratory where the role of vitamin K was being explained. Nobel Prize winners came to speak on their research...
on a frequent basis. It just felt normal to him that a graduate student should make important discoveries. If you didn’t do it, you felt almost inferior. At the time he didn’t realize that he had made a “once in a lifetime” discovery. Now he does!

**Literature Cited**


