THE JOURNAL OF NUTRITION®

OFFICIAL ORGAN OF THE
AMERICAN INSTITUTE OF NUTRITION

RICHARD H. BARNES, *Editor*
Graduate School of Nutrition
Cornell University, Savage Hall
Ithaca, New York

HAROLD H. WILLIAMS
Associate Editor

E. NEIGE TODHUNTER
Biographical Editor

VOLUME 90

SEPTEMBER – DECEMBER 1966

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PENNSYLVANIA

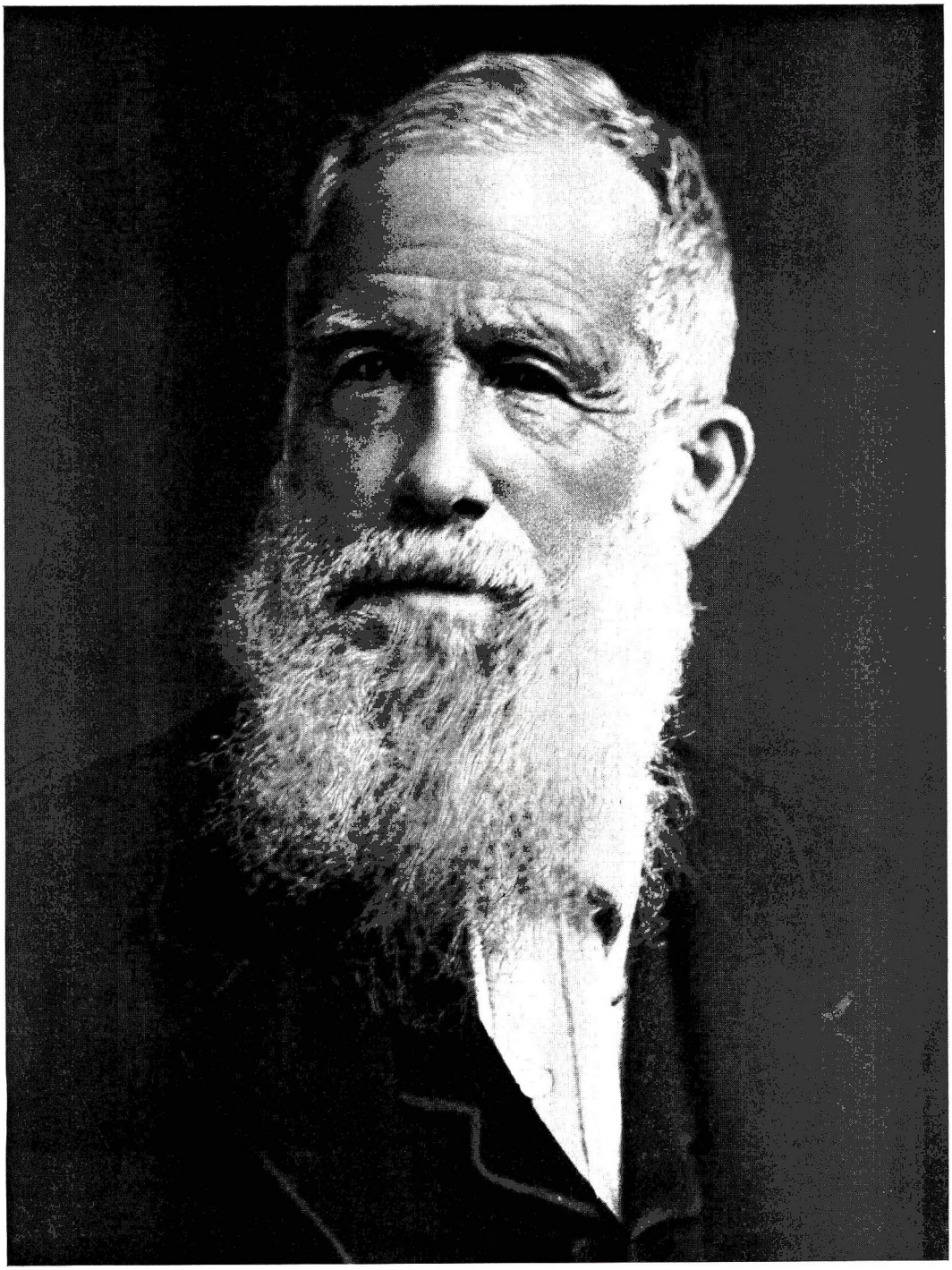
Contents

No. 1 SEPTEMBER 1966

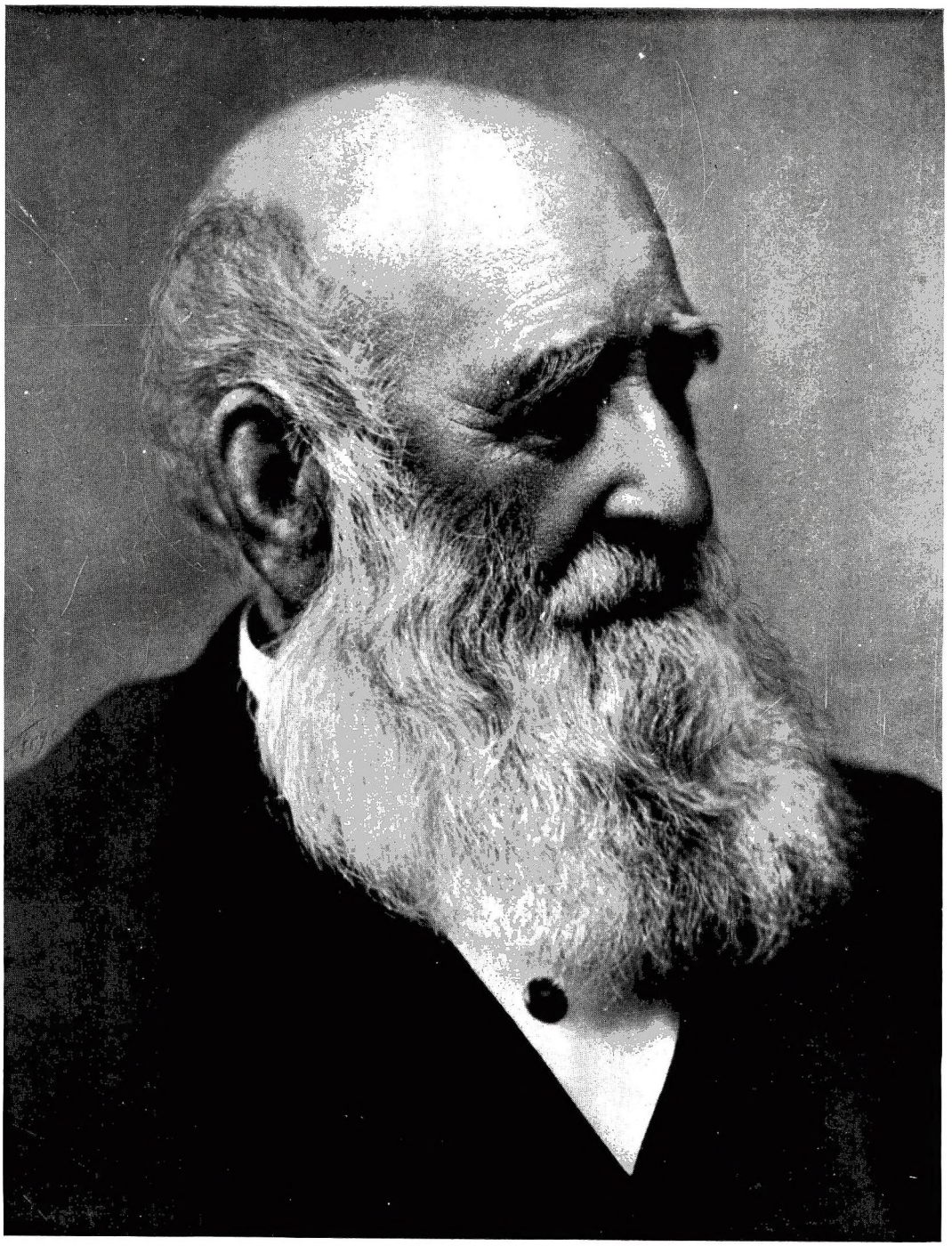
No. 2 OCTOBER 1966

No. 3 NOVEMBER 1966

No. 4 DECEMBER 1966



John Bennet Lawes



JOSEPH HENRY GILBERT

John Bennet Lawes

(December 28, 1814 — August 31, 1900)

Joseph Henry Gilbert

(August 1, 1817 — December 23, 1901)

— Biographical Sketches

In recalling for the *Journal of Nutrition* something of the lives and achievements of Lawes and Gilbert, it is wholly fitting to do so in a single biography for although they were very dissimilar, unalike in upbringing, temperament and outlook, they were united by their devotion to scientific enquiry, and their contribution to knowledge of the nutrition of plants and animals was truly a joint enterprise. Factually one was the employer of the other, but this is not the relationship evident from their many publications, which show them as scientific colleagues collaborating as equals, which they did productively and harmoniously for the remarkable period of more than half a century.

The interests and abilities of the one perfectly complemented those of the other. Lawes was essentially a practical man, but a very unusual one in being a nonconformist with an enquiring mind and great insight, who was imbued with the desire that agricultural practices should be based on established principles instead of untested traditions. Gilbert was more the academic chemist, meticulous in all he did, cautious and painstaking, methodically analyzing and measuring everything to produce the mass of facts and figures he needed before he would commit himself to any conclusion. The broad concept of the field experiments that first made Rothamsted famous was Lawes', but their long continuance and the detailed information they provided over the years reflects the influence of Gilbert.

Lawes had already revealed his unexpected talents and interests before 1843, when Gilbert joined him, but how far these talents would have taken him in his scientific enquiries without Gilbert, it is impossible to guess. Gilbert's career is the more easily understood. A studious child, he went to the university to study chemistry and having done so, when Lawes needed a chemist, it was reasonable for him to take the post. What remains inexplicable is why Lawes with his upbringing should have developed an interest in research and wanted to employ a chemist. It is idle to speculate on the reasons because he also was at a loss himself to explain his interests, as this revealing quotation shows: "As I had no male relations, my mother was the only person to influence me in my pursuits, and she was violently opposed both to science or business, although at the same time devoted to me. Home influence and education all tended to make me in pursuits an ordinary country gentleman, in politics a conservative, in religion an ordinary member of the Church of England accepting as truth all that they teach. Whereas for some causes to be enquired into and explained, I have been largely engaged in manufacturing pursuits, devoted to scientific investigation, very liberal in politics, and in religion although firmly and thoroughly believing in the truth of the Christian religion and ready to accept it as the guide of my life as far as I can understand it and being at the same time

a regular attendant at the service of the Church of England, still I cannot admit the right of that church or of any other church to teach dogmatically what truths are necessary for my salvation." From this, let us look briefly at his early life, knowing that we shall find little in it to account for his scientific curiosity, radical ideas or business acumen.

BEFORE 1843

John Bennet Lawes was born on 28 December 1814 in the Rothamsted Manor House, Harpenden, which his ancestors had occupied for many generations. His father, whose friendship with the Prince Regent had proved very costly, died when he was only eight, and his family then moved into Rothamsted Lodge, which was their home for several years. After attending two preparatory schools where, he says, he learned very little and was always in mischief and disgrace, he went to Eton, where "I learnt just enough to escape punishment but no more" and "most of my pursuits were more or less mischievous, such as digging mice out of the fields and putting them into my tutor's house." He left without regret and went to Oxford University, where he stayed for two years "learning little and following no particular pursuits. I did not go up for my degree." Although at that time Oxford had little to offer a man whose interests were scientific rather than classical, Lawes did attend some lectures by the Professor of Chemistry and he may have learnt of the plans for experiments in the Botanic Gardens to compare crops grown continuously with the same ones grown in rotation. While a school boy he showed some interest in chemistry, for during one of his holidays in Paris he not only helped to build the barricades during the revolution of 1830, which he thought "great fun," but also bought various chemicals, which "caused such destruction to my clothes and the furniture that my mother got rid of them as quickly as possible."

When Lawes left Oxford at the age of 20, the tenant of Rothamsted was insolvent, so he and his mother again lived there. He says, "My education was therefore supposed to have finished and I was to set up as a country gentleman. I had no idea or wishes about farming, but the home farm was vacant and therefore I took it. Up to this period, I had formed no opinions of my own on any subject." This statement we must accept and there is certainly no evidence that he was then attracted by agriculture; that he had decided to dabble in chemistry, however, is obvious, for as soon as he arrived at Rothamsted he had one of the best bedrooms fitted out as a laboratory, where he started experimenting, and he spent much time reading books on chemistry. The writings of A. T. Thomson, the first professor of Materia Medica and Therapeutics at University College, London, impressed Lawes, who sowed many kinds of drug plants on the farm and extracted from them opium and other active principles. He also worked for a time in Thomson's laboratory and while there showed his first interest in commercial enterprises; after persuading Thomson to patent a process for making calomel and corrosive sublimate, he turned an old barn on his farm into a factory where he made many tons of these substances. This venture was a commercial failure, but gave Lawes experience that proved useful to him later.

Lawes seems first to have seen a connection between chemistry and agriculture in 1837 from a chance remark to him by Lord Dacre that bones were useful as a manure for turnips on some fields but useless on others. This particularly roused his interest because he had spent a good deal of money on bones without seeing any reward from applying them to his fields. At about this time also he was offered free a lot of spent animal charcoal, which he treated with the sulphuric acid he had for making chlorine and found that the product was an effective manure.

During the next few years he did many experiments with plants in pots and in his fields, testing the effects of bones, burnt bones and mineral phosphates decomposed by various acids. This was the birth of superphosphate, and by 1842 Lawes was well enough convinced of its value to take out a patent for it. In 1842 he also married Caroline Fountaine, an amateur artist of considerable ability. Their son

Charles, who became well known as an athlete and sculptor, was born in 1843 and their daughter Caroline in 1844. Much against the wishes of his own family and his wife, who did not approve of him entering any trade, but least of all the manure trade, in 1843 he opened a factory to make superphosphate, and so was born the fertilizer industry. His family's fears that the venture would ruin him proved unfounded, for the business prospered and despite costly lawsuits with other manufacturers who infringed his patents, he was able to sell it in 1882 to the Lawes Chemical Manure Company for £300,000. However, at first he had to practice strict economy and for four years let Rothamsted Manor; it says much for his enthusiasm for research that, with his factory making such demands on his capital, he should nevertheless have paid Gilbert to conduct the experiments at Rothamsted.

From 1843 Lawes in effect led two lives. During part of the week he was a manufacturer, running not only an expanding fertilizer business, but also factories to produce citric and tartaric acids. A later venture that did not prosper was the growing of sugar cane, and sugar processing in Australia. During the rest of the week he collaborated with Gilbert in agricultural research, the activity in which he found the greater satisfaction and to which he gave increasingly more of his time as the years went by.

Joseph Henry Gilbert was born at Hull, Yorkshire, on 1 August 1817. His father was an eminent Congregational minister and his mother was widely known as a writer of hymns and songs for children; of their four sons and three daughters, he was the only one to become a scientist. His schooling at Nottingham and Mansfield was interrupted by a shooting accident, which destroyed the sight of one eye, damaged the other and impaired his general health for some years. He later triumphed over these disabilities, but they delayed his education and he was 24 years old before he went to the university, first for a year at Glasgow and then to University College, London, where in the laboratory of A. T. Thomson he first met Lawes. At both places he mainly studied analytical chemistry, but also *materia medica* and botany. He learned German as a preliminary to going in 1840 to Giessen, the then Mecca of chemistry, to work under Liebig, with whom he was later to disagree so vehemently over the nitrogen nutrition of plants. After taking his Ph.D. degree at Giessen, he returned for a brief second spell at University College, London, before he went to Manchester to work on the dyeing and printing of calico. While there he was recommended by Thomson to Lawes and in June 1843 moved to Rothamsted, where he worked until he died in 1901. In 1850, he married Eliza Forbes Laurie, but she died two years later, and in 1855 he married Maria Smith, who survived him. He had no children by either marriage.

Unlike Lawes, who had many other activities than agricultural research, Gilbert devoted his whole life and energies to this. He not only supervised the conduct of the Rothamsted experiments and all the chemistry done in connection with them, but he also was their main exponent, both in the written and spoken word. Both men were equally willing to demonstrate the experiments to visitors but Lawes was less willing than Gilbert to address scientific meetings or attend social functions.

1843 ONWARDS: CROP NUTRITION

We have already seen that Lawes had started to experiment on crop nutrition before Gilbert joined him. Also, as the first of the large-scale field trials, with roots on Barnfield, was laid down in the spring of 1843, it is unlikely Gilbert helped in its design. However, with Gilbert's arrival, which is taken as the foundation date of Rothamsted Experimental Station, the scope of the work greatly increased and its type changed. Results were no longer simply observed and yields weighed, but everything was now carefully measured and analyzed. How this was done with such accuracy in the old barn that served as a laboratory until 1855 is difficult to imagine.

The autumn of 1843 saw the start of their most famous field experiment, with winter wheat on Broadbalk field, which still continues. This, although of different

design, was of the same general pattern as with the root crop on Barnfield and was later followed with other main agricultural crops. The same crop was grown year after year and usually each plot was given the same treatment every year. The main treatments were no manure; farmyard manure; nitrogen only; minerals only; minerals plus nitrogen. But there were additional treatments, testing different kinds and amounts of nitrogen, different times of applying it, and various combinations of inorganic materials. In addition to the plots given the same treatment annually, Broadbalk contained two plots in which the treatments alternated, one getting nitrogen only in the year when the other got minerals only, with the procedure reversed the next year. A unique feature of the Broadbalk experiment was the installation of drains, one to each plot, to allow the losses of nutrients by leaching into the subsoil to be measured.

The main question that interested Lawes and Gilbert was the relative importance for the growth of crops of nitrogenous manures and minerals, i.e., the constituents of the ash of crops, mostly compounds of phosphorus, potassium, sodium and magnesium. In seeking to answer this question, they were stimulated not only by its practical importance for farming, but by Liebig's assertion that crops could get all the nitrogen they needed from ammonia in the air and to yield, fully needed to be manured with minerals only. The Rothamsted experiments, particularly with cereals, soon showed the fallacy of Liebig's mineral theory, for yields were small without organic or inorganic nitrogenous manures and minerals produced appreciable effects only when nitrogen was also given. However, Liebig was unconvinced and his adverse criticisms of the Rothamsted experiments led to controversy, which became increasingly heated as he minimized the importance of nitrogen while Lawes and Gilbert produced more and more evidence of its paramount importance except with legumes. There is, indeed, a touch of irony in the fact that Rothamsted work began by demonstrating the value of superphosphate, from which Lawes mainly made his fortune, whereas later so much was done that established the greater benefits from nitrogenous fertilizers.

How strongly Lawes felt early on in the controversy will be shown by a quotation. This, too, serves well to indicate his philosophy and practical approach to research. "The theory advanced by Liebig, that 'the crops on a field diminish or increase in exact proportion to the diminution or increase of the mineral substances conveyed to it in manure,' is calculated so seriously to mislead the agriculturist that it is highly important that its fallacies should be generally known. The contempt which the practical farmer feels for the science of agricultural chemistry arises from the errors which have been committed by its professors. They have endeavored to account for, and sometimes to pronounce as erroneous, the knowledge which ages of experience have established; and they have attempted to generalize without the practical data necessary to accomplish their end with success. Agriculture will eventually derive the most important assistance from chemistry, but before it can propose any changes in the established routine of the farmer, it must, by a series of laborious and costly experiments, explain this routine in a satisfactory manner.

"Although the experimental results which have been detailed undoubtedly prove that to produce agricultural crops of corn, nitrogen must be supplied to the soil in some form or other, two important questions still remain unanswered, namely, first, what amount of ammonia will be required to produce a given amount of corn? or, in other words, what amount of nitrogen must the farmer accumulate in his soil to obtain each bushel of corn beyond the natural produce? Secondly, what are the most economical means at his disposal for securing the necessary supply? The solution of these questions is within the reach of careful experiment and calculation."

Although Liebig questioned the work at Rothamsted, farmers were rapidly impressed by its value and showed their appreciation by subscribing to a testimonial fund to Lawes which was used to build in 1855 the first Rothamsted laboratory. At its opening, Lawes paid public tribute to

Gilbert, saying: "To Dr. Gilbert I consider a debt of gratitude is due from myself and from every agriculturist in Great Britain. It is not every gentleman of his attainments who would subject himself to the caprice of an individual, or risk his reputation by following the pursuit of a science which has hardly a recognized existence. For twelve years our acquaintance has existed, and I hope twelve more years will find it continuing." It was on this occasion that Lawes first mentioned his intention to provide for the maintenance of the work after his death. He implemented this intention by setting aside from the sale of his manure business £100,000, which in 1889 he transferred together with the buildings to the Lawes Trustees. Under the Trust Deed a management committee was set up with members appointed by the Royal Society of London, the Royal Agricultural Society, the Linnean Society and the Chemical Society. This committee continues to be the governing body of Rothamsted Experimental Station.

Lawes' hope for the continuation of his association with Gilbert was more than fulfilled, for they worked together for another 45 years after 1855, steadily amassing valuable information about such various problems as the relative needs of different crops for different nutrients ("for the production of increased growth, *nitrogenous manures* had the *most characteristic* effect upon the cereals; *potass* on the *leguminous crops;* and *phosphates* on turnips"); the effects of different nutrients on yield and quality of crop; the interactions between different nutrients; the different amounts of nutrients taken from the land by different crops; the interaction between different crops grown in rotation; the effect of fallow and green-manure crops on yields of subsequent crops; the effects of different manurial regimens on the supply of nutrients in the soil.

From Broadbalk they were able to compile a balance sheet showing what happened to applied manures, how much came off in the wheat grain and straw, how much was retained in the soil and how much was lost in the drainage. Nitrogen was the main nutrient lost, and the plots given alternating dressings of nitrogen and minerals showed how transient was the effect of inorganic nitrogen, for although yields were large in the years when nitrogen was given, in the alternate years the plots yielded little more than those that never received nitrogen. A still more important feature of Broadbalk was the demonstration that yields could be as large with inorganic as with organic manures. It is difficult now to recapture the reactions at the time to being told that a few hundredweights of powder from a factory could produce the same results as many tons of farmyard manure, but there were the results for all to see and to convince the disbelievers. Although there are a few people who still attribute almost mystical value to organic manures, later work has done nothing except strengthen Lawes' and Gilbert's conclusions that the nutritive value of organic manures lies solely in their content of nitrogen and minerals, and that any other effects on crop growth are indirect, by changing soil structure or the water-holding capacity of the soil.

The ability of nitrogen to increase leaf growth was plainly evident without measurement, but Gilbert's detailed chemical analyses also provided much new information on the way it affected leaf constitution, not only increasing the content of nitrogenous substances, but also of other nutrients and of sugars and starch. They further showed that these large effects on the constitution of leaves was not reflected in the constitution of the grain, for although wheat yields were much increased by nitrogenous manure, the composition of grain on plots with and without nitrogen differed little. From the continuation of the experiments over many years, Lawes and Gilbert also noted that responses to the same manuring varied greatly, with the results depending much on the weather. In attributing all the effects of weather on crop growth directly to nutrition, they were in error, for many reflect effects on the incidence of pests and diseases, but they drew some shrewd conclusions. For example, they noted how nitrogen was leached from the soil during wet winters and the need to allow for this in manuring, something many farmers still fail to do.

Their experiment on old pasture, which like the one with wheat on Broadbalk still continues, was outstanding, not simply for showing how manuring affected the yield of hay, but much more so for its dramatic demonstration of how differential manuring changed the composition of the sward. Park Grass originally had a rich flora of grasses, legumes and weeds, as the unmanured plot still has. Fertilizers rapidly altered the proportions of these three components; nitrogen suppressed the legumes and weeds, whereas potash and phosphate without nitrogen increased the legumes. Ammonium sulphate soon produced a sward that was almost wholly grass, but the species depended on whether it was given alone or with phosphate and/or potash, and the extent to which it acidified the soil. For example, below pH 4.1, *Agrostis tenuis* dominated where only nitrogen was given, but *Holcus lanatus* where potash and phosphate were added; between pH 4 and 6, there were more species, with *Alopecurus pratensis* the most common given full manuring but supplanted by *Festuca rubra* on plots lacking phosphate and potash; above pH 6, the sward was still more mixed and no species dominated, but *A. pratensis* again was on plots given phosphate and potash.

In denying Liebig's "minerals only" theory and in their insistence on the paramount importance to crops of nitrogen manuring, Lawes and Gilbert were guided solely by the results of their experiments and observations. They well knew that the growing of legumes left a residue of nitrogen in the soil for succeeding crops, and they were not prejudiced against the idea that plants might assimilate nitrogen from the air. Indeed, when writing in 1847 about sources of ammonia, Lawes said "by cultivating turnips and the leguminous plants, a large amount of this substance is collected by them from the atmosphere."

The explanation of the extra nitrogen gained by leguminous plants eluded them, but they abandoned the idea of assimilation from the air because of the results of critical experiments done, in conjunction with a visiting American scientist, Evan Pugh, on plants in pots. The plants were grown in burnt soil and kept in an enclosed system to prevent any external forms of combined nitrogen reaching them. Everything was analysed in detail and the results showed clearly that neither legumes nor other plants gained nitrogen from the air. Indeed, in these circumstances legumes grew less well than other plants. The experiments were magnificently done but the precautions taken to make the results reliable chemically made them irrelevant to what happens in field soils; by burning the soil before use, they killed any *Rhizobium* sp. and so not only destroyed their chances of discovering the main natural source of combined nitrogen in the soil, but set themselves off on many fruitless searches for other explanations of the action of leguminous crops. Gilbert was present at the meeting in 1886 when Hellriegel and Willgarth reported the symbiotic association between *Rhizobium* and legumes that leads to nitrogen fixation in the root nodules. It says much for his receptive mind and energy that, although over 70, he immediately began a series of experiments that not only confirmed but extended the results of Hellriegel and Willgarth.

Although most of Lawes' and Gilbert's work was done at Rothamsted, they played a leading role in designing experiments at Woburn, where in 1876 the Royal Agricultural Society of England established a research station, which is now a part of Rothamsted. The central problem there was to measure the residual value of manures and of food fed to cattle, so that outgoing tenants could be properly compensated, but Lawes and Gilbert also took the opportunity of duplicating on the light land there many of the experiments done on the heavy land at Rothamsted. At the request of the Government they also undertook a major series of experiments in which they assessed the manurial value of sewage. Here it is possible only to indicate the scope of their work, and impossible to summarise the detailed results on crop nutrition they reported in more than 100 publications, mostly in the *Journal of the Royal Agricultural Society* and the *Philosophical Transactions of the Royal Society*.

ANIMAL NUTRITION

The work of Lawes and Gilbert on animal nutrition is almost as notable as their work with crops and fertilizers, and would have ensured them a place in the history of science had they done nothing else. There are several reasons why it is less widely known than their work on crop nutrition. First, although they studied animal nutrition during many years, the subject was a major activity only in the middle years of their collaboration and was not continued at Rothamsted after their deaths. Secondly, the results could be obtained only from their published papers, in records of weights and analytical measurements, not as in the fertilizer experiments where large effects were clearly demonstrated simply by looking at the growing crops. Thirdly, although their work produced much new information and, equally important, destroyed several myths, it did not have the immediate practical consequences of their work on crop nutrition.

Their work started in 1848 when they compared the fattening capacity of different breeds of sheep in normal farming conditions, by measuring the ratio of food eaten to live-weight increase, but it soon extended to more thorough work, not only with sheep, but also with pigs and oxen. At this time the relative importance and roles of different constituents of food for animals was unestablished and a subject of controversy. However, it was widely accepted that the most important factor was the amount of nitrogenous substances and that there was need to know only this to assess the relative value of different foods. Fat in the animal was assumed by most people to come from fat or nitrogenous substances in the food; in the controversy over this, Lawes and Gilbert found themselves on the side of Liebig, and their experiments on the fattening of pigs produced the evidence to support his assertion that fat in the animal body can be synthesized from carbohydrate in the food. However, in another controversial subject, the source of energy for muscular effort, they were again in conflict with Liebig, who maintained that it came from muscle substance, whereas they considered it came mainly from non-nitrogenous materials. They advanced various reasons in support of their ideas, but regarded as conclusive the results of their experiments in which pigs fed very different amounts of protein excreted nitrogen roughly in proportion to the amount they were fed and quite independently of their muscular activity.

Lawes and Gilbert were also the first to show that not all proteins were of equal nutritive value. This they did by feeding pigs on either lentil meal containing 4% protein or barley meal containing 2%. From the total food eaten, they calculated the nitrogen intake and, after the pigs had been on the different diets for a while, they measured the amount of nitrogen excreted. The pig given lentil meal excreted more than twice as much urea as the one given barley meal, showing that the proportions of the total nitrogen in the two foods retained and converted into pig meat differed greatly. The significance of their conclusion, however, for long went unappreciated by nutritionists, who continued to assume that all proteins were equal.

As with the wheat on Broadbalk field, so with the animals, Lawes and Gilbert attempted to compile a balance sheet, showing the fate of food eaten, whether it was excreted or retained in the body and when retained in what form and whether it was used to sustain the animal or to add to its weight. This work entailed enormous numbers of dry-matter measurements and determinations of the ash and nitrogen in the food, feces and urine. Probably their most laborious piece of work, however, was to determine the composition of whole bodies of animals of different ages and in different conditions of fatness. They separated and weighed the amounts of different organs or parts in 2 calves, 2 heifers, 14 bullocks, 1 lamb, 249 sheep and 59 pigs. In 1 calf, 2 bullocks, 1 lamb, 4 sheep and 2 pigs, they also analyzed each part and organ to find the proportions of water, minerals, fat and nitrogenous substances. This work not only provided the first factual information on the composition of farm animals and how their composition changes with age and with degree of fatness, but their results for

long remained the standard textbook figures.

Interested as they were in both crops and animals, their work extended beyond the effects of various animal foods on the growth and composition of the animals to their effects on the manurial value of the animal's excreta. They concluded that, for the fattening of cattle, provided the diet was not deficient in nitrogenous substances, richness in digestible carbohydrates was the most important, whereas for the manure to be valuable the diet needed to be rich in nitrogen. Their wide-ranging interests are shown in Bulletin no. 22 of the U.S. Department of Agriculture, published in 1895, where Gilbert summarized the main points of their work with animals under the following seven headings:

"(1) The amount of food and of its several constituents consumed in relation to a given live weight of animal within a given time.

(2) The amount of food and of its several constituents consumed to produce a given amount of increase in live weight.

(3) The proportion and relative development of the different organs or parts of different animals.

(4) The proximate and ultimate composition of the animals in different conditions as to age and fatness, and the probable composition of their increase in live weight during the fattening process.

(5) The composition of the solid and liquid excreta (the manure) in relation to that of the food consumed.

(6) The loss of expenditure of constituents by respiration and the cutaneous exhalations; that is, in the mere sustenance of the living meat-and-manure-making machine.

(7) The yield of milk in relation to the food consumed to produce it, and the influence of different descriptions of food on the quantity and on the composition of the milk."

Although impressive, this list of their interests is far from complete. It needs supplementing at least to the extent of saying they also compared the feeding value of hay and silage, and, at the request of the Government, they studied the effect of malting on the nutritive value of barley. Malt was generally believed to be the more nutritious, but they disproved this by measuring the loss of dry matter during malting and showing that, per unit of dry matter, malted and unmalted barley had the same food value. Their attention to detail and desire to put everything to the test is well evidenced by their experiments with condiments, showing these added nothing to the nutritive value of food.

THE JUBILEE

Neither Lawes nor Gilbert sought honours but severally and jointly they received them in quantity. Lawes was created a Baronet and Gilbert received a Knighthood. Each was given an honorary degree from several universities and was made an honorary member of many academies. Each was elected to Fellowship of the Royal Society and jointly they were awarded a Royal Medal. The Royal Society of Arts also jointly awarded them its greatest honor, the Albert Gold Medal.

A unique occasion was the Jubilee Celebration, held at Rothamsted on 29 July 1893, when a very distinguished company, headed by the President of the Board of Agriculture, gathered to do them honor. Unlike the occasion in 1855, when the Testimonial Laboratory was opened and it was left to Lawes to pay tribute to Gilbert, this one honored them jointly. Lawes was presented with his portrait, Gilbert with a silver salver, and congratulatory addresses were read from many learned societies. The main testimonial took the form of a granite monolith, inscribed "To commemorate the completion of fifty years of continuous experiments (the first of their kind) in agriculture conducted at Rothamsted by Sir John Bennet Lawes and Joseph Henry Gilbert."

From the address to Lawes, signed by the Prince of Wales on behalf of subscribers to the Jubilee Fund, who came from the world over, we may fittingly quote a paragraph: "The Memorial which is now erected, will, it is hoped, preserve your joint names in honored remembrance for centuries to come, while the portrait will hand down to future generations the like-

ness of one of the most disinterested as well as the most scientific of our public benefactors." And from his address to Gilbert: "If the institution of the various investigations and experiments carried out at Rothamsted has been due to Sir John Lawes, their ultimate success has been in a great measure secured by your scientific skill and unremitting industry.

"A collaboration such as yours with Sir John Lawes, already extending over a period of upwards of fifty years, is unexampled in the annals of science. I venture to hope for an extended prolongation of these joint labors, and trust that the names of Lawes and Gilbert, which for so many years have been almost inseparable, may survive in happy conjunction for centuries to come."

Lawes and Gilbert could not have had such full and active lives had they not been unusually hale. Writing of Lawes, his colleague R. Warington said: "When past 85 he still exhibited few of the infirmities of old age;" and of his interests and personality, Warington said: "He was a keen observer and knew the experimental fields better than any of the Rothamsted workers. Not the fields only, but the birds and every living thing on the estate. The large amount of business he was able to get through was in no small degree due to his calm and cheerful temperament, which no disaster seemed to disturb. This quiet, self-contained temperament sometimes appeared as reserve or even shyness, and led to a reluctance to accept public positions and to take part in public functions; but his work doubtless gained by his refusal to expend his energy on outside occupations. The reserve we have mentioned was, however, a mood rather than a character, and disappeared the instant he was appealed to by any scientific or benevolent question. To speak to him of agricultural science would at once open the storehouse of thought and lead to a discourse of ready eloquence, interspersed with shrewd observations and humorous remarks."

Gilbert, too, showed few infirmities, for soon after the Jubilee Celebrations he visited the U.S.A. to give lectures under the provision made by Lawes in his Trust Deed for the purpose. His six lectures were published in 1895 as Bulletin no. 22 of the United States Department of Agriculture. Major H. E. Alvord, then chairman of the executive committee of the Association of American Agricultural Colleges and Experiment Stations, wrote: "The lectures comprise the only condensed, carefully prepared, and authorized review of the famous investigations by Lawes and Gilbert for half a century at Rothamsted. They constitute an extremely valuable and truly unique contribution to the literature of experimental agriculture." Major Alvord's use of the word "condensed" was correct but could be misleading; the Bulletin runs to 316 pages of not very large print and has 85 tables; its preparation was no mean feat for a man nearing 78, and the manner of his writing shows no falling off in vigour or delight in controversy.

Both men remained active till the end of their lives. Lawes died on 31 August 1900, after a brief illness. The end of their long association was a great blow to Gilbert and although, with his characteristic perseverance, he kept the Rothamsted experiments going for another year, his health then failed and he died on 23 December 1901. Like Lawes, he was buried in the churchyard at Harpenden.

Their unique partnership did more than simply produce new information on the nutrition of crops and animals. It revolutionized the manuring of crops and it set a tradition for accuracy in agricultural research. They were modest about their achievements, realising not only the many problems that remained unsolved, but that other methods than theirs would be needed to solve them. Let Lawes have the last word; writing in 1888 about Liebig's book published in 1840, on Agricultural Chemistry, he said: "Nearly fifty years have passed since that book was written. It was a bold work; and for some years afterward everyone could give confident opinions upon all subjects relating to agriculture — but where are we now? Have we a foundation laid, and can we say that such a thing exists as a science of agriculture? Another half-century will doubtless show more rapid progress, as there are so many more brains at work on

the subject in various parts of the world; but when we consider that almost every other science contributes its share to form what we call the science of agriculture, those who follow the pursuit must expect plenty of hard work and be content with a moderate amount of success."

F. C. Bawden, M.A., F.R.S.,*Director*
Rothamsted Experimental Station
Harpenden, Herts, England

Effect of Age, Vitamin B_6 Deficiency, Isoniazid and Deoxypyridoxine on the Urinary Taurine of the Rat

NINA H. MERCER,[1] PHYLLIS P. BOWEN AND FRANCES A. JOHNSTON
Department of Food and Nutrition, New York State College of Home Economics, Cornell University, Ithaca, New York

ABSTRACT The object of the present study was to find the effects of isoniazid (INH) and deoxypyridoxine (DOP) as well as a vitamin B_6 deficiency on urinary taurine excretion. Groups of male rats 38 to 47 days of age were fed 5 diets: adequate in vitamin B_6, adequate in vitamin B_6 with INH added, deficient in vitamin B_6, and deficient in vitamin B_6 with INH or DOP added. The urinary taurine of male rats fed adequate vitamin B_6 remained at about 2 mg/100 g body weight/day up to 50 to 55 days of age, then increased rapidly to about 7 mg at 75 to 95 days of age. The urinary taurine of rats fed diets deficient in vitamin B_6 remained at 1 to 2 mg over a 7-week period. The urinary taurine of rats fed diets adequate in vitamin B_6 plus INH and of those fed diets inadequate in vitamin B_6 plus DOP was significantly higher than that of the controls; the taurine also tended to be higher when rats were fed vitamin B_6-deficient diets plus INH. The results are contrary to expectation in view of the known inhibitory effects of the 2 compounds on vitamin B_6.

The object of the present study was to learn whether urinary taurine can be used to follow the development of vitamin B_6 inhibition caused by isoniazid (INH) or deoxypyridoxine (DOP). Urinary taurine proved to be useless for this purpose. Observations of value to persons working on taurine metabolism, however, were made, namely: urinary taurine increased with age in male rats fed diets adequate in vitamin B_6, but, for deficient rats, urinary taurine did not increase with age and continued at the same low level for 7 weeks; and urinary taurine excretion increased with the administration of the vitamin B_6-inhibiting compounds, INH and DOP.

METHODS

Five experiments were carried out using 60, 24, 24, 24 and 16 male rats of the Holtzman strain, of ages 46, 42, 39, 38 and 47 days, respectively; the mean weights on the corresponding days were 144, 131, 123, 128 and 151 g. In each experiment the rats were paired by weight when they were divided into groups for different treatments.

The basal diet contained: (in per cent) "vitamin-free" casein,[2] 18; glycine, 1; methionine, 0.2; corn oil, 5; salt mix USP XIV,[3] 4; agar, 2; a vitamin preparation containing no pyridoxine,[4] 2.2; and enough sucrose to make 100; thus, the basal diet was adequate except for vitamin B_6. In experiment 1, 4 mg pyridoxine·HCl/kg of food were fed to the group which was to have adequate vitamin B_6 but in the other experiments when pyridoxine·HCl was added only 2 mg were incorporated to provide adequacy. In experiment 1 the group of rats given INH was fed 1.5 g of INH/kg of food but in the other experiments 1 g was used. In experiment 1 the group given DOP was fed 100 mg of DOP/kg of diet; in experiment 5, the amount was increased to 1 g.

In all 5 experiments the animals were pair-fed to the animal which ate the least. In experiments 1 and 2, in which the rats were arranged in groups of four with each

Received for publication December 15, 1965.

[1] Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

[2] The casein preparation contained 0.63 mμg of vitamin B_6/g. The basal diet therefore contained 0.113 mg vitamin B_6/kg.

[3] Salt mixture USP XIV, Nutritional Biochemicals Corporation, Cleveland.

[4] The vitamin mixture supplied in each 45.5 kg (100 pounds) of diet: (in grams) vitamin A (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75; riboflavin, 1.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35. (Vitamin Diet Fortification Mixture in Dextrose, with pyridoxine·HCl omitted, obtained from Nutritional Biochemicals Corporation.)

rat in the group fed a different diet, 3 animals were pair-fed to the fourth. The mean daily food consumptions per rat were 8.5, 9.8, 12.0, 9.2 and 9.8 g, respectively.

The rats were housed in metabolism cages. Urine was collected each day, filtered and combined into 3-day composites for each rat except in experiment 4 when the urine of 3 rats was composited each day. The composites were stored in a freezer. All values for taurine are expressed as a daily mean for one rat over a 3-day period except for experiment 4 where the values are a daily mean per rat for 3 rats. The procedure for the determination of taurine in the urine was an adaptation by Mercer [5] of the method of Sörbo (1) with a substitution of the ion-exchange column for one described by Garvin (2). By this procedure amino acids present in the urine because of food spillage were removed. The method involved the selected passage of the zwitterion of taurine through an anion and a cation exchange resin and quantitative spectrophometric estimation of taurine in the effluent utilizing the color reaction between taurine and ninhydrin.

Experiment 1. Sixty rats were distributed equally among 4 groups. One group was fed the basal vitamin B_6-deficient diet, the other three were given the basal diet plus pyridoxine·HCl, or INH, or DOP. They were arranged in 15 groups of four each for control of the food intake. The experiment was continued for 7 weeks. INH was discontinued after 28 days and DOP after 17 days. Two groups of four were killed each week for biological determinations not reported in this paper. Before the rats were killed, urine was collected for two 3-day periods from most of the groups.

Experiment 2. Twenty-four rats were divided into 4 groups of 6 each. For one week all groups received the basal diet plus pyridoxine·HCl. Thereafter 2 groups were fed the basal vitamin B_6-deficient diet with INH added to one of them, and 2 groups were fed diets made adequate in vitamin B_6 by the addition of pyridoxine·HCl with INH added to one of them. At the end of the fifth week INH administration was discontinued for the group of rats not receiving pyridoxine·HCl. The experiment was continued for 7 weeks following the preliminary week. Taurine was determined in the preliminary week and in weeks 2, 4 and 7.

Experiment 3. Twenty-four rats were fed the basal diet plus pyridoxine·HCl; 12 of them were also given INH. Urine was collected for two 3-day periods starting on the tenth and seventeenth days.

Experiment 4. Twenty-four rats were fed the basal diet; INH was added to the diets of half of the animals. Urine was collected on days 12, 13 and 14.

Experiment 5. Sixteen rats were fed the basal diet; DOP was added to the diets of half the animals. They were maintained with the diet for 3 weeks. Urine was collected for 3 days during each week.

RESULTS AND DISCUSSION

Effect of age on urinary taurine. When male rats 38 to 47 days of age were fed diets adequate in vitamin B_6 (exps. 1, 2 and 3) urinary taurine, in general, remained at about 2 mg/24 hours/100 g body weight until 50 to 55 days of age; the range was from 1.40 ± 0.36 mg [6] to 2.06 mg [7] (table 1). The amounts increased with age from 3.75 ± 0.30 mg to 7.08 ± 0.65 mg for rats 56 to 58 days and 75 to 95 days of age respectively (table 1).

An increase in urinary taurine at 50 to 55 days may possibly be controlled by hormones. That sex hormones influence taurine excretion is suggested by the work of Chatagner and Bergeret (3) who observed that the decarboxylation of cysteinesulfinic acid was 2 to 3 times faster in liver homogenates from male than from female rats. Sloane-Stanley (4) observed that the decarboxylation of cysteic acid was twice as rapid in male rats as in females. These decarboxylations are steps in the conversion of sulfur amino acids to taurine; thus, it might be assumed that taurine formation follows a similar pattern. Another possible explanation for an increase in taurine excretion at 50 to 55 days of age is a decrease in thionase

[5] Mercer, N. H. 1963 Application of a method for the determination of urinary taurine to the urine of rats fed vitamin B-deficient diets and diets containing B_6 antagonists. Master of Science Thesis, Cornell University, Ithaca, New York.

[6] SE of mean.

[7] Mean for only 2 animals.

TABLE 1

Effect of vitamin B_6 deficiency, isoniazid (INH) and deoxypyridoxine (DOP) on the excretion of taurine in the urine of rats [1]

Age	Time fed exp. diet	Taurine						
		Diet with vitamin B_6 [2]		Diet with no vitamin B_6				
		No INH or DOP	INH	No INH or DOP	INH	INH removed	DOP	DOP removed
days	*days*	*mg/24 hr/100 g body wt/3-day period*		*mg/24 hr/100 g body wt/3-day period*				
				Experiment 1				
47 through 53	1 through 7	2.06(2) [3]		1.98(2)	1.01(2)		1.09(2)	
54 through 63	8 through 17	5.76 ± 0.65 [4](5)		2.25 ± 0.48(5)	1.76(2)		2.16(3)	
54 through 63	8 through 17					after 10–14 days 1.65(3)		after 10–14 days 1.81(3)
64 through 74	18 through 28	4.38 ± 0.20(6)		1.92 ± 0.17(6)	3.88 ± 0.55(5)			after 17 days 2.21 ± 0.36(6)
75 through 95	29 through 49	7.08 ± 0.65(11)		2.07 ± 0.31(11)		after 28 days 2.75 ± 0.33(7)		after 17 days 1.94 ± 0.19(12)
				Experiment 2				
				(days 35–41 preliminary, all rats given vitamin B_6 with no inhibitor)				
[35 through 41]	[1 through 7]	[1.71 ± 0.24(6)]	[1.92 ± 0.31(6)]	[1.31 ± 0.11(6)]	[1.38 ± 0.22(6)]			
49 through 55	8 through 14	1.40 ± 0.36(6)	2.56 ± 1.00(6)	1.06 ± 0.11(6)	1.60 ± 0.49(6)			
56 through 62	22 through 28	5.35 ± 0.55(6)	6.78 ± 1.03(6)	1.99 ± 0.33(6)	3.23 ± 0.47(6)			
84 through 90	43 through 49	6.31 ± 0.49(5)	8.75 ± 0.74(5)	1.02 ± 0.24(5)		after 35 days 1.61 ± 0.31(5)		
				Experiment 3				
49 through 51	10 through 12	1.79 ± 0.23(12)	1.91 ± 0.17(12)					
56 through 58	17 through 19	3.75 ± 0.30(12)	5.13 ± 0.27(12)					
				Experiment 4				
50 through 52	12 through 14			1.73 ± 0.32(12)	2.57 ± 0.28(12)			
				Experiment 5				
47 through 53	1 through 7			2.53 ± 0.40(8)			1.69 ± 0.16(8)	
54 through 60	8 through 14			2.39 ± 0.40(8)			4.49 ± 0.52(8)	
61 through 67	15 through 21			2.48 ± 0.20(8)			6.21 ± 0.44(8)	

[1] Urine was collected from the same rats on succeeding days in all experiments except in experiment 1.
[2] Pyridoxine hydrochloride.
[3] The numbers in parentheses indicate the number of rats included in the mean.
[4] SE of mean.

(cysteine desulfhydrase) activity which would result in a decrease in the conversion of cysteine to pyruvate and glucose; thus the amount of cysteine available for metabolism to taurine would be increased. Thompson and Guerrant (5) observed that weanling rats exhibited a maximal thionase activity; 21 days later the enzyme activity began to decrease. Whether the change in thionase is related to hormonal secretions is not known.

The increase of urinary taurine with a increase in age should be taken into account when designing experiments in which the object is to determine whether certain treatments affect the amount of taurine in urine. Treatments of rats over 55 days of age could be expected to give taurine values of greater magnitude.

Effect of vitamin B_6 deficiency on urinary taurine. The amount of taurine in the urine of male rats fed vitamin B_6-deficient diets was similar to that of those fed adequate vitamin B_6, until 50 to 55 days or age; after that age the urinary taurine of the vitamin B_6-deprived animals did not increase although it increased for rats receiving adequate vitamin B_6 (exps. 1, 2, 3; table 1). The amount of taurine excreted by rats deprived of vitamin B_6 remained throughout 4 experiments (exps. 1, 2, 4, 5; table 1) at about 2 mg/kg/100 g body weight for rats up to 95 days of age (7 weeks of feeding), except in experiment 2 when the values at times decreased to about 1 mg (table 1).

The failure of urinary taurine to increase with age for male rats fed vitamin B_6-deficient diets may be related to retarded sexual development or may be attributed to a limitation in the amount of pyridoxal phosphate present. This coenzyme is needed for the activity of cysteinesulfinic acid and of cysteic acid decarboxylase. Blaschko et al. (6), using a paper chromatography method have shown that in pyridoxine-deficient rats the decarboxylase activity in the liver is lost and that taurine disappears from the urine. Later, with an improved method, Hope (7) observed that after 14 weeks of deficiency, some taurine still remained in the urine of rats. The present study was not continued for as long a time but the additional observation was made that the amount excreted remains on an approximate plateau through a 7-week feeding period. Hope suggests two possible sources of persistent small amounts of taurine: ". . . since the liver enzyme disappears in the early stages of deficiency, the taurine present in the urine in the later stages is probably dependent upon the activity of the decarboxylase present in nervous tissue, which persists under these conditions. . . . Taurine may also be derived from the cysteamine moiety of coenzyme A."

Effect of INH on urinary taurine. INH increased the excretion of taurine in the urine of male rats fed diets adequate in vitamin B_6 for 3 weeks or longer and tended to increase taurine excretion when the diets were deficient in vitamin B_6.

In one of two experiments (exp. 2) in which the diet was adequate in vitamin B_6, when values for weeks 2, 4 and 7 were combined, the taurine of rats given INH was higher than that of counterparts not receiving the drug (table 1) although the increase was of low statistical significance ($P < 0.05$).[8] In the other experiment (exp. 3), rats that received INH for 17 to 19 days (table 1) excreted significantly more taurine ($P < 0.01$).

In 3 experiments (exps. 1, 2, 4), after the rats had been fed a diet deficient in vitamin B_6, the urinary taurine excretion tended to be greater for rats fed INH than for rats that did not receive the drug. In 2 experiments (exps. 1, 2) at the end of 28 days the amount of urinary taurine excreted by rats given INH exceeded that of their partners, although the difference was of low statistical significance (exp. 1, $P < 0.05$; exp. 2, $P < 0.10$). In the third experiment (exp. 4) continued for 2 weeks only, rats given INH excreted more taurine than the controls: 2.57 ± 0.28 mg/24 hours/100 g body weight and 1.73 ± 0.32 mg, respectively. The level of significance was low ($P < 0.07$). The fact that in all 3 experiments the values for rats given INH were higher tends to substantiate the belief that a real increase occurred. Futhermore, when the administration of INH was discontinued (exps. 1, 2) taurine excretion declined (table 1).

[8] All tests of significance were made by paired *t* tests except where specified as group *t* tests.

In unpublished work carried out in this laboratory the taurine content of the urine of 4 young men was observed to increase during INH administration.

Two groups of workers, Marcucci and Mussini (8) and Yoshikawa et al. (9) reported that INH decreased the urinary taurine of rats. Both groups administered many times the amounts of INH used in the present study.

The increase in urinary taurine caused by INH may have been brought about by an increase of coenzyme in the liver. An increase in the pyridoxal phosphate content of the livers of the rats fed INH in experiments 2 and 3 occurred and has been reported by Sevigny et al. (10, exps. B and C). In experiment B, when the diet was adequate in vitamin B_6, INH increased pyridoxal phosphate from 8.1 ± 0.3 to 10.9 ± 0.5 μg/g; when the diet was deficient in vitamin B_6, INH increased pyridoxal phosphate from 2.2 ± 0.1 to 3.6 ± 0.4 μg/g. In experiment C, when the diet was adequate in vitamin B_6, INH increased pyridoxal phosphate from 7.1 ± 0.3 to 10.8 ± 0.5 μg/g. In the present study, some of the pyridoxal phosphate in the livers of the rats fed INH may have been present as isonicotinyl pyridoxal phosphate hydrazone. However Gonnard and Fenard (11), Torchinsky (12), Makino et al. (13) and Bonavita and Scardo (14) have shown that the pyridoxal phosphate in the hydrazone can be used to activate several vitamin B_6-requiring apoenzymes. Possibly INH causes an increase of taurine because of a toxic effect unrelated to its anti-vitamin effect. Possibly, too, INH causes the excretion of taurine from preformed tissue taurine rather than causing the formation of additional taurine.

Another possible cause of the increase in taurine excretion by INH is its action on the adrenal cortex. Both ACTH and cortisone are known to increase the amount of taurine in urine. Whether INH increases the excretion of cortical hormones is, however, questionable. An increase in certain cortical hormones following treatment with INH has been observed by some but not all workers. In addition, Wiesel (15) obtained evidence by experiments in vitro that INH markedly retards the inactivation of cortisone by the liver.

Effect of DOP on urinary taurine. In experiment 5, DOP increased the urinary taurine of male rats fed the basal vitamin B_6-deficient diet. At the end of 2 weeks the increase was considerable (table 1); at the end of 3 weeks it was highly significant ($P < 0.01$). Some supporting evidence was supplied in experiment 1 in which the mean excretion for taurine for 3 rats increased during 10 days when DOP (in a smaller dose than in experiment 5) was being administered.

No studies of the effect of DOP on the amount of taurine present in urine were found in the literature.

The cause of the increase in taurine may have been an increase in the amount of pyridoxal phosphate present in the livers of the rats. The liver of the rats fed DOP in experiment 5 contained more pyridoxal phosphate than those of their counterparts not fed the drug. This observation has been reported by Johnston et al. (16, exp. D). Stoerk (17) has reported a similar observation.

ACKNOWLEDGMENTS

Technical assistance in the determination of taurine by Mrs. Esserline Gatewod is gratefully acknowledged. We wish to express our thanks to Sister Saint Jeanne de Jesus Sevigny, Ph.D., who conducted experiment 1 for other purposes, for her contribution of material to use in the present study.

LITERATURE CITED

1. Sörbo, B. 1961 A method for the determination of taurine in urine. Clini. Chim. Acta, *6:* 87.
2. Garvin, J. E. 1960 A new method for the determination of taurine in tissues. Arch. Biochem. Biophys., *91:* 219.
3. Chatagner, F., and B. Bergeret 1956 Décarboxylation de l'acide cystéinesulfinique par le foie et le cerveau du rat mâle, du rat femelle et du rat femelle ovariectomisé. Bull. Soc. Chim. Biol., *38:* 1159.
4. Sloane-Stanley, G. H. 1949 Amino-acid decarboxylases in rat liver. Biochem. J., *45:* 556.
5. Thompson, R. Q., and N. B. Guerrant 1953 Effect of dietary protein and vitamin B_6 on hepatic thionase activity. J. Nutr., *50:* 161.
6. Blaschko, H., S. P. Datta and H. Harris 1953 Pyridoxin deficiency in the rat: liver L-cysteic acid decarboxylase activity and urinary amino-acids. Brit. J. Nutr., *7:* 364.

7. Hope, D. B. 1957 The persistence of taurine in the brains of pyridoxine-deficient rats. J. Neurochem., *1:* 364.
8. Marcucci, F., and E. Mussini 1958 Sulla eliminazione di acido cisteico et de taurina dopo trattomento con isoniazid. Boll. Soc. Ital. Biol. Sperim., *34:* 422.
9. Yoshikawa, K. T., T. Aoki, K. Matoba and I. Matsumo 1960 Change of the taurine excretion in the urine by the administration of INAH to rats. Nara Igaku Zaschi., *11:* 71.
10. Sevigny, J., S. L. White, M. L. Halsey and F. A. Johnston 1966 The effect of isoniazid on the loss of pyridoxal phosphate from, and its distribution in, the body of the rat. J. Nutr., *88:* 45.
11. Gonnard, P., and S. Fenard 1962 Cerebral glutamic acid decarboxylase and pyridoxal-5-phosphate hydrazones. J. Neurochem., *9:* 135.
12. Torchinsky, Y. M. 1963 The mode of interaction of the isonicotinoyl hydrazone of pyridoxal phosphate with aspartate-glutamate apotransaminase. Biochem. Biopyhs. Res. Commun., *10:* 401.
13. Makino, K., Y. Ooi, M. Matsuda, M. Tsuji, M. Matsumoto and K. Kuroda 1962 Some notes on the coenzyme-activity of phosphopyridoxal derivates for the brain glutamic decarboxylase. Biochem. Biophys. Res., Commun., *9:* 246.
14. Bonavita, V., and V. Scardi 1959 Studies on glutamic-oxaloacetic transaminase. The coenzymatic role of the isonicotinylhydrazone of pyridoxal-5-phosphate. Biochem. Pharmacol., *2:* 58.
15. Wiesel, L. L. 1956 Investigation of the synergism of isonicotinic acid hydrazide and cortisone. I. Effect of isonicotinic acid on the metabolism of cortisone by liver tissue. Amer. J. Med. Sci., *232:* 412.
16. Johnston, F. A., S. L. White, M. L. Halsey and J. Sevigny 1966 The effect of deoxypyridoxine on the amount of pyridoxal phosphate in the livers and leukocytes of rats and on leukocyte number, size and type. J. Nutr., *88:* 51.
17. Stoerk, H. C. 1950 Desoxypyridoxine observations in "acute pyridoxine deficiency." Ann. N. Y. Acad. Sci., *52:* 1302.

Effect of Coprophagy on Protein Utilization in the Rat [1]

B. R. STILLINGS [2] AND L. R. HACKLER
New York State Agricultural Experiment Station, Cornell University at Geneva, New York

ABSTRACT Diets differing widely in protein quality were fed to male weanling rats, and the effect of coprophagy on protein utilization was determined. In a metabolism study in which animals consumed 5 g daily of diets containing 10% protein, preventing coprophagy decreased biological value and nitrogen absorption, and increased urinary nitrogen excretion and metabolic fecal nitrogen. In a growth study in which animals consumed diets containing 10% protein ad libitum, coprophagy prevention resulted in lower protein efficiency ratios and weight gains, but had little effect on feed intake.

The practice of coprophagy by the rat is well known, and Barnes et al. (1) have estimated that rats recycle approximately 50% of their feces. The possible effect which this practice might have on nutrient utilization and requirements has been the subject of many previous reports. The effect of preventing coprophagy in in mature rats on protein digestion was studied by Barnes et al. (2), who reported that the practice of coprophagy did not influence to any measurable extent the digestibility of protein. Data on the effect of coprophagy on nitrogen utilization and retention are not available. Since the rat is used in the majority of studies dealing with protein and amino acid nutrition, it was deemed advisable to conduct studies on the effect of coprophagy on protein utilization.

EXPERIMENTAL METHODS

Proteins known to vary widely in quality were used to study the effect of coprophagy on protein utilization. The sources included zein, wheat gluten and whole egg. In addition, 4 samples of heat-treated soya protein were used. Each was mixed with 1.5 times its weight of water, autoclaved at 121° for 5, 40, 120, or 240 minutes and then freeze-dried. Two amino acid mixtures were also used, the compositions of which are given in table 1. The balanced amino acid mixture was essentially that of Rama Rao et al. (3), and this was substantially modified to produce the imbalanced mixture. The composition of the basal diet is shown in table 2. All protein sources were incorporated in amounts to produce 10% protein diets, which were calculated to be isocaloric. Diets were stored in metal containers at 1° and used within a few days in a metabolism experiment and in 3 months in a growth study.

The biological value of these diets was determined in a metabolism experiment and computed essentially according to the procedure outlined by Mitchell (4, 5). Male rats of the Holtzman strain, 21 days old, were maintained with a 15% casein diet for 2 days and then were assigned on a weight basis to one of 9 groups. The groups, in turn, were assigned at random to the 9 diets. Each group contained 4 animals for which coprophagy was prevented in two and allowed in the two remaining. Coprophagy was prevented by fecal collection cups similar to those illustrated by Barnes et al. (6).

During the experiment, animals were maintained in metal metabolism cages which facilitated separation and collection of urine and feces. Feed was mixed with an amount of water equal to approximately 60% of the diet weight and offered to the animals once daily.

The metabolism study consisted of 4 collection periods of 7 days each. Prior to each collection period, animals were fed experimental diets for at least 5 days, and they consumed a constant amount

Received for publication March 12, 1966.

[1] Approved for publication by the Director of the New York State Agricultural Experiment Station, Geneva, New York, as Journal Paper no. 1476, March 4, 1966.

[2] Postdoctoral Fellow on National Institutes of Health Training Grant 2 G-950, Spec. (1) E. H. Present address: Bureau of Commercial Fisheries, Technological Laboratory, College Park, Maryland 20740.

TABLE 1

Amino acid composition of amino acid diets

Amino acid	"Balanced" amino acid diet	"Imbalanced" amino acid diet
	% of diet	*% of diet*
Histidine	0.25	0.50
Arginine	0.28	0.14
Lysine	0.90	0.09
Tryptophan	0.11	0.22
Isoleucine	0.55	0.27
Valine	0.55	0.27
Leucine	0.70	1.55
Threonine	0.50	1.00
Methionine	0.16	0.08
Cystine	0.34	0.07
Phenylalanine	0.42	0.84
Tyrosine	0.30	0.30
Aspartic	1.041	1.041
Alanine	0.484	0.484
Serine	1.009	1.009
Proline	1.943	1.943
Glycine	0.312	0.312
Glutamic	3.411	3.411
Total	13.26	13.53

TABLE 2

Composition of 10% protein diets

	%
Protein source [1,2] / Dextrose [2]	77.70
Sucrose	12.00
Cellulose [2,3]	5.00
Salt mix [3,4]	4.00
Vitamin mix [5]	1.00
Corn oil [6,7]	10.00
Choline citrate	0.30

[1] Protein sources: (% of diet) zein, 10.88; wheat gluten, 12.20; soya assay protein heated 5, 40, 120, and 240 minutes at 120°, 11.70, 11.44, 11.38, and 11.54, respectively; whole egg, 21.48; imbalanced amino acids, 13.53; and balanced amino acids, 13.26.

[2] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Non-nutritive fiber (cellulose type).

[4] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition., *14:* 273.

[5] Vitamin mix composition: (mg/100 g diet) thiamine·HCl, 1.0; riboflavin, 1.5; pyridoxine·HCl, 0.5; Ca DL-pantothenate, 2.0; nicotinic acid, 3.0; biotin, 0.03; folic acid, 0.2; menadione, 0.4; inositol, 7.5; vitamin B_{12} (0.1% in mannitol), 4.0; *p*-aminobenzoic acid, 2.5; ascorbic acid, 0.4; crystalline vitamin A, 2.0 (500 USP units/mg); vitamin D_2, 0.2 (500 USP units/mg); and *dl*-α-tocopherol, 12.0; (250 USP units/g).

[6] Mazola corn oil, Corn Products Company, New York.

[7] Whole egg contained 35.2% ether extract; therefore, 2.4% corn oil added to whole egg diet.

the last 3 days. In the first and third periods, animals consumed 10% protein diets, whereas in the second and fourth periods they were fed an equal amount of a 3.4% whole egg diet which was isocaloric and otherwise identical to the basal diet (table 2).

During the first and second periods, coprophagy was prevented in one-half the animals and allowed in the remaining half. Throughout the third and fourth periods fecal collection cups were switched to animals that had been allowed to practice coprophagy during the first 2 periods. Feces were collected daily and dried at 50°. Urine samples were preserved with H_2SO_4 and KF. Samples of feed, feces and urine were analyzed for nitrogen by the macro-Kjeldahl procedure.

In a second study, the effect of coprophagy on weight gain, feed consumption and protein efficiency ratio was determined. Twenty-three-day-old male weanling rats of the Holtzman strain were allotted on a weight basis to 10 groups. Groups were then assigned at random to the 9 diets used in the first study and to a tenth diet which contained a 20-fold increase in niacin but which was otherwise identical to the zein diet.

A total of 8 animals was assigned to each diet, with coprophagy prevented in four and allowed in the 4 remaining animals. An attempt was made to nullify possible effects which the physical presence of the fecal collection cups might have on the responses of the animals. Fecal collection cups with the open end pointing away from the animals were attached midway down the tails of animals allowed to practice coprophagy. Thus, fecal collection cups were attached to all animals, but in one-half of the animals the cups were attached in a manner which allowed coprophagy.

During the second study the animals were fed ad libitum for 28 days, and feed intake and weight gain data were recorded. Protein efficiency ratio (PER) was calculated by dividing the grams of weight gained by the grams of protein consumed. Data from both studies were analyzed statistically by an analysis of variance (7).

RESULTS AND DISCUSSION

Significant depressions in biological value of dietary proteins were observed when rats were prevented from practicing coprophagy (table 3). The average biological value for all diets was reduced from 60% to 50% when coprophagy was prevented. Decreases were observed for

TABLE 3

Effect of coprophagy prevention on protein utilization by weanling rats fed 10% protein diets [1]

	Wheat gluten	Whole egg	Soya protein, heated at 121°, min				Imbalanced amino acid	Balanced amino acid	Mean
			5	40	120	240			
				Metabolic nitrogen, mg/g feed					
Coprophagy allowed	1.09	1.26	1.08	1.02	1.08	1.03	1.05	1.08	1.08
Coprophagy prevented	1.30	1.29	1.42	1.35	1.31	1.39	1.27	1.35	1.33
Difference	0.21*	0.03	0.34**	0.33**	0.23*	0.36**	0.22*	0.27*	0.25**
				Nitrogen absorption, % of nitrogen intake					
Coprophagy allowed	99.9	98.2	98.7	97.8	95.8	93.8	99.6	100.0	98.0
Coprophagy prevented	98.8	96.1	98.2	96.0	94.6	91.8	99.8	100.0	97.0
Difference	−0.1	−2.1**	−0.5	−1.8**	−1.2	−2.0**	0.2	0.0	−1.0**
				Endogenous nitrogen, mg/10 g feed $W^{0.75}$					
Coprophagy allowed	1.59	1.29	1.58	1.37	1.24	1.46	1.18	1.31	1.38
Coprophagy prevented	1.06	1.39	1.38	1.67	1.17	1.38	0.94	1.36	1.29
Difference	−0.53	0.10	−0.20	0.30	−0.07	−0.08	−0.24	0.05	−0.09
				Urinary food nitrogen, % of nitrogen intake					
Coprophagy allowed	55.3	20.1	32.9	35.2	38.2	42.2	66.6	28.5	39.9
Coprophagy prevented	56.6	38.1	43.6	37.9	40.3	48.3	76.9	52.8	49.3
Difference	1.3	18.0**	10.7*	2.7	2.1	6.1	10.3*	24.3**	9.4**
				Biological value, %					
Coprophagy allowed	44.7	79.6	66.8	65.0	60.1	55.1	33.1	71.6	59.5
Coprophagy prevented	43.4	60.4	55.6	60.6	57.5	47.4	23.9	47.2	49.5
Difference	−1.3	−19.2**	−11.2*	−4.4	−2.6	−7.7	−9.2*	−24.4**	−10.0**

[1] Nitrogen × 6.25.
* $P < 0.05$.
** $P < 0.01$.

all diets; however, significant differences were found only for diets containing the balanced amino acid mixture ($P < 0.01$), whole egg ($P < 0.01$), imbalanced amino acid mixture ($P < 0.05$), and soya protein heated 5 minutes ($P < 0.05$).

With the exception of the imbalanced amino acid diet, significant differences were found only for diets with the highest biological value. Biological value is known to be affected by level of feed intake; however, with the exception of the zein and imbalanced amino acid diets all animals consumed 5 g of feed daily. In a subsequent study in which each animal consumed 4.5 g daily of the imbalanced amino acid diets, no significant difference was found in biological value between the coprophagic and noncoprophagic rats.

The differences obtained in biological value between coprophagic and noncoprophagic rats were reflected in weight gains of animals. As shown in table 4, the average weight gains during the first and third collection periods were consistently higher for animals practicing coprophagy. In addition, diets which gave the largest differences in biological value also tended to result in wider differences in weight gains.

The interaction which was observed between quality of diet and coprophagy on biological value is difficult to explain; however, 2 possibilities can be presented. First, although all animals were fed equal amounts of feed at the same time each day, those rats assigned to the higher quality diets tended to consume their feed in less time than those fed the lower quality diets. Also there may have been a difference between diets in the degree to which animals practiced coprophagy. For example, if coprophagic rats assigned to the whole egg diet consumed more of their feces than similar animals assigned to the wheat gluten diet, greater differences due to coprophagy would be expected with the former diet than with the latter.

The depression in biological value which occurred when coprophagy was prevented was the result of an increased urinary nitrogen excretion (table 3). It appears that some factor(s) in the feces was required for optimal utilization of the absorbed nitrogen. Until additional studies are conducted, any attempt to arrive at an adequate explanation can be only speculative.

Preventing coprophagy also resulted in a slight but statistically significant ($P < 0.01$) depression in nitrogen absorption. Although the practical significance of this small difference is questionable, the results are in accord with those obtained in the rabbit by Thacker and Brandt (8), who reported that coprophagy prevention decreased apparent protein digestibility. However, Barnes et al. (2) reported that protein digestibility in the rat was uninfluenced by preventing coprophagy. In our study, nitrogen absorption was high for all diets, which possibly was attributable to the low level of feed intake. Nitrogen absorption decreased, however, in the soya protein diets as the time of autoclaving increased.

The metabolic fecal nitrogen was significantly increased for all animals in which coprophagy was prevented, with the exception of those which previously had been fed the 10% whole egg-protein diet (table 3). Metabolic and endogenous nitrogen were determined in the second and fourth periods with all animals consuming 5 g of the 3.4% whole egg-protein diet. Urinary endogenous nitrogen was not significantly affected by coprophagy prevention. Barnes et al. (2) also observed small increases in metabolic nitrogen in rats prevented from practicing coprophagy. They suggested that nitrogen derived from the intestinal tract might be digested if recycled through the small intestine, which would result in lower

TABLE 4

Effect of coprophagy prevention on average weight gain during first and third collection periods of metabolism experiment

Diet	Coprophagy allowed	Coprophagy prevented
	g/rat/week	
Wheat gluten	2.9	2.6
Whole egg	7.2	1.9
Soya protein, 5 min [1]	6.0	2.0
Soya protein, 40 min	5.3	1.9
Soya protein, 120 min	5.6	3.9
Soya protein, 240 min	4.4	0.5
Imbalanced amino acid	0.6	−0.2
Balanced amino acid	5.9	1.8

[1]Minutes heated at 121°.

metabolic nitrogen values in rats practicing coprophagy.

The results obtained with the zein diet are not included in table 3 because animals failed to consume the entire amount of feed offered, and one-half of the animals died near the end of the second experimental period. After 10 days, rats consuming the zein diet and allowed to practice coprophagy exhibited apparent vitamin deficiency symptoms, namely, rough hair coat, porphyrin-caked whiskers, diarrhea and general unthriftiness. Additional vitamin supplements were administered orally; however, the animals did not survive. Animals fed the zein diet that were prevented from practicing coprophagy showed no abnormalities during the first and second periods; but, when allowed to practice coprophagy during the third and fourth periods, similar vitamin deficiency symptoms were exhibited. It is possible that the animals were deficient in niacin; however, it is difficult to explain the occurrence of symptoms only in animals allowed to practice coprophagy.

In view of the possibility of a niacin deficiency in the zein diet, the second study included a tenth diet which was identical to the zein diet except that it contained a 20-fold increase in niacin. As shown in table 5, all animals consuming the zein diets lost weight, although none showed the symptoms exhibited in the first experiment. Only slight differences between the 2 zein diets were noted in weight gain and feed consumed, but animals fed both diets and in which coprophagy was prevented tended to consume more feed. From these results a conclusion cannot be drawn to explain the symptoms exhibited by animals in the first study.

Slight depressions in weight gains for all diets, except that with whole egg, were observed when coprophagy was prevented (table 5). The average decrease for all diets was 11%. Barnes et al. (6) reported a 15 to 25% reduction in growth with regular fecal collection cups and a 5 to 8% decrease with cups attached which still permitted coprophagy. It can be assumed, therefore, that 10 to 17% of the decrease was due to failure of the animals to prac-

TABLE 5

Effect of coprophagy prevention on weight gain, feed intake, and protein efficiency ratio by weanling rats fed 10% protein diets [1]

Diet	Average daily gain			Average daily feed intake			Protein efficiency ratio		
	Coprophagy allowed	Coprophagy prevented	Difference	Coprophagy allowed	Coprophagy prevented	Difference	Coprophagy allowed	Coprophagy prevented	Difference
	g	*g*	*g*	*g*	*g*	*g*			
Zein	−0.62	−0.63	0.01	4.67	5.01	0.34	—	—	—
Zein + niacin [2]	−0.71	−0.73	0.02	4.43	5.19	0.76*	—	—	—
Wheat gluten	0.58	0.36	−0.22*	7.23	6.88	−0.35	0.78	0.49	−0.29*
Whole egg	4.19	4.31	0.12	11.19	11.97	0.78*	3.68	3.54	−0.14
Soya protein, 5 min [3]	2.33	2.17	−0.16	10.99	11.07	0.08	2.05	1.91	−0.14
Soya protein, 40 min	2.34	2.12	−0.22	10.94	10.58	−0.36	2.07	1.95	−0.12
Soya protein, 120 min	1.88	1.59	−0.29	9.99	10.15	0.16	1.86	1.55	−0.31*
Soya protein, 240 min	1.28	1.12	−0.16	8.34	8.61	0.27	1.51	1.27	−0.24*
Imbalanced amino acid	−0.28	−0.33	−0.05	4.77	4.96	0.19	—	—	—
Balanced amino acid	3.87	3.29	−0.58*	12.17	11.30	−0.87*	3.08	2.85	−0.23*
Mean	1.49	1.33	−0.16*	8.47	8.57	0.10	2.15	1.94	−0.21**

[1] Nitrogen × 6.25.
[2] Niacin content increased 20 times over that of other diets.
[3] Minutes heated at 121°.
* $P < 0.05$.
** $P < 0.01$.

tice coprophagy, and the decrease obtained in our study falls within this range.

Slight and inconsistent differences were noted on the effect of coprophagy on feed intake (table 5). When coprophagy was prevented animals consumed slightly more of the balanced amino acid diet but less of the whole egg diet.

Preventing coprophagy resulted, however, in a reduction in PER (table 5). The PER for each diet was less for animals prevented from practicing coprophagy, and the average PER for all diets was significantly depressed ($P < 0.01$). These results are not in accord with those of Barnes et al. (6), who reported no effect on PER by the prevention of coprophagy. In the present study, the magnitude of the differences in PER was not as great as that for biological value, especially with the higher quality diets. This may have been due to the difference in feed intake and a difference in the degree to which animals practiced coprophagy in the 2 experiments. In addition, animals allowed to practice coprophagy in the metabolism study were not fitted with tail cups as they were in the growth study.

Decreases in PER and those noted in biological value provide evidence that prevention of coprophagy reduces the utilization of dietary protein. Investigations should be made to determine the degree to which rats practice coprophagy when fed diets differing in protein quality and to determine what effect level of energy and feed intake have on extent of coprophagy practice and on the utilization of protein when coprophagy is prevented. In addition, studies are required to explain the increased urinary nitrogen excretion when coprophagy is prevented.

ACKNOWLEDGMENT

The authors are indebted to Allison Beinert for technical assistance during these studies.

LITERATURE CITED

1. Barnes, R. H., G. Fiala, B. McGehee and A. Brown 1957 Prevention of coprophagy in the rat. J. Nutr., 63: 489.
2. Barnes, R. H., E. Kwong and G. Fiala 1958 Effects of the prevention of coprophagy in the rat. III. Digestibility of protein and fat. J. Nutr., 65: 251.
3. Rama Rao, P. B., H. W. Norton and B. C. Johnson 1964 The amino acid composition and nutritive value of proteins. V. Amino acid requirements as a pattern for protein evaluation. J. Nutr., 82: 88.
4. Mitchell, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., 58: 873.
5. Mitchell, H. H. 1944 Determination of the nutritive value of the proteins of food products. Ind. Eng. Chem. (Anal. ed.), 16: 696.
6. Barnes, R. H., G. Fiala and E. Kwong 1963 Decreased growth rate resulting from prevention of coprophagy. Federation Proc., 22: 125.
7. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.
8. Thacker, E. J., and C. S. Brandt 1955 Coprophagy in the rabbit. J. Nutr., 55: 375.

Amino Acid Activation in the Liver of Growing Rats Maintained with Normal and with Protein-deficient Diets

A. MARIANI, P. A. MIGLIACCIO, M. A. SPADONI AND M. TICCA
Istituto Nazionale della Nutrizione, Città Universitaria, Rome, Italy

ABSTRACT The activity of the amino acid-activating enzymes was measured in the liver of growing rats maintained with a normal and with a protein-deficient diet. The determinations were based on the isotope exchange between ATP and PP labeled with ^{32}P. The changes in the DNA content of the cell nucleus were also determined. Studies of changes in DNA content, and hence changes in ploidy during growth, indicated that in normal rats the activity of the amino acid-activating enzymes increased when expressed per average nucleus, whereas it remained constant and unaffected by growth when expressed per unit weight of DNA. On the contrary, in rats maintained with a protein-deficient diet, in which the arrest of growth causes an arrest of ploidy, the activity of the amino acid-activating enzymes increased both when expressed per average nucleus and per unit weight of DNA. We suggest that under these conditions a control mechanism is brought into action by changes in the amount of amino acids in circulation. This mechanism is independent of the increase in the DNA content of cell nucleus, and results, for growing rats maintained with a protein-deficient diet, in a preferential utilization of the amino acids for the synthesis of proteins.

Following a prolonged protein fast the liver of the adult rat exhibits a higher activity of all the amino acid-activating enzymes, both when expressed per unit weight of liver protein and per unit weight of liver DNA-P (1, 2). This may be interpreted as an attempt on the part of the liver to control the metabolism of amino acids by redirecting the latter from catabolic to anabolic processes.

Previous experiments carried out on adult rats, with isotopically labeled amino acids, have indicated that a prolonged protein fast results in the preferential utilization of amino acids by the hepatic cells (3). The same result has also been obtained in investigations on rats maintained after weaning with a protein-deficient diet for a prolonged period (4, 5).

The present experiments were carried out to determine whether in growing rats a protein-deficient diet results in a similar higher activity of the amino acid-activating enzymes.

In view of the changes in the ploidy of the hepatic cells during growth and of the effect of the diet on these changes (6–9), it was considered advisable to undertake a preliminary investigation on the DNA content of the average nucleus, in the liver of growing rats kept under our experimental conditions. In protein-deficient rats we had found no increase with age of the DNA content of the average nucleus, whereas in normally growing rats the DNA per average nucleus increased as a consequence of the formation of new polyploid cells. We decided, therefore, to express the results relative to amino acid activation not only per unit weight of DNA-P, but per average nucleus as well. The results obtained show that the activity of the amino acid-activating enzymes increases in protein-deficient rats, both when expressed per unit weight of DNA-P and per average nucleus.

EXPERIMENTAL

Locally bred male albino rats of the Wistar strain were taken immediately after weaning and fed ad libitum a diet containing 25 and 5% casein (normal and protein-deficient diets, respectively) (table 1). They were then decapitated 10, 20 and 30 days later, the liver was immediately excised, and washed with an ice-cold solution of Tris (hydroxymethyl)

Received for publication March 11, 1966.

TABLE 1

Composition of experimental diets

	Normal diet	Protein-deficient diet
	%	%
Casein	25	5
Rice starch	34	44
Sucrose	28.7	38.7
DL-Methionine	0.3	0.3
Salt mixture [1]	5	5
Olive oil	5	5
Cod liver oil	1	1
Vitamin mixture [2]	1	1

[1] Each 100 g of the salt mixture supplied: (in grams) calcium carbonate, 38.140; cobalt chloride, 0.002; copper sulfate, 0.048; ferrous sulfate, 2.700; magnesium sulfate, anhydrous, 5.730; manganese sulfate, 0.445; potassium iodide, 0.079; potassium phosphate, monobasic, 38.900; zinc chloride, 0.026; sodium chloride, 13.930; (Jones, J. H., and C. Foster. J. Nutrition, 24: 245, 1943, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

[2] Each 1,000 g of the vitamin mixture supplied: (in grams) p-aminobenzoic acid, 2; vitamin B_1, 0.2; vitamin B_2, 0.4; vitamin B_6, 0.2; Ca pantothenate, 0.8; niacin, 2; inositol, 2; folic acid, 0.04; biotin, 0.024; vitamin B_{12}, 0.001; vitamin C, 2; menadione sodium bisulfite, 0.2; vitamin E, 1,000 IU; choline chloride, 40; vitamin A, 200,000 IU; vitamin D_2, 20,000 IU; sucrose to make 1,000 g.

amino-methane (Tris) (pH 7.4) and KCl in concentrations of 0.02 and 0.05 M, respectively. A suitable amount of the liver was homogenized in the Tris-KCl buffer at 0° by a motor-driven Potter homogenizer fitted with a Teflon pestle.

Determination of enzymatic activity. The homogenate was centrifuged in a Martin-Christ ultracentrifuge at 105,000 × *g* for one hour. The temperature inside the tubes was kept below 4° since the activity of the enzymes activating the amino acids decreases at a higher temperature. Suitable equal portions of the supernatant were then treated with Carbowax [1] (20 M) according to the technique described by Pennington (10). This was carried out to remove the endogenous amino acids. The activation of the amino acids was then determined by measuring the rate of isotope exchange between ^{32}PP and ATP by the method of De Moss and Novelli (11). Incubation was carried out as described previously (2). The radioactivity was determined with the aid of a gas-flow end-window counter.

Preparation of the suspension for nuclei count and DNA determination. The liver was homogenized manually for 2.30 minutes in a Potter apparatus with 4 volumes of a 0.88 M sucrose solution. The homogenate was then filtered through gauze and diluted in a ratio of 1:20 with a 3% solution of acetic acid containing 0.2% of methyl green. The cells were counted directly in a Bürker cell counter (12). The DNA determination was carried out on a suitable amount of the homogenate by the colorimetric method of Webb and Levy (13). The results of the above 2 determinations were then used to calculate the average DNA content per cell nucleus. To check this method, we used the method of Chauveau et al. (14) as modified by Di Girolamo et al. (15) (but substituting 0.25 M sucrose with 0.88 M sucrose) to isolate cell nuclei and subjected this preparation to a cell count and DNA determination as described above. The value thus obtained for the DNA content per cell nucleus was in satisfactory agreement with the corresponding value for the above homogenate.

RESULTS

DNA content of the nuclei. The values for the number of nuclei, the DNAP content per nucleus and per gram of fresh tissue are shown in table 2. In neonatal rats, the number of nuclei and the DNAP content per unit weight of liver were the highest, the DNA content per nucleus being approximately the same as the value calculated (16) for the diploid cells in rats. Twenty days after birth, however, the DNAP conent per unit weight of liver decreased as a result of cell enlargement, although the DNAP content per cell nucleus remained unchanged.

In rats fed a diet containing 25% of casein (normal diet, normal growth), owing to the formation of tetraploid and octaploid nuclei, the DNA content per nucleus in the hepatic cells increased and reached the value of adult rats. However, in rats fed a diet containing only 5% of casein (protein-deficient diet, strongly retarded growth), the DNA content per nucleus remained statistically the same as that at the time of the weaning.

The results obtained for the activation of the amino acids were then correlated both with the nuclei and with the DNA. In the first case, we expressed the enzymatic activity per average cell on the

[1] Union Carbide Chemical Co., Charleston, W. Va.

TABLE 2

Effect of protein content of the diet on the number of nuclei and on DNAP content of the liver of growing rats

Age	Rat no.	Body wt	Liver wt	Nuclei	DNAP	
days		*g*	*g*	*millions/g liver wt millions/total liver*	*μg/g liver wt*	*μg/total liver*	*μμg/nucleus*
0	30	5.1	—	426 ± 20.7 —	343 ± 8.9	—	0.809 ± 0.037
(at birth)							
28	15	49 ± 3.1	1.943 ± 0.140	333 ± 24.2 647 ± 43.3	242 ± 17.7	470 ± 57.6	0.727 ± 0.085
(at weaning)							
				25% casein diet			
38	16	87 ± 6.6	3.693 ± 0.228	216 ± 13.4 798 ± 41.4	232 ± 13.5	856 ± 66.5	1.074 ± 0.117
48	15	116 ± 6.9	5.225 ± 0.130	169 ± 7.8 883 ± 49.7	196 ± 11.8	1024 ± 89.0	1.160 ± 0.086
58	23	149 ± 6.4	5.652 ± 0.270	167 ± 8.5 943 ± 54.2	211 ± 14.3	1192 ± 88.5	1.263 ± 0.063
				5% casein diet			
38	15	48 ± 2.0	2.169 ± 0.265	328 ± 22.9 711 ± 85.4	251 ± 11.9	544 ± 24.5	0.765 ± 0.079
48	19	54 ± 1.7	2.403 ± 0.077	299 ± 23.4 718 ± 55.2	221 ± 11.0	531 ± 21.4	0.739 ± 0.068
58	26	60 ± 4.4	2.681 ± 0.167	260 ± 15.2 697 ± 48.8	224 ± 15.1	600 ± 92.9	0.862 ± 0.065

assumption that the cytoplasmic mass of the binuclear cells (10 to 20% of the hepatic cells of rats) (17) is twice that of mononuclear cells. To correlate the enzymatic activity with the DNA content, we assumed that the DNA content of the diploid cells is constant. Thus, the values expressed per unit weight of DNA refer to the enzymatic activity per hypothetical diploid cell, i.e., per amount of protoplasmic mass corresponding to a diploid amount of DNA (18).

Activation of the amino acids as a function of growth and diet. Figure 1 shows the variation in the enzymatic activity in rat liver during a period of 30 days after weaning, that is, up to the age of 58 days, the values being expressed as enzymatic activity per nucleus and hence per average cell. Since the amino acid activation sometimes varied from one series of experiments to another, we placed in each series a rat from each age group. The amino acid activation at the time of weaning was used as an internal check of the effective variations in enzymatic activity with age and diet. Figure 1 shows that the amino acid activation increased during the first 10 days after weaning in both series of experiments. In the normal rats, no appreciable variation was observed for the rest of the experimental period. The rats fed the protein-deficient diet, however, although showing no change in the next 10 days, subsequently showed a highly significant increase in the amino acid activation.

The values for the activation of the amino acids are shown in figure 2 as a function of the DNA content and hence in a manner proportional to the diploid set of chromosomes. The values for normal rats did not change with age. This indicates that the increase in the activity per nucleus during the first 10 days after weaning is proportional to an increase in the ploidy. Since the ploidy did not increase in the deficient rats, the curve for these is parallel to that described previously. At the age of 58 days, the deficient rats showed a definite increase in the activity of enzymes activating the amino acids, the activity being about 90% higher than in normal rats.

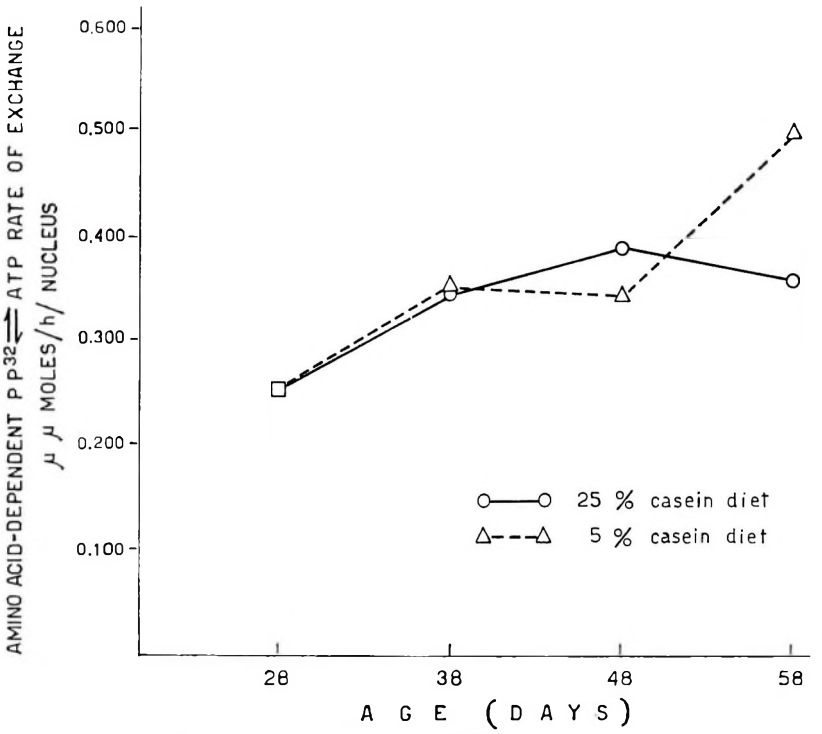


Fig. 1 Variation of the amino acid-dependent ^{32}PP ⇋ ATP rate of exchange/nucleus as a function of age. Each point on the graph is the average of values from a group of 5 rats. Assay conditions: 0.2 ml enzyme preparation (between 2.1 and 4.3 mg supernatant protein in each incubation mixture); 100 μmoles Tris-KCl buffer (pH 7.4); 50 μmoles KF; 10 μmoles ATP, disodium salt, (adjusted to pH 7.4); 10 μmoles pyrophosphate ^{32}P; 10 μmoles $MgCl_2$; complete amino acid mixture (adjusted to pH 7.4), 4 μmoles of each of the following amino acids; alanine, arginine, aspartic acid, cysteine, cystine, glycine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Cystine and tyrosine did not dissolve completely. Total volume 1 ml. Incubated at 37° for 15 minutes.

DISCUSSION

In rats fed normal diet (25% casein), the growth of the liver in the first month after weaning is characterized by an increase in the number and size of cells (see table 2). This has emerged from the calculation of the number of nuclei per gram of liver and in the whole liver. This result does not therefore take into account the increase in the binuclear cells during growth. However, the resulting error was not large, considering that the cytoplasmic mass of the binuclear cells is about twice as great as that of the mononuclear cells. In the first 10 days after weaning, the increase in the number and size of the cells was accompanied by an increase in the DNA content of the average nucleus and therefore by an increase of ploidy up to a value which then remained constant. The increase in the ploidy was in turn accompanied by a proportional increase in the activity of the enzymes activating the amino acids and presumably also in the level of these enzymes. It appeared, therefore, that the level of the activating enzymes is related to a unit weight of DNA corresponding to a diploid set of chromosomes and that this level remains constant and unaffected by the growth of the animals.

In contrast, the animals whose growth was strongly retarded by feeding a protein-deficient diet, showed during the first 10 days after weaning, a slight increase in the weight of the liver resulting almost entirely from an increase in the number of the cells, after which cell division was

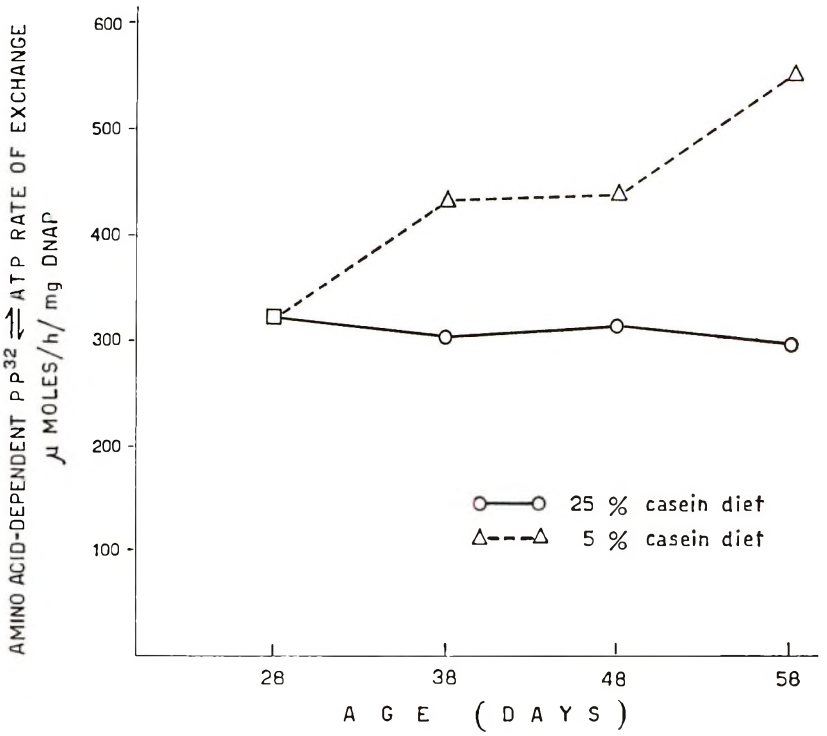


Fig. 2 Variation of the amino acid dependent $^{32}PP \rightleftharpoons ATP$ rate of exchange/DNAP as a function of age. Each point on the graph is the average of values from a group of 5 rats. Assay conditions as in figure 1.

arrested and the cells grew in size, though they remained much smaller than the normal cells. The ploidy did not increase, as in normal rats, but a considerable increase was observed in the level of the enzymes activating the amino acids. This increase does not, therefore, depend on the increase in the DNA content per nucleus, as it does during the growth of the normal rats. Instead, it results from a different control mechanism probably actuated by a change in the pool of circulating amino acids, similar to the situation observed in adult rats subjected to a prolonged protein fast. This increase of the enzymes activating the amino acids may account for the preferential utilization of the amino acids for the synthesis of proteins, which has been found in rats fed a protein-deficient diet after weaning.

LITERATURE CITED

1. Mariani, A., M. A. Spadoni and G. Tomassi 1963 Effect of protein depletion on amino acid activating enzymes of rat liver. Nature. *199:* 378.
2. Gaetani, S., A. M. Paolucci, M. A. Spadoni and G. Tomassi 1964 Activity of amino acid activating enzymes in tissues from protein-depleted rats. J. Nutr., *84:* 173.
3. Gaetani, S., A. Mariani, M. A. Spadoni and G. Tomassi 1961 Distribuzione della C^{14}-lisina nel plasma, fegato, cuore e muscolo gastrocnemio di ratti in deplezione proteica. Boll. Soc. Ital. Biol. Sperim., *37:* 1685.
4. Waterlow, J. C. 1959 Effect of protein depletion on the distribution of protein synthesis. Nature, *184:* 1875.
5. Bendicenti, A., A. Mariani, A. M. Paolucci and M. A. Spadoni 1959 L'influenza del contenuto proteico della dieta sulla distribuzione della S^{35}-metionina nei tessuti di ratti in accrescimento. Boll. Soc. Ital. Biol. Sperim., *35:* 1997.
6. Wilson, J. W., and E. H. Leduc 1948 The occurrence and formation of binucleate and multinucleate cells and polyploid nuclei in the mouse liver. Amer. J. Anat., *82:* 353.
7. Campbell, R. M., H. W. Kosterlitz 1952 The absence of dietary effects on the DNA content of liver nuclei of the adult rat. Science, *115:* 84.

8. Ely, J. O., and M. H. Ross 1951 Deoxyribonucleic acid content of rat liver nuclei influenced by diet. Science, *114:* 70.
9. Umana, R. 1965 Effect of protein malnutrition on the DNA content of rat liver. J. Nutr., *85:* 169.
10. Pennington, R. J. 1960 Amino acid activating enzymes in muscle. Biochem. J., *77:* 205.
11. Demoss, J. A., and G. D. Novelli 1956 An amino acid dependent exchange between ^{32}P-labeled inorganic pyrophosphate and ATP in microbial extracts. Biochim. Biophys. Acta, *22:* 49.
12. Price, J. M., E. C. Miller, J. A. Miller and G. M. Weber 1950 Studies on intracellular composition of livers from rats fed various amino acids. Cancer Res. *10:* 18.
13. Webb, J. M., and H. B. Levy 1955 A sensitive method for the determination of deoxyribonucleic acid in tissues and microrganisms. J. Biol. Chem., *213:* 107.
14. Chauveau, J. Y. Moulé and C. R. Rouiller 1956 Isolation of pure and unaltered liver nuclei: morphology and biochemical composition. Exp. Cell Res., *11:* 317.
15. Di Girolamo, A., E. C. Henshaw and H. H. Hiatt 1964 Messenger ribonucleic acid in rat liver nuclei and cytoplasm. J. Mol. Biol., *8:* 479.
16. Vendrely, R. 1955 In: The Nucleic Acids, eds., E. Chargaff and J. N. Davidson, vol. 2. Academic Press, New York, p. 155.
17. Iype, P. T., P. M. Bhargava and A. D. Tasker 1965 Some aspects of the chemical and cellular composition of adult rat liver. Exp. Cell Res., *40:* 233.
18. Petermann, M. L., and M. G. Hamilton 1958 The influence of age, sex, pregnancy, starvation and other factors, on the cytoplasmic ribonucleoproteins of rat liver. J. Biophys. Biochem. Cytol., *4:* 771.

Utilization of D-Amino Acids for Growth by *Drosophila melanogaster* Larvae [1]

B. W. GEER
Department of Biology, Knox College, Galesburg, Illinois

ABSTRACT The capacity of *Drosophila melanogaster* larvae to utilize the D-forms of the essential amino acids for growth in place of their L-isomers was examined. D-Phenylalanine, D-methionine, and D-histidine, in decreasing order, were the only D-amino acids which were readily used for growth by larvae. The D-forms of arginine, lysine, and valine stimulated larval growth when fed with suboptimal quantities of their L-isomers. D-Isoleucine and D-threonine had little, if any, effect on larval growth. At the levels tested, D-leucine and D-tryptophan inhibited the growth of D. *melanogaster* larvae. Feeding either glycine, L-cystine, or L-tyrosine in a diet containing the L-isomers of the essential amino acids and L-glutamic acid stimulated larval growth. Larvae appear capable of using D-cystine for growth in place of L-cystine. The ability of *D. melanogaster* larvae, *Tetrahymena pyriformis*, and the rat to utilize D-amino acids for growth was compared. Points of evolutionary interest were discussed.

Insects require, in general, the same 10 amino acids in the diet for growth that are required by the rat (1). The essential amino acids are usually supplied in the diet as their L-isomers although purified diets often contain mixtures of the D- and L-isomers of some amino acids. The capacity to utilize D-amino acids in lieu of their L-enantiomorphs has been examined in 2 insects. Fraenkel and Printy (2) found that *Tribolium confusum* utilizes fully or partially the D-forms of methionine, phenylalanine, and possibly, lysine, whereas DeGroot (3) found *Apis mellificia* capable of utilizing D-methionine, D-phenylalanine, and, to some extent, D-histidine.

Drosophila melanogaster larvae, in addition to the 10 essential amino acids, are reported to benefit by supplementation of the diet with L-cystine and glycine (4).[2] The present study was conducted to find which of the D-enantiomorphic forms of the essential amino acids can be utilized for growth by *D. melanogaster* larvae in place of their L-isomers. The nutritive values of L-cystine, L-tyrosine, and glycine for larval growth were also examined.

METHODS AND MATERIALS

The standard medium (table 1) was the same as that used in previous studies (5–7) except that all amino acids, unless being tested, were fed as the L-enantiomorph. Cultures were prepared by dispensing 5 ml of medium in 23-g (6-dram) shell vials, plugging with cotton, and sterilizing by autoclaving. Eggs, which had been sterilized by methods described previously (5), were added to the culture vials by aseptic techniques. The number of eggs was limited so that each culture contained 40 to 60 larvae when the eggs hatched. The eggs were collected from Riverside-Canton-S hybrid females that had been mated to Oregon-R males. Larvae of the 3 *D. melanogaster* strains and those derived by means of the three-way cross between the strains have been used in other nutrition studies, (5–9). Cultures were maintained at 23.8 ± 1.1° and 45% relative humidity with a 10-hour day and 14-hour night.

The growth requirement for an L-amino acid and the utilization of its D-isomer were tested by a five-part experiment in which the media were prepared and autoclaved at the same time and the larvae for all cultures were derived from eggs collected over the same period of time. In one part of the series larvae were fed a

Received for publication April 20, 1966.
[1] This investigation was supported by National Science Foundation Grants GB-2239 and GB-4838.
[2] Rudkin, G. T., and J. Schultz 1947 Evolution of nutritional requirements in animals: amino-acids essential for *Drosophila melanogaster*. Anat. Rec., *99*: 613 (abstract).

TABLE 1

Composition of the standard test medium

	mg
Amino acids	
L-Arginine·HCl	80
L-Cystine	30
L-Glutamic acid	840
Glycine	40
L-Histidine·HCl	100
L-Isoleucine	300
L-Leucine	200
L-Lysine·HCl	190
L-Methionine	80
L-Phenylalanine	130
L-Threonine	200
L-Tryptophan	50
L-Tyrosine	80
L-Valine	280
Other components	
Agar	1500
Sucrose	1000
Yeast ribonucleic acid	100
Cholesterol	30
Thiamine·HCl	0.2
Nicotinic acid	1.2
Riboflavin-5′-phosphate, Na	1.0
Calcium pantothenate	1.6
Pyridoxine·HCl	0.25
Biotin	0.03
Folic acid	1.0
Choline chloride	8.0
$FeSO_4$	1.0
$CaCl_2$	1.29
$MgSO_4 \cdot 7H_2O$	24.6
$MnSO_4 \cdot H_2O$	1.29
$NaHCO_3$	100
KH_2PO_4	183
Na_2HPO_4	189
	ml
Water to	100

[1] The vitamins, yeast nucleic acid, cholesterol, choline chloride, agar, sucrose, and all L- and D-amino acids except D-cystine, D-isoleucine, D-lysine, and D-lysine were obtained from Nutritional Biochemicals Corporation, Cleveland. The D-forms of cystine, isoleucine, lysine, and threonine were purchased from Sigma Chemical Company, St. Louis. The salts were provided by J. T. Baker Chemical Company, Phillipsburg, New Jersey.

diet lacking the amino acid; larvae in a second part were fed an optimal level of the L-amino acid; and a diet containing an equivalent amount of the D-form of the amino acid was fed to larvae in a third part. The 2 remaining parts of the series were designed to determine whether the D-counterpart can spare the dietary requirement for the L-amino acid. In part four, a suboptimal level of the L-isomer was fed; depending upon the amino acid, the amount of L-isomer fed was 10 to 20% of the optimal quantity. In the remaining part of the experiment, a suboptimal amount of the L-amino acid was fed, but it was supplemented with a quantity of the D-form sufficient to raise the total amount of the amino acid to the same level as in the optimal diet (part 2). The diets fed to larvae in parts one and four were supplemented with L-glutamic acid to make all diets isonitrogenous. Optimal levels of the L-amino acids were the quantities in the standard diet. This amino acid mixture had been compared with several others and was found to be the most adequate of the mixtures for *D. melanogaster* larval growth (7).

Larval growth was assessed by several criteria: larval dry weight, survival of larvae to pupation and to eclosion, and the duration of the larval growth period. For dry-weight determinations, larvae from 5 cultures were heat-killed after a 10-day growth period, dried at 100° overnight, and weighed. To minimize the weighing error, the larvae from each culture were dried and weighed together. The number of larvae included in each dry-weight sample ranged from 200 to 300.

Survival of larvae to pupation was determined by counting the number of larvae to hatch from eggs in each culture and then scoring daily the numbers of larvae to pupate. The number of larvae to become adults was subsequently determined for each culture. The duration of the larval growth period was the time in days that elapsed between the inoculation of cultures with eggs and the time of pupation. Each culture was scored every day and the number of new pupae recorded. From these data the mean length of the growth period for larvae fed the experimental diet was calculated. Each determination of larval survival and growth period length was based upon the observation of at least 400 larvae.

RESULTS

Readily utilized D-amino acids. Three of the D-amino acids replaced their L-counterparts to some extent in the diets of *D. melanogaster* larvae (table 2), D-phenylalanine being the most effective. Larvae fed D-phenylalanine survived to pupation and to eclosion almost as well and grew at a rate slightly less rapid than larvae fed an equivalent amount of L-phenylalanine.

TABLE 2

D-Amino acids that are readily utilized for growth by D. melanogaster larvae

Supplement	Larvae to pupate	Larvae to become adults	Growth period [1]	Dry wt at 10 days [2]
mg/100 ml	*%*	*%*	*days*	*mg × 100*
	Phenylalanine-deficient medium			
None	0	0		
130 mg L-Phenylalanine	93.0	86.9	12.9 ± 1.0	24.4
130 mg D-Phenylalanine	93.0	83.2	13.0 ± 1.0	21.5
15 mg L-Phenylalanine	69.3	66.5	19.4 ± 1.5	4.3
15 mg L-Phenylalanine and 115 mg D-phenylalanine	88.7	77.5	12.7 ± 1.1	23.6
	Methionine-deficient medium			
None	0	0		
80 mg L-Methionine	91.4	79.3	12.2 ± 1.2	21.3
80 mg D-Methionine	71.8	64.6	12.1 ± 1.8	17.0
10 mg L-Methionine	64.3	58.5	18.6 ± 2.5	8.4
10 mg L-Methionine and 70 mg D-methionine	73.0	67.9	12.5 ± 1.8	23.8
	Histidine-deficient medium			
None	0	0		
100 mg L-Histidine·HCl	93.2	82.4	12.0 ± 1.1	23.6
100 mg D-Histidine·HCl	9.7	2.6	28.9 ± 3.3	2.7
15 mg L-Histidine·HCl	84.1	72.2	17.1 ± 1.9	9.6
15 mg L-Histidine·HCl and 85 mg D-histidine·HCl	87.5	74.8	12.6 ± 1.1	22.2

[1] Figures represent mean ± SD.
[2] Each figure is the mean of the larvae from 5 cultures.

The D-form of methionine was utilized more than 70% as effectively as its L-isomer, this being true for all the growth characteristics measured. The growth period was as short for D-methionine-fed larvae as for L-methionine-fed larvae, on the average, but there was more variation between larvae. At 10 days, D-methionine-fed larvae were smaller than those fed L-methionine and fewer survived to pupation and to eclosion.

D-Histidine replaced L-histidine in the diet of *D. melanogaster* larvae to a limited extent. Although larvae fed only D-histidine grew very slowly, a small percentage survived to eclosion. Adults raised with a D-histidine diet were successfully mated. D-Histidine exerted a marked sparing effect on the growth requirement for L-histidine when the D- and L-forms were fed in the same diet; D-histidine increased the weights of 10-day-old larvae and shortened the larval growth period.

Moderately utilized D-amino acids. D-Arginine, D-lysine and D-valine stimulated the growth of *D. melanogaster* larvae when fed with suboptimal quantities of their L-form but failed to promote measurable growth when fed as the sole dietary source of the amino acid (table 3).

D-Arginine exerted a marked sparing effect on the dietary requirement for its L-isomer. When D-arginine was fed with a growth-limiting quantity of L-arginine, the frequencies of larvae to pupate and to become adults were several times greater than when larvae were fed only a limiting amount of L-arginine. Also, the observed weights of 10-day-old larvae and the lengths of the larval growth periods further evidenced the sparing effect of D-arginine for the L-arginine growth requirement.

D-Lysine spared the growth requirement for its L-isomer. When both D- and L-lysine were fed, nearly twice as many larvae survived to pupation and to eclosion when fed a suboptimal level of L-lysine. Furthermore, 10-day-old larvae fed D- and L-lysine had weights more than twice the magnitude of larvae fed L-lysine. The larval growth period was also much shorter when the D-form was present in the diet with L-lysine.

TABLE 3

D-Amino acids that spare the growth requirement of D. melanogaster larvae for their L-isomer

Supplement	Larvae to pupate	Larvae to become adults	Growth period [1]	Dry wt at 10 days [2]
mg/100 ml	%	%	*days*	*mg × 100*
	Arginine-deficient medium			
None	0	0		
80 mg L-Arginine·HCl	87.8	83.4	12.4 ± 1.1	22.6
80 mg D-Arginine·HCl	0	0		
16 mg L-Arginine·HCl	6.5	2.0	29.9 ± 2.2	1.7
16 mg L-Arginine·HCl and 64 mg D-arginine·HCl	40.9	21.6	24.8 ± 1.9	4.2
	Lysine-deficient medium			
None	0	0		
190 mg L-Lysine·HCl	87.1	82.9	11.9 ± 1.1	23.0
190 mg D-Lysine·HCl	0	0		
20 mg L-Lysine·HCl	44.8	35.5	28.6 ± 2.0	1.7
20 mg L-Lysine·HCl and 170 mg D-lysine·HCl	73.5	63.8	20.8 ± 2.0	4.0
	Valine-deficient medium			
None	0	0		
280 mg L-Valine	84.8	81.3	12.1 ± 1.1	22.5
280 mg D-Valine	0	0		
30 mg L-Valine	82.8	72.4	18.2 ± 1.8	11.2
30 mg L-Valine and 250 mg D-valine	79.7	75.8	15.5 ± 1.7	15.6

[1] Figures represent mean ± SD.
[2] Each figure is the mean of the larvae from 5 cultures.

Under the test conditions D-valine exerted only a slight sparing influence on the L-valine growth requirement. Viabilities of larvae fed either a suboptimal level of L-valine or the same level of L-valine supplemented with D-valine were essentially the same. However, the mean weight of 10-day-old larvae and the growth period were significantly [3] altered by the presence of D-valine in the diet with L-valine ($P < 0.01$ for both values).

Unavailable D-amino acids. D-Isoleucine can not replace L-isoleucine in the diet and it is doubtful whether it spares the growth requirement for L-isoleucine (table 4). When considered individually the differences between larval survival frequencies, larval dry weights, and growth periods are questionable due to a great deal of overlapping of individual determinations. Nevertheless, when diets containing 2 suboptimal quantities of L-isoleucine and L-isoleucine plus D-isoleucine other than that shown in table 4 were tested, small but consistent differences were noted between the growth performances of larvae fed the test diets. The D-preparation of isoleucine was a mixture of D-isoleucine and D-alloisoleucine; thus neither diastereomer effectively spared the L-isoleucine growth requirement.

D-Threonine had little effect upon growth. Larvae maintained on a suboptimal level of L-threonine grew as well as those fed the suboptimal amount of L-threonine supplemented with D-threonine (table 4).

Growth-inhibiting D-amino acids. D-Leucine and D-tryptophan inhibited the growth of *D. melanogaster* larvae at the levels fed in the test diets (table 5). D-Leucine exerted the greater inhibitory influence. When larvae were fed D-leucine with a suboptimal quantity of L-leucine, less than half as many larvae became adults as when fed only the limiting amount of L-leucine. The period of larval growth was lengthened by 6.4 days and the 10-day-old larva dry weight was reduced by 80% when D-leucine was fed with L-leucine.

[3] Student's *t* test.

TABLE 4

D-Amino acids that do not effectively spare the growth requirement of D. melanogaster larvae for their L-isomer

Supplement	Larvae to pupate	Larvae to become adults	Growth period [1]	Dry wt at 10 days [2]
mg/100 ml	%	%	*days*	*mg × 100*
	Isoleucine-deficient diet			
None	0	0		
300 mg L-Isoleucine	92.4	84.9	11.6 ± 1.1	23.8
300 mg D-Isoleucine	0	0		
30 mg L-Isoleucine	48.1	35.3	19.8 ± 3.7	4.0
30 mg L-Isoleucine and 270 mg D-isoleucine	56.5	43.8	19.2 ± 3.1	5.1
	Threonine-deficient diet			
None	0	0		
200 mg L-Threonine	89.1	77.4	12.5 ± 1.3	26.1
200 mg D-Threonine	0	0		
25 mg L-Threonine	60.3	52.7	18.4 ± 1.8	5.9
25 mg L-Threonine and 175 mg D-threonine	55.8	49.3	16.4 ± 2.3	6.3

[1] Figures represent mean ± SD.
[2] Each figure is the mean of the larvae from 5 cultures.

TABLE 5

D-Amino acids that inhibit the growth of D. melanogaster larvae

Supplement	Larvae to pupate	Larvae to become adults	Growth period [1]	Dry wt at 10 days [2]
mg/100 ml	%	%	*days*	*mg × 100*
	Leucine-deficient medium			
None	0	0		
200 mg L-Leucine	87.3	84.3	12.1 ± 1.1	23.3
200 mg D-Leucine	0	0		
40 mg L-Leucine	81.7	74.5	16.0 ± 1.6	11.1
40 mg L-Leucine and 160 mg D-leucine	36.6	31.7	22.4 ± 5.3	2.1
	Tryptophan-deficient medium			
None	0	0		
50 mg L-Tryptophan	86.6	82.3	11.8 ± 1.0	24.6
50 mg D-Tryptophan	0	0		
5 mg L-Tryptophan	83.9	69.3	20.4 ± 2.4	6.9
5 mg L-Tryptophan and 45 mg D-tryptophan	52.6	41.7	23.5 ± 2.5	2.5

[1] Figures represent mean ± SD.
[2] Each figure is the mean of the larvae from 5 cultures.

The antagonism of D-tryptophan to larval growth affected the viability, dry weight, and growth rate of larvae. D-Tryptophan reduced by one-third the frequency of larvae to become adults, whereas the larval growth period was extended by 3.1 days and the dry weight of 10-day-old larvae was reduced to two-thirds that of larvae fed only the limiting quantity of L-tryptophan.

Cystine, glycine, and tyrosine. Whether D-cystine can be utilized for growth by *D. melanogaster* larvae in place of L-cystine can only be determined with difficulty because L-cystine is not required for larval growth (table 6). Addition of L-cystine to the diet improved larval survival to the adult stage significantly [4] ($P < 0.05$) but did not shorten the growth period; in fact, the growth period was extended slightly. That D-cystine was utilized for growth was evidenced by the

[4] Student's *t* test.

improved viabilities of larvae fed either D-cystine or D-cystine plus a suboptimal amount of L-cystine as compared with those fed no cystine or a suboptimal amount of L-cystine. However, only when the latter diet was fed was the improvement in larval survival to the adult stage significant [5] ($P < 0.05$). In the cystine experiment there were no significant differences between the growth periods or the dry weights of larvae fed the different test diets.

Addition of glycine to the diet improved larval growth (table 7). The frequencies of larvae to pupate and to become adults were higher; the larvae were one-third larger at 10 days; and the growth period was shortened by 2 days when glycine was fed.

L-Tyrosine had little effect on larval viability or the length of the growth period (table 7). Larval dry weight, however, was increased when L-tyrosine was fed.

DISCUSSION

D-Methionine and D-phenylalanine are effective replacements for their L-enantiomorphs in the diets of *D. melanogaster*, *T. confusum* (2), and *A. mellificia* (3). Drosophila, like Apis, is able to utilize D-histidine to a moderate degree in place of L-histidine.

Fraenkel and Printy (2) obtained inconsistent results when testing D-lysine in the diet of Tribolium. In one experiment D-lysine appeared to be effective but in a second experiment it was an ineffective substitute for its L-counterpart. D-Lysine spares the growth requirement for L-lysine when fed to *D. melanogaster* larvae but it cannot promote the growth of larvae appreciably when fed as the only source of dietary lysine. D-Lysine is an ineffective nutrient for Apis (3).

In the present experimentation D-valine and D-arginine spared the dietary require-

[5] Student's t test.

TABLE 6

Utilization of cystine for growth by D. melanogaster larvae

Supplement	Larvae to pupate	Larvae to become adults	Growth period [1]	Dry wt at 10 days [2]
mg/100 ml	%	%	*days*	*mg × 100*
		Cystine-deficient medium		
None	78.7	68.5	11.6 ± 1.4	23.7
30 mg L-Cystine	88.5	83.2	12.3 ± 1.3	24.3
30 mg D-Cystine	81.6	75.2	11.9 ± 1.2	23.4
5 mg L-Cystine	75.2	69.9	11.8 ± 1.5	25.2
5 mg L-Cystine and 25 mg D-cystine	84.7	81.0	12.5 ± 1.3	22.8

[1] Figures represent mean ± SD.
[2] Each figure is the mean of the larvae from 5 cultures.

TABLE 7

Utilization of glycine and tyrosine for growth by D. melanogaster larvae

Supplement	Larvae to pupate	Larvae to become adults	Growth period [1]	Dry wt at 10 days [2]
mg/100 ml	%	%	*days*	*mg × 100*
		Glycine-deficient medium		
None	82.7	61.2	14.3 ± 1.8	16.7
40 mg Glycine	88.5	83.2	12.3 ± 1.3	24.3
		Tyrosine-deficient medium		
None	92.5	82.4	14.0 ± 1.8	17.3
80 mg L-Tyrosine	92.0	84.1	13.5 ± 1.2	21.7

[1] Figures represent mean ± SD.
[2] Each figure is the mean of the larvae from 5 cultures.

ments for their L-isomers when fed to Drosophila larvae. In addition, a very slight sparing effect for the requirement for L-isoleucine may be attributed to D-isoleucine. Thus, the D-forms of lysine, valine, arginine, and possibly isoleucine can be utilized for growth by Drosophila larvae but not to the extent that the larvae are able to utilize D-phenylalanine, D-methionine, or D-histidine. D-Tryptophan and D-leucine inhibit the growth of *D. melanogaster* larvae, whereas D-threonine has no apparent growth influence. Both Apis and Tribolium are incapable of utilizing the D-forms of valine, arginine, isoleucine, leucine, threonine, or tryptophan for growth in place of their L-isomers (2,3).

Comparisons (table 8) between the results of the present experimentation and studies of the protozoan *Tetrahymena pyriformis* (10)[6] and of the rat (11) yield interesting but only approximate relationships due to the differences in methods and growth criteria used: 1) D-Methionine is the only D-amino acid that can be readily utilized for growth by all 3 organisms, whereas D-arginine can be used for growth to some degree by each. Several differences are notable. 2) The rat utilizes D-tryptophan nearly as well as its L-counterpart for growth, whereas D-tryptophan is an ineffective growth-promoting substance for Tetrahymena and Drosophila. 3) D-Lysine is utilized to some degree by Tetrahymena and Drosophila but not by the rat. 4) D-Phenylalanine is readily utilized by the rat and Drosophila but is not utilized by Tetrahymena. 5) D-Leucine is used for growth to a moderate extent by the rat but has no growth-promoting activity for Tetrahymena or Drosophila. 6) D-Histidine and D-valine are utilzied to different extents for growth by the rat and Drosophila but are ineffective for Tetrahymena.

TABLE 8

Utilization of D-amino acids for growth of T. pyriformis, D. melanogaster, and the rat [1]

D-Amino acid	Tetrahymena [2]	Drosophila	Rat [3]
Arginine	++	+	+
Histidine	−	++	+
Isoleucine	−	±	−
Leucine	−	−	+
Lysine	++	+	−
Methionine	++	++	++
Phenylalanine	−	++	++
Threonine	−	−	−
Tryptophan	−	−	++
Valine	−	+	±

[1] ++ indicates that the D-amino acid is readily utilized for growth, + indicates that the D-amino acid is moderately available for growth, ± shows that utilization of the D-amino acid for growth is meager, − shows that the D-amino acid is not used for growth.

[2] Taken from the observations of Kidder and Dewey (10) and Elliott et al. (see footnote 6 in text).

[3] Taken from a review by Berg (11).

These comparisons raise some questions of evolutionary interest. For example, the ability to utilize D-lysine for growth appears to have been lost during the evolution of the mammals. That this is the case is suggested by observations on the rat (11), the mouse (12), and man (13). None of these mammals are able to use D-lysine in place of its L-form.

Also, the capacity to utilize D-tryptophan efficiently appears to be an almost unique trait of the rat. The mouse (12) and chick (14) utilize D-tryptophan only to a slight degree; Drosophilia, Apis, Tribolium, and Tetrahymena can not utilize D-tryptophan for growth.

Tetrahymena does not share the ability to utilize D-phenylalanine with the insects or vertebrates. This suggests that the insects and vertebrates might possibly have inherited the ability to utilize D-phenylalanine from a common ancestor.

The rat and Drosophila share the ability to utilize D-histidine for growth, a quality not possessed by the mouse (12) but exhibited by Apis. Thus, the capacity to utilize D-histidine is established in certain insects but may be rare in mammals.

That D-valine is utilized by the rat and Drosophila but not by Tetrahymena is of questionable significance. Drosophila stands alone among the insects with its ability to utilize D-valine for growth and the rat holds an equally unique position among the vertebrates that have been tested. The ability of the rat to utilize D-valine for growth is meager (15), whereas the mouse (16) and man (17) are unable to use D-valine for growth. The growth-promoting activity of D-valine for Drosophila, as shown in the present experimentation, is limited.

[6] Elliott, A. M., J. F. Hogg and C. Wu 1952 Utilization of D-amino acids by *Tetrahymena geleii*. Federation Proc., *11*: 207 (abstract).

The growth-promoting activities of some of the D-amino acids, such as D-leucine, for the rat have been difficult to detect and to quantify because of the growth antagonisms between certain of the D-forms. DL-Norleucine, for example, inhibits the growth response of the rat to D-leucine, whereas D-leucine reduces the growth response to D-valine (15). Presence in the diet of any of several D-amino acids inhibits the growth-stimulating activity of D-histidine, and the presence *en masse* of several of the poorly invertible D-forms antagonizes the growth promotion of the rat by the D-isomers of methionine, phenylalanine, and tryptophan (18, 19). Diets used in the cited studies have in some cases included portions of one or more D-amino acids, usually as DL-mixtures, other than the one being tested and this must be regarded as a limitation and possible source of error in the above comparisons.

Protozoans, *T. pyriformis* in particular, are being used more and more in nutritional studies (20). The amino acid requirements of Tetrahymena (21) are similar to those of the higher invertebrates and vertebrates but it should be kept in mind that there are differences between the amino acid metabolisms of these groups of organisms, as shown by the comparisons of the previous paragraphs.

Drosophila appears capable of utilizing D-cystine for growth in place of its L-form. It is difficult to assess this quality because near-optimal development is possible when cystine is omitted from the diet. However, addition of either L- or D-cystine improves larval viability, D-cystine being slightly less effective than L-cystine. Experiments of du Vigneaud et al. (22) showed that D-cystine cannot replace L-cystine for the purposes of growth in the rat diet.

The means by which D-amino acids promote growth are unknown although enzymes capable of converting D-amino acids to L-amino acids have been demonstrated in rat tissue (11, 23). The stimulation of growth by D-amino acids when fed with suboptimal levels of their L-forms, such as in the present study, is possibly due to the utilization of the D-amino acids as sources of nonessential nitrogen. However, in the current study the D-amino acid in question would have to be a better source of nonessential nitrogen than L-glutamic acid, which was used to make all diets isonitrogenous and which was included in all diets in large quantities. Therefore, since the L-form of the amino acid was the limiting factor in the test diets and not the quantity of nonessential amino acid, it is more probable that the growth-stimulating capacities of the D-amino acids in the current experiments were due to the conversion of the D-amino acids to their L-isomers.

Each of the 3 nonessential amino acids examined in the present investigation improved the growth of *D. melanogaster* larvae when added to the diet. L-Cystine, although necessary for optimal larval viability, is not essential for an optimal rate of growth by *D. melanogaster* larvae. Cystine apparently does not stimulate larval growth by sparing the methionine requirement. The pathway from cystine to methionine does not appear to operate in Drosophila since neither homocysteine nor homocystine can replace methionine to a measurable extent in larval diets (5). Furthermore, larval growth is not improved by the addition of methionine in quantities exceeding that of the standard diet.

L-Tyrosine is needed by *D. melanogaster* larvae to grow at the optimal rate. Whether tyrosine spares the growth requirement for phenylalanine in Drosophila has not been examined. Golberg and de Meillon (24) found *Aëdes aegypti* capable of growth with a diet lacking either phenylalanine or tyrosine but not both of these amino acids, indicating that this insect is capable of the interconversion of phenylalanine and tyrosine.

Glycine is necessary for both optimal larval viability and the optimal rate of larval growth of *D. melanogaster*. Glycine has growth-promoting activity for many of the Diptera. Omission of glycine from the diets of *A. aegypti* (24), *Agria affinis* (25), and *Calliphora erythrocephala* (26) retards growth. *Phormia regina* is the only Dipteran studied that does not require glycine for optimal growth (27, 28).

ACKNOWLEDGMENTS

Grateful appreciation is extended to Julia Ricker and Jerrie Tietge for their technical assistance.

LITERATURE CITED

1. Gilmour, D. 1961 The Biochemistry of Insects. Academic Press, New York.
2. Fraenkel, G., and G. E. Printy 1954 The amino acid requirements of the confused flour beetle, *Tribolium confusum*, Duval. Biol. Bull., *106:* 149.
3. DeGroot, A. P. 1953 Protein and amino-acid requirements of the honey bee (*Apis mellificia L.*). Physiol. Comp. Oecol., *3:* 197.
4. Hinton, T., D. T. Noyes and J. Ellis 1951 Amino acids and growth factors in a chemically defined medium for *Drosophila*. Physiol. Zool., *24:* 335.
5. Geer, B. W., and G. F. Vovis 1965 The effects of choline and related compounds on the growth and development of *Drosophila melanogaster*. J. Exp. Zool., *158:* 223.
6. Geer, B. W., and J. G. Ricker 1965 The growth effects of carnitine, deoxycarnitine, and sulfocholine for Drosophila, Neurospora, and Saccharomyces. Growth, *29:* 405.
7. Geer, B. W. 1966 Comparison of some amino acid mixtures and proteins for the diet of *Drosophila melanogaster*. Trans. Illinois Acad. Sci., *59:* 3.
8. Geer, B. W. 1963 A ribonucleic acid-protein relationship in Drosophila nutrition. J. Exp. Zool., *154:* 353.
9. Geer, B. W. 1964 Inheritance of the dietary ribonucleic acid requirement of *Drosophila melanogaster*. Genetics, *49:* 787.
10. Kidder, G. W., and V. C. Dewey 1951 The biochemistry of ciliates in pure culture. In: Biochemistry and Physiology of Protozoa, vol. 1, ed., A. Lwoff. Academic Press, New York, pp. 324–400.
11. Berg, C. P. 1959 Utilization of D-amino acids. In: Protein and Amino Acid Nutrition, ed., A. A. Albanese. Academic Press, New York, pp. 57–96.
12. Celander, D. R., and C. P. Berg 1953 The availability of D-histidine, related imidazoles, and D-tryptophan in the mouse. J. Biol. Chem., *202:* 339.
13. Rose, W. C., A. Borman, M. J. Coon and G. F. Lambert 1955 The amino acid requirements of man. X. The lysine requirement. J. Biol. Chem., *214:* 579.
14. Morrison, W. D., T. S. Hamilton and H. M. Scott 1956 Utilization of D-tryptophan by the chick. J. Nutr., *60:* 47.
15. Gerulat, B. F., and C. P. Berg 1960 Growth promotion by D-valine and D-leucine. Arch. Biochem. Biophys., *88:* 273.
16. Bauer, C. D., and C. P. Berg 1943 The amino acids required for growth in mice and the availability of their optical isomers. J. Nutr., *26:* 51.
17. Rose, W. C., R. L. Wixom, H. B. Lockhart and G. F. Lambert 1955 The amino acid requirements of man. XV. The valine requirement: summary and final observations. J. Biol. Chem., *217:* 987.
18. Kamath, S. H., and C. P. Berg 1964 Antagonism of poorly invertible D-amino acids toward growth promotion by readily invertible D-amino acids. J. Nutr., *82:* 237.
19. Kamath, S. H., and C. P. Berg 1964 Antagonism of the D-forms of the essential amino acids toward the promotion of growth by D-histidine. J. Nutr., *82:* 243.
20. Hutner, S. H., and L. Provasoli 1964 Comparative physiology: nutrition. Ann. Rev. Physiol., *27:* 19.
21. Holz, G. G. 1964 Nutrition and metabolism of ciliates. In: Biochemistry and Physiology of Protozoa, vol. 3, ed., S. H. Hutner. Academic Press, New York, pp. 199–243.
22. du Vigneaud, V., R. Dorfmann, and H. S. Loring 1932 A comparison of the growth-promoting properties of *d-* and *l*-cystine. J. Biol. Chem., *98:* 577.
23. Greenstein, J. P., and M. Winitz 1961 Chemistry of the Amino Acids, vol. 1, John Wiley and Sons, New York, p. 320.
24. Goldberg, L., and B. de Meillon 1948 The nutrition of the larva of *Aëdes aegypti* Linnaeus. Biochem. J., *43:* 379.
25. House, H. L. 1954 Nutritional studies with *Pseudosarcophaga affinis* (Fall.), a dipterous parasite of the spruce budworm, *Choristoneura fumiferana* (Clem.). I. A chemically defined medium and aseptic-culture technique. Can. J. Zool., *32:* 331.
26. Sedee, D. J. W. 1954 Qualitative amino acid requirements of larvae of *Calliphora erythrocephala* (Meigen). Acta Physiol. Pharmacol. Neerl., *3:* 262.
27. McGinnis, A. J., R. W. Newburgh, and V. H. Cheldelin 1956 Nutritional studies on the blowfly, *Phormia regina* (Meig.). J. Nutr., *58:* 309.
28. Hodgson, E., V. H. Cheldelin and R. W. Newburgh 1956 Substitution of choline by related compounds and further studies on amino acid requirements in nutrition of *Phormia regina* (Meig.). Can. J. Zool., *34:* 389.

Mechanism of Suckling Rat Hypercholesterolemia: Dietary and Drug Studies [1,2,3]

R. A. HARRIS,[4] J. E. MacNINTCH [5] AND F. W. QUACKENBUSH
Department of Biochemistry, Purdue University, Lafayette, Indiana

ABSTRACT The objective of this study was to determine the cause(s) of the hypercholesterolemia which is known to occur in suckling rats. A semi-purified, milk-simulating diet fed to 21-day-old rats upon weaning was found to maintain the hypercholesterolemic condition. With this diet, the mechanism of suckling rat hypercholesterolemia was investigated by testing the effect of dietary levels of lactose, bulk, polyunsaturated fatty acids, cholesterol and fat. The condition appears to be dependent upon the high fat content of rat milk but independent of the dietary bulk, polyunsaturates, and carbohydrate source. Isocaloric diets were used to establish the dependency upon the fat content of diet. Feeding experiments suggest that dietary cholesterol is not necessary for maintaining hypercholesterolemia for short periods of time, but isotopic balance studies show that the dam contributes a portion of the cholesterol found in the plasma of suckling rats. Ethyl linoleate, α-*p*-chlorophenoxyisobutyrate (CPIB), benzmalecene, and β-diethylaminoethyl diphenylpropyl acetate hydrochloride were found not to be effective as hypocholesterolemic agents in the suckling rat hypercholesterolemia. L-Thyroxine was active in lowering plasma cholesterol in this system, but it increased liver free and total cholesterol. Liver wet weight, dry weight, and protein content increased in response to CPIB.

Serum cholesterol levels have been observed to be much higher in suckling rats than in weaned rats (1, 2). Hypercholesterolemia also has been observed in suckling rabbits (3) and shown to be dependent upon the high triglyceride and cholesterol content of rabbit milk (4). Carroll (2) observed rat milk to be relatively low in cholesterol and also noted hepatic biosynthesis of sterol to be suppressed in the suckling rat. Many drugs which normally function as hypocholesterolemic agents fail to produce a response in the suckling rat hypercholesterolemia (1).

A better understanding of control mechanisms for plasma cholesterol in the suckling animal may help to reveal the reasons for suppressed hepatic cholesterol biosynthesis and lack of response to certain hypocholesterolemic agents. The experiments presented here were designed to test the role of dietary factors in suckling rat hypercholesterolemia. Specifically, the contributions of lactose, bulk, polyunsaturated fatty acids, cholesterol and fat were investigated. The response to various hypocholesterolemic agents is also reported.

EXPERIMENTAL

Materials. Ethyl linoleate of 97+% purity was prepared from safflower oil [6] as described previously (5). Test substances obtained commercially included the following: ethyl palmitate,[7] 99+% purity; cholesterol-4-^{14}C,[8] 4.38 mCi/mmole; and L-thyroxine, cholesterol and sodium glycocholate.[9] The other test substances used were: ethyl α-*p*-chlorophenoxyisobutyrate (CPIB ethyl ester;[10] β-diethylaminoethyl diphenylpropyl acetate hydrochloride (SK&F 525A);[11] sodium [N-(1-methyl-2,3-di-*p*-chlorophenylpropyl) maleamate] benz-

Received for publication March 24, 1966.

[1] Journal paper no. 2831 Purdue University Agricultural Experiment Station, Lafayette, Indiana.

[2] This study was supported in part by Public Health Service Grants no. HE 08424 and T1 GM 1195.

[3] Presented in part before the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1965 (Federation Proc., 24: 1078, 1081, 1965, abstracts).

[4] Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

[5] Present address: Biochemistry Department, Bowman Gray Medical School, Winston-Salem, North Carolina.

[6] Contributed by Pacific Vegetable Oil Corporation, Richmond, California.

[7] Eastman Organic Chemicals, Rochester, New York.

[8] Tracerlab, Waltham, Massachusetts.

[9] Nutritional Biochemicals Corporation, Cleveland.

[10] Ayerst Laboratories, Inc., New York.

[11] Smith, Kline and French Laboratories, Philadelphia.

malecene);[12] hydrogenated coconut oil;[13] and sardine oil.[14].

Diets. The semi-purified diets are shown in table 1. Diet M-47 (47% fat) simulated the dry-weight composition of rat milk as described by Luckey et al. (6). Diets M-24 and M-1 24% and 1% fat, respectively, were isocaloric with diet M-47 since minerals and vitamins were added to each in constant ratio to carbohydrate and fat calories as described previously (5). Lactating females consumed ad libitum the colony diet which consisted chiefly of ground yellow corn, wheat middlings and meat meal or diet G when a well-defined, semi-purified diet was desired. Diet F was similar to diet G but 1% cholesterol and 0.5% sodium glycocholate were added to produce a hypercholesterolemia.

Dietary studies. Suckling female rats, 19 to 21 days old and weighing 39 to 45 g, were obtained from our colony of Wistar-strain rats. Groups of 6 weanlings were fed various diets ad libitum and killed after 2 or 5 days. In certain studies the diet of the lactating females was changed at parturition from the colony diet and diet G with either hydrogenated coconut oil or ethyl linoleate as the fat source. The pups of these litters were killed at 21 days of age.

TABLE 1

Composition of diets

Diets [1,2]	M-47	M-24	M-1	F	G
	%	%	%	%	%
Fat	47	24	1	2	2
Casein [3]	32	27	21	15	15
Glucose monohydrate [4]	—	—	—	76	77
Lactose	17	46	75	—	—
Cellulose [5]	—	—	—	1.7	1.7
Cholesterol	—	—	—	1.0	—
Sodium glycocholate	—	—	—	0.5	—

[1] Minerals and vitamins were added to each diet in constant ratio to carbohydrate and fat calories (5).
[2] Diets (see text for complete description:
M-47, 47% fat, simulating dry-weight composition of rat milk
M-24, 24% fat, isocaloric with diet M-47
M-1, 1% fat, isocaloric with diet M-47
F, semi-purified + 1% cholesterol and 0.5% sodium glycocholate
G, semi-purified
Colony, ground yellow corn, wheat middlings and meat meal.
[3] Casein was extracted continuously for 72 hours with boiling ether to remove fat.
[4] Cerelose, Corn Products Company, Argo, Illinois.
[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.

To compare the effects of sardine oil, ethyl linoleate and hydrogenated coconut oil on hypercholesterolemia, weanling rats were fed these fats in diet F for 8 weeks.

Drug studies. Since initial studies indicated that the degree of hypercholesterolemia in suckling rats from our colony varied greatly between litters but not between littermates, the method described by Bizzi et al. (1) for the assay of hypocholesterolemic agents in suckling rats was modified. Litters of 10 rats, 7 days old, were marked to divide them into 2 equal groups with respect to body weight and sex; all were left with their dams receiving the colony diet. Daily, for 7 days, one group of 5 was force-fed, by tube, the compound being tested for hypocholesterolemic activity, and the control group of 5 was similarly fed an equal volume of saline. All were killed and analyzed at 13 days of age.

Hypercholesterolemic adult rats (250–300 g), obtained by feeding weanling rats diet F for 6 weeks, were fed various compounds incorporated in the diet for 7 days.

Polyunsaturated fatty acids of rat milk. Methyl esters were prepared with diazomethane (7) from the saponifiable fraction of a chloroform-methanol extract (8) of the milk curd obtained from the stomachs of 14-day-old suckling rats of several litters from the colony diet. The fatty acids were identified by comparison of retention times with those of known standards on 2 columns (152-cm and 304-cm [5-ft and 10-ft] diethylene glycol succinate on firebrick; Barber-Coleman Model 10 gas chromatograph).

Cholesterol-4-^{14}C pellet study. The procedure for producing an isotopic steady state in the rat was essentially that described by Wilson (9). A gelatin pellet of 100 mg of cholesterol-4-^{14}C (50,000 cpm/mg) was implanted dorsally under the skin of a mature female rat; 4 weeks later the female was mated. One of its pups was killed at parturition, before nursing. Three remaining pups were killed at 15 days of age, after 30 minutes of nursing subsequent to a 1-hour fast, and the milk curds from their stomachs were pooled for analysis. The specific activities of

[12] Merck, Sharp and Dohme, West Point, Pennsylvania.
[13] Procter and Gamble Company, Cincinnati.
[14] Maine Sardine Industry, Bangor, Maine.

cholesterol from the whole blood, plasma and milk of the dam and from the pooled plasma of the suckling rats were determined. The percentage of cholesterol in the plasma of the pups which originated from the female was estimated from suitable equations.

Analysis. Lipids were extracted from plasma and tissues as described previously (8). Cholesterol was precipitated (after hydrolysis for the total) as the digitonide which was washed as described by Sperry and Webb (10). The digitonides were dissolved in acetic acid for colorimetric determination and in methanol for assay of radioactivity. Aliquots of the methanolic solutions of the digitonides were added to 15 ml of a toluene scintillation fluid (0.1 g/liter 1,4-bis-2-(5-phenyloxazole)-benzene and 4 g/liter 2,5-diphenyloxazole) and assayed for radioactivity with a liquid scintillation counter (Packard). Samples were routinely corrected for quenching with an internal standard of cholesterol-4-^{14}C. Protein was determined by the Folin-Ciocalteu method as modified by Lowry et al. (11).

RESULTS

Although the plasma cholesterol levels of both newly weaned and young adult rats in our inbred Wistar colony commonly range between 50 and 70 mg/100 ml, the level in suckling rats at 13 days was found to be 157 ± 7 mg/100 ml and at 21 days, 138 ± 10 mg/100 ml (mean of 5–7 and SE). No increase at 21 days was observed when the dam was fed a diet containing hydrogenated coconut oil as the only fat source. A decrease in plasma cholesterol level between 13 and 21 days is characteristic of this hypercholesterolemia (2).

The colony diet, consisting principally of ground cereal grains, is well supplied with unsaturated fatty acids. The lipid extract from milk curd, pooled from the stomachs of suckling rats from the colony, contained 6.1% of 18:2 [15] and 0.1% of 20:4 by gas chromatographic analysis.

When suckling rats were weaned at 20 days of age and fed the colony diet, plasma cholesterol fell quickly to near normal values (table 2). Diet G with either ethyl linoleate or hydrogenated coconut oil produced comparable responses at 2 days; however, linoleate produced lower values after 5 days ($P < 0.001$). Fasting produced a linear fall in plasma cholesterol levels over the 5-day period. Diet M-47, which simulated the dry weight composition of rat milk, was found to maintain the hypercholesterolemia at a level (table 2) which is characteristic of 21-day-old suckling rats. When 2 groups of suckling rats were weaned with colony diet for 48 hours and then forced to either (a) resume nursing lactating females or (b) consume diet M-47, both groups returned to a state of hypercholesterolemia after 96 hours (suckling 142 ± 4 [16] mg/100 ml; diet M-47, 119 ± 2 mg/100 ml).

Several alterations in the basic composition of diet M-47 produced no significant effect (table 3). These include cholesterol addition to the diet at the level found in rat milk; replacing a portion of the hydrogenated coconut oil with ethyl linoleate; substitution of sucrose for lactose; and the addition of 20% bulk to the diet in the form of cellulose.[17] However, replac-

[15] Carbon chain length:number of double bonds.
[16] SE of the mean for 6 animals.
[17] Cellu Flour, Chicago Dietetic Supply House, Chicago.

TABLE 2

Effect of colony and purified diets on hypercholesterolemia of the suckling rat

Diet [1,2]	Type of fat	Plasma total cholesterol	
		2 days	5 days
		mg/100 ml	*mg/100 ml*
Colony	—	69 ± 4 [3]	68 ± 3
G	ethyl linoleate	77 ± 3	48 ± 4
G	hydrogenated coconut oil	63 ± 4	73 ± 3
Fasting	—	61 ± 5	34 ± 12
M-47	hydrogenated coconut oil	143 ± 7	130 ± 6

[1] Suckling rats were weaned with the respective diets and killed after 2 or 5 days.
[2] See table 1, footnote 2, for description of diets.
[3] Average ± SE of mean for 6 rats/group.

TABLE 3

Effect of various alterations in diet M-47 upon hypercholesterolemia in the suckling rat

Alteration [1]		Added at expense of	Plasma total cholesterol	
			2 days	5 days
	%		*mg/100 ml*	*mg/100 ml*
None	—	—	143 ± 7 [2]	130 ± 6
Cholesterol	0.06	—	134 ± 4	142 ± 12
Ethyl linoleate	2.0	HCO [3]	131 ± 8	152 ± 10
Sardine oil	24	HCO	187 ± 21	247 ± 29
Sucrose	17	lactose	107 ± 8	125 ± 6
Cellulose [4]	2.0	—	128 ± 8	139 ± 7
Cellulose	20	—	133 ± 9	132 ± 8

[1] Alterations are based upon diet M-47 (see table 1, footnote 2 for description) with hydrogenated coconut oil as the fat source.
[2] Average ± SE for 6 rats/group.
[3] HCO, hydrogenated coconut oil.
[4] Cellu Flour.

ing half of the hydrogenated coconut oil in diet M-47 with sardine oil produced a hypercholesterolemic response ($P < 0.01$ at 5 days, compared with diet M-47). However, the sardine oil and ethyl linoleate used in this study produced lower plasma cholesterol levels than hydrogenated coconut oil (191 ± 19, 109 ± 14 and 324 ± 64 mg/100 ml, respectively) after 8 weeks when fed at 2% (diet F) to weaned rats.

Removing half of the fat from diet M-47 to give diet M-24 did not produce a significant response, but diet M-1 (1% fat as hydrogenated coconut oil) reduced plasma cholesterol to normal (table 4). The addition of bulk to the diet or substitution of sucrose for lactose did not increase the hypocholesterolemic response. Levels were slightly higher with the sucrose diets, when compared with lactose ($P < 0.05$).

Ethyl palmitate (10 mg/day) and ethyl linoleate (10–25 mg/day), force-fed for 7 days, had no effect upon plasma and liver cholesterol levels of suckling rats (table 5). L-Thyroxine was an effective hypercholesterolemic agent, as shown previously by Bizzi et al. (1). However, in the present study, L-thyroxine increased both free and total cholesterol of the liver significantly. None of the other drugs lowered the plasma cholesterol. Benzmalecene significantly increased plasma free cholesterol. CPIB and SK&F 525A increased total lipids. CPIB decreased liver cholesterol levels, but this decrease was coincident with an increase in liver size. In another experiment CPIB significantly ($P < 0.001$) increased the liver wet weight, dry weight and protein content.

When these same agents were assayed with groups of 6 or 7 adult rats that had dietary induced hypercholesterolemia, L-thyroxine and SK&F 525A were very effective in reducing plasma cholesterol levels. CPIB was less effective and benzmalecene was without significant effect. Initial and final plasma cholesterol levels were: L-thyroxine, 342 to 121; SK&F 525A, 272 to 109; CPIB, 291 to 193; benzmalecene, 265 to 225; and no treatment, 373 to 352 mg/100 ml. The compounds were fed for a 7-day period, thyroxine at 0.003% and all others at 0.15% of the diet.

Data from the cholesterol-4-^{14}C pellet experiment showed that 37% of the cholesterol in the plasma of the suckling rats, and 30% of the cholesterol in the pup killed at parturition, had originated in the dam. The equation used for the plasma was:

$$\%\ \text{Plasma cholesterol from dam} = 100 \times \frac{\text{specific activity of suckling rats' plasma cholesterol}}{\text{specific activity of milk cholesterol}}$$

derived from

$$\%\ \text{Plasma cholesterol from dam} = 100 \times \frac{\text{cholesterol (mg) in plasma originating from milk}}{\text{total cholesterol (mg) in suckling rat's plasma}}$$

where

$$\text{Cholesterol (mg) in plasma originating from milk} = \frac{\text{counts/min in suckling rat's plasma cholesterol}}{\text{specific activity of milk cholesterol}}.$$

A similar equation was used to calculate the percentage for the pup killed at parturition. Calculations were based upon the

TABLE 4

Effect of various diets which were isocaloric with diet M-47 upon hypercholesterolemia in the suckling rat

Diet [1]	Alteration [2]		Added at expense of	Total plasma cholesterol at 5 days
		%		*mg/100 ml*
M-47	None	—	—	142 ± 4 [3]
M-24	None	—	—	148 ± 9
M-1	None	—	—	55 ± 5
M-1	Cellulose [4]	20	—	61 ± 5
M-1	Sucrose	75	lactose	76 ± 4
M-1	Sucrose + cellulose	75 20	lactose	83 ± 5

[1] See table 1, footnote 2, for description of diets.
[2] Alterations are based upon Diet M-1 with hydrogenated coconut oil as the fat source.
[3] Average ± SE for 6 rats/group.
[4] Cellu Flour.

TABLE 5

Plasma and liver responses of suckling rats to various test substances

Test substance [1]	Level force-fed	Liver wt / Body wt	Cholesterol found in				Total liver lipid
			Plasma		Liver		
			Total	Free	Total	Free	
	mg/day		*mg/100 ml*		*mg/g*		%
Control	(saline)	0.028	224	60	2.85	2.32	4.08
Ethyl palmitate	10	0.027	220	58	2.99	2.34	4.31
Control	(saline)	0.029	172	50	2.70	2.34	4.54
Ethyl linoleate	10	0.030	157	45	2.80	2.37	4.77
Control	(saline)	0.036	179	56	—	—	—
Ethyl linoleate	25	0.036	185	53	—	—	—
Control	(saline)	0.028	109	34	2.72	2.21	4.30
L-Thyroxine	0.01	0.030	80 [2]	28	3.51 [3]	2.83 [3]	4.69
Control	(saline)	0.028	145	41	2.81	2.63	4.38
CPIB [4]	10	0.041 [3]	151	48	2.36 [3]	2.30 [3]	5.07 [3]
Control	(saline)	0.028	147	36	2.87	2.58	4.15
Benzmalecene	2	0.032	161	75 [2]	2.85	2.77	4.26
Control	(saline)	0.030	187	49	2.97	2.21	4.54
SK & F 525A [5]	2	0.034	180	50	3.39	2.34	5.99 [3]

[1] Five control animals (1 week old) were force-fed saline daily for 1 week. Five littermates received the test substance.
[2] P between 0.05 and 0.01 (probably significant).
[3] $P < 0.01$ (highly significant).
[4] Ethyl α-p-chlorophenoxyisobutyrate.
[5] β-Diethylaminoethyl dephenylpropyl acetate hydrochloride.

assumption that the dam was in an isotopic steady state throughout the experiment. The ratios of specific activity of dam's plasma cholesterol to the dam's whole blood cholesterol and to the dam's milk cholesterol, 0.91 and 1.01, respectively, indicated equilibration between milk and tissue cholesterol of the dam.

DISCUSSION

In diet M-47, which simulates the dry weight composition of rat milk and which was found to maintain rats in a state of hypercholesterolemia, the most important factor appears to be a high fat content. Friedman and Byers (4) observed hypercholesterolemia of the suckling rabbit to

be dependent upon the cholesterol furnished by the milk, and the high fat or triglyceride level of normal rabbit milk was also clearly shown to be necessary for maintaining the rabbit in a state of hypercholesterolemia. Since the cholesterol content of milk from the rat is much lower than that from the rabbit and since hypercholesterolemia was sustained with the high fat, cholesterol-free diet (M-47), the primary factor in hypercholesterolemia of the suckling rat appears to be high dietary fat. Dietary and prenatal transfer of cholesterol may be important factors for initially building up a high plasma cholesterol level in the newborn rat. In the present study, rats weaned with the colony diet were rapidly cleared of hypercholesterolemia; however, it was restored by changing to diet M-47. These results indicate that cholesterol from the milk is not essential for this hypercholesterolemia.

The amount of cholesterol ingested by suckling rats in a 24-hour period can be estimated. The cholesterol content of rat milk on a dry weight basis is approximately 0.06% (2). Newly weaned rats (21 days old) fed diet M-47 consume approximately 4.2 g of diet/day. If suckling rats consume a comparable number of calories, the milk would provide 2.5 mg of cholesterol to each rat/day. Therefore, even though the cholesterol content of milk is low, the 21-day-old suckling rat consumes more cholesterol daily than is present in his entire plasma.[18] The cholesterol-4-^{14}C pellet study showed that approximately one-third of the plasma cholesterol of the suckling rats in this experiment originated from the female. Evidently both milk consumption and endogenous biosynthesis contribute significant amounts of sterol to the suckling rat hypercholesterolemia.

The milk of both the rat and the rabbit is characterized by a high fat content, 40 to 50% of the dry weight. Lower lipid levels are found in the milk of species which are known not to have hypercholesterolemic suckling young. Among those studies only whale milk, in which lipid makes up 37% of the wet weight (12), has a higher level. Plasma cholesterol levels of suckling whales have not been reported.

The hypercholesterolemia of the suckling rat is distinctive in its failure to respond to either ethyl linoleate or to other polyunsaturates. Sardine oil, which is known to have hypocholesterolemic properties (13), produced an increase in plasma cholesterol when fed in diet M-47 to the suckling rat, perhaps due to the more pronounced effect of its cholesterol (15 mg/g).

Turnover of cholic acid has been shown to be slow in rats fed diets containing lactose or diets low in bulk (14). Rats fed diets which tend to promote excretion of greater quantities of bile acids appear also to have lower serum cholesterol levels (14–16). Bloomfield (17) observed the quantity of bile acid eliminated to correlate with the quantity of fecal residue produced by a diet. He concluded that a cholesterol-accumulating diet should be well-refined, high in calories, and low in fecal residue. These are characteristics of rat milk and diet M-47 both of which yield slow rates of cholic acid excretion.[19] A large fecal residue is probably a factor in the rapid lowering of plasma cholesterol when suckling rats are weaned with a colony diet; this lowering may be related to the observed increase in cholic acid excretion rates on weaning.[20] However, the presence of lactose and lack of bulk in rat milk were shown in this study not to be important factors in maintaining hypercholesterolemia in the suckling rat. Diet M-1, in contrast with diet M-47, rapidly diminished plasma cholesterol levels, yet the cholic acid excretion rates were similar.[21] Therefore, the importance of a slow rate of cholic acid excretion in maintaining hypercholesterolemia in the suckling rat is uncertain.

In agreement with Bizzi et al. (1) L-thyroxine was found to be an effective plasma cholesterol-lowering agent in the suckling rat; however, since liver cholesterol was increased the thyroxine may have altered the partition of cholesterol between plasma and tissue. The failure of

[18] 2.4 mg of cholesterol based upon 3.2 ml plasma/100 g, 50 g rat, and 150 mg cholesterol/100 ml plasma.
[19] Harris, R. A., C. L. Villemez, Jr. and F. W. Quackenbush 1965 Dietary studies on suckling rat hypercholesterolemia. Federation Proc., 24: 1081 (abstract).
[20] See footnote 19.
[21] See footnote 19.

other hypocholesterolemic agents to lower plasma cholesterol levels in suckling rats suggests that steps which are rate-limiting in the lowering in this system are different from those which limit in dietary-induced hypercholesterolemia of adult rats.

The increase in liver total lipid with CPIB may have been the result either of an accumulation of the drug or of an increase in lipid. The increase in liver wet weight, dry weight and protein content upon CPIB treatment of suckling rats may reflect an increase in synthesis of drug metabolizing enzymes (18). An increase in liver weight of CPIB-treated, mature rats has been reported (19).

Carroll (2) observed subnormal cholesterol biosynthesis from labeled acetate in the suckling rat. This is characteristic of rats fed diets containing cholesterol (20, 21). Evidence recently has been presented which suggests that bile acids are the metabolites of cholesterol responsible for the physiological regulation of cholesterol biosynthesis. Bloomfield (17) observed that total body sterol biosynthesis was independent of cholesterol feeding at low dietary levels of cholesterol. Fimognori and Rodwell (22) observed a suppression of mevalonate biosynthesis by bile acids. Since the cholic acid turnover in suckling rats is very slow compared with that of adult animals fed a colony diet,[22] hepatic bile acids are probably elevated in the suckling rat to the extent that biosynthesis of sterol is suppressed. Therefore, milk cholesterol as such may not contribute to the suppression of cholesterol biosynthesis in the suckling rat.

LITERATURE CITED

1. Bizzi, A., E. Veneroni and S. Garattini 1963 Hypercholesteremia in suckling rats. J. Atheroscler. Res., *3:* 121.
2. Carroll, K. K. 1964 Acetate incorporation into cholesterol and fatty acids by livers of fetal, suckling, and weaned rats. Can. J. Biochem., *42:* 79.
3. Bragdon, J. H. 1952 Spontaneous atherosclerosis in the rabbit. Circulation, *5:* 641.
4. Friedman, M., and S. O. Byers 1961 Effects of diet on serum lipids of fetal, neonatal, and pregnant rabbits. Amer. J. Physiol., *201:* 611.
5. Quackenbush, F. W., and M. Pawlowski 1960 Effects of purified linoleic ester on cholesterol in the rat. J. Nutr., *72:* 196.
6. Luckey, T. D., T. J. Mende and J. Pleasants 1954 The physical and chemical characterization of rat's milk. J. Nutr., *54:* 345.
7. Schlenk, H., and J. L. Gellerman 1960 Esterification of fatty acids with diazomethane on a small scale. Anal. Chem., *32:* 1412.
8. Harris, R. A., and D. Gambal 1963 Fluorimetric determination of total phospholipids in rat tissues. Anal. Biochem., *5:* 479.
9. Wilson, J. D. 1964 The quantification of cholesterol excretion and degradation in the isotopic steady state in the rat: the influence of dietary cholesterol. J. Lipid Res., *5:* 409.
10. Sperry, W. M., and M. Webb 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
12. Garton, G. A. 1963 The composition and biosynthesis of milk lipids. J. Lipid Res., *4:* 237.
13. Nicolaysen, R., and R. Ragard 1961 Effect of various oils and fats on serum cholesterol in experimental hypercholesterolemic rats. J. Nutr., *73:* 299.
14. Portman, O. W. 1960 Nutritional influence on the metabolism of bile acids. Amer. J. Clini. Nutr., *8:* 462.
15. Danielsson, H., and B. Gustafsson 1959 On serum cholesterol levels and neutral fecal sterols in germ-free rats. Bile acids and acids and steroids. Arch. Biochem. Biophys., *83:* 482.
16. Wells, W. W., and S. C. Anderson 1959 The increased severity of atherosclerosis in rabbits on a lactose-containing diet. J. Nutr., *68:* 541.
17. Bloomfield, D. K. 1963 Dynamics of cholesterol metabolism. I. Factors regulating total sterol biosynthesis and accumulation in the rat. Proc. Nat. Acad., Sci., *50:* 117.
18. Conney, A. H., C. Darison, R. Gastel and J. J. Burns 1960 Adaptive increases in drug-metabolizing enzymes induced by phenobarbital and other drugs. J. Pharmacol., *130:* 1.
19. Best, M. M., and C. H. Duncan 1964 Hypolipemia and hepatomegaly from ethyl chlorophenoxyisobutyrate (CPIB) in the rat. J. Lab. Clini. Med., *64:* 634.
20. Tomkins, G. M., H. Sheppard and I. L. Chaikoff 1953 Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol. J. Biol. Chem., *201:* 137.
21. Frantz, I. D., Jr., H. S. Schneider and B. T. Hinkelman 1954 Suppression of hepatic cholesterol synthesis in the rat by cholesterol feeding. J. Biol. Chem., *206:* 465.
22. Fimognori, G. M., and V. W. Rodwell 1964 Cholesterol biosynthesis: mevalonate synthesis inhibited by bile salts. Science, *174:* 1038.

[22] See footnote 19.

Effect of Carbohydrate and Fat Intakes upon the Activities of Several Liver Enzymes in Rats, Guinea Piglets, Piglets and Calves [1,2]

R. L. BALDWIN, M. RONNING, C. RADANOVICS AND G. PLANGE
Department of Animal Husbandry, University of California, Davis, California

ABSTRACT The effects of 4 diets containing a wide range of carbohydrate and fat upon the activities of a number of liver enzymes in rats, guinea piglets, calves and piglets were investigated. The results indicated that large adaptive enzymatic changes occurred in rats fed high carbohydrate diets but not in guinea piglets, piglets and calves. The activities of most of the enzymes studied changed in logarithmic relationship to the ratio, carbohydrate calories-to-carbohydrate + fat calories, in the diets indicating that the enzymatic adaptations occurred in response to changes in proportions of energy contributed by carbohydrate and fat rather than to changes in the amounts of these components per se. The results indicated that calves, guinea piglets and piglets have much lower capacities for adaptation to high carbohydrate, low fat diets than rats, due either to species differences in rates of physiological development after birth or to other inherent species differences. The activities of all the enzymes whose functions have been related to fat synthesis were much lower in calves than in rats.

The activities of a number of liver enzymes are affected markedly by changes in the carbohydrate content of rat diets (1, 2). Several of these enzymatic changes have been investigated in detail and their probable significance with respect to carbohydrate and fat metabolism and dietary requirements for micronutrients (1–8) have been discussed. In view of the considerable difficulties that have been encountered in attempts to develop high carbohydrate, low fat diets suitable for study of the vitamin and mineral requirements of young calves and the apparent metabolic involvment in these difficulties (9, 10), a preliminary study was made of the effect of carbohydrate and fat intakes upon the activities of several enzymes in calf livers. Only minor changes in enzymatic activities were observed when the fat content of the calf diets was varied from zero to 15% (11), suggesting that the difficulties encountered in the development of high carbohydrate diets might be due to a lack of metabolic adaptation in calves fed these diets. The present study was undertaken to investigate this possibility further and to determine whether the apparent lack of enzymatic response to high carbohydrate diets was unique to the young calf.

METHODS AND MATERIALS

Male weanling rats of the Sprague Dawley strain, 3-day-old male albino guinea piglets, 2-day-old male Holstein calves and 3-day-old male and female Duroc piglets were either purchased commercially or obtained from the University herds.

The rats were fed diets A, B, C and D (table 1) upon arrival and were maintained with these diets for 4 weeks prior to killing for sampling. The rates of gain of the rats were 4.2, 4.6, 5.0 and 5.1 g/day with diets A through D, respectively, and no difficulties with respect to feeding and management were encountered.

The guinea piglets were fed a commercial guinea pig ration for 2 days after arrival and then fed the 4 experimental diets (table 1). Several of the guinea piglets fed each diet lost weight during the first few days following the switch to the experimental diets, but all animals recovered their initial weights within 3 to 4 days. To maintain adequate intakes, it was neces-

Received for publication April 11, 1966.
[1] Supported by Public Health Service Research Grant no. AM05745 from the National Institute of Arthritis and Metabolic Diseases.
[2] Taken in part from a Master's Thesis submitted by C. Radanovics.

TABLE 1

Composition of diets [1]

	Diet A	Diet B	Diet C	Diet D
	g/100 g of basic diet			
Glucose monohydrate [2]	39.3	32.8	26.2	—
Lard	—	2.5	5.0	15.1
Non-fat milk solids	34.7	34.7	34.7	34.7
Dried whey	21.2	21.2	21.2	21.2
Non-nutritive cellulose [3]	4.8	8.8	12.9	30.0
Vitamin and salt mixes [4]				
Cal. CHO/Cal. CHO+fat	0.98	0.90	0.80	0.55

[1] Diets calculated to be isonitrogenous and isocaloric.

[2] Cerelose, Corn Products Company, Argo, Illinois.

[3] Non-nutritive cellulose was not added to calf and piglet diets which were fed as semi-purified milks (see text). Proportions of other ingredients in diet were not altered.

[4] The vitamins and minerals were added to the basic diet and varied with species as follows: for the rat diets, 0.6 g of a mineral mix containing 39.8% dipotassium phosphate, 25.4% Ca carbonate, 14.1% sodium chloride, 6.3% dibasic Ca phosphate, 2.3% ferric citrate, 0.63% manganese sulfate, 8.6% magnesium sulfate, 0.9% zinc sulfate, 0.068% potassium iodide, 0.125% copper sulfate, 0.02% zinc chloride, 0.004% cobalt chloride and 1.4% magnesium oxide and 0.3 g of a vitamin mix containing 3,000 IU vitamin A, 375 IU vitamin D, 10 mg *dl*-α-tocopherol powder (250 IU vitamin E/g) and 0.1 mg menadione, were added/100 g of the basic diets; for the guinea piglet diets: 4.75 g of a mineral mix containing 29.4% dipotassium phosphate, 25.2% Ca carbonate, 18.5% sodium chloride, 10.5% magnesium sulfate, 12.6% Ca phosphate, 2.9% ferric citrate, 0.6% manganese sulfate, 0.08% potassium iodide, 0.03% copper sulfate, 0.03% zinc chloride and 0.01% cobalt chloride and 0.6 g of a vitamin mix containing 1.6 mg each of thiamine·HCl, riboflavin, and pyridoxine·HCl, 4 mg sodium pantothenate, 10 mg niacin, 0.06 mg biotin, 1.0 mg folic acid, 0.004 mg vitamin B_{12}, 300 IU vitamin A, 12 mg α-tocopherol, 0.2 mg menadione, 10 mg p-aminobenzoic acid, 200 mg ascorbic acid, 0.004 mg calciferol (vitamin D) and 300 mg choline chloride were added/100 g of the basic diets; for the calves and piglets 0.8 g of a salt mix containing 82.5% trace mineralized salt, 9.4% magnesium oxide, 7.6% ferric citrate and 0.5% copper sulfate and 0.3 g of the vitamin mix used in the rat diets were added/100 g of the basic diets. The trace mineralized salt contained not less than: (in per cent) NaCl, 96.5; manganese, 0.20; iron, 0.16; copper, 0.033; cobalt, 0.01; iodine, 0.007; and zinc, 0.005.

sary to pellet the food for the guinea piglets. The guinea piglets were fed the experimental diets for 4 weeks prior to killing for sampling. Their rates of gain were 1.1, 1.0, 2.2 and 3.0 g/day with diets A through D, respectively. The poor growth responses of the guinea piglets were recognized in the course of preliminary experiments and were a matter of concern because the poor growth might affect the validity of the data. Better growth response could be obtained by decreasing the carbohydrate content of the diets. However, since one of the goals of the investigation was study of the nature and extent of the enzymatic adaptations to high carbohydrate diets and since the poor growth may have been a reflection of a lack of adaptation, the carbohydrate content of the diets was not decreased.

The calves and piglets were fed 2 of the experimental diets (A and D) beginning at 2 and 3 days of age, respectively. The diets were prepared as semi-purified milks and fed isocalorically and isonitrogenously to the calves in amounts equivalent to usual levels of whole milk feeding. The calves were maintained with the experimental diets for an average of 4 weeks prior to final sampling at killing. In addition, the calves were sampled by biopsy during the 4-week feeding period; the results were similar to those obtained in the final samples. The average rates of gain of the calves were 0.39 and 0.42 kg/day with diets A and D, respectively. The piglets were fed ad libitum and appeared to respond well to the experimental diets during preliminary studies but during the main study many of the piglets suddenly became sickly and died between the third and fourth weeks of the feeding period. No specific cause could be determined and numerous attempts to control the problem were unsuccessful. Therefore, most of the results reported were determined in samples collected after killing of piglets between 2 and 3 weeks of age, and only a few data are presented. The animals represented were growing well and appeared healthy and vigorous at the time of killing.

Results of routine blood analyses for hexose, pyruvate, oxyhemoglobin and hematocrit did not appear to be affected by differences in the diets.

Liver samples were stored in ice during transport to the laboratory and were prepared for enzymatic analysis according to methods reported previously (12). Enzyme assays and auxilliary clinical techniques were carried out according to procedures described elsewhere (12, 13). Liver slices prepared with a Stadie tissue slicer were incubated under 95% oxygen and 5% CO_2 in Krebs bicarbonate buffer with 0.5 μCi of either glucose-1-^{14}C or glucose-6-^{14}C for 90 minutes in a shaking water bath. The reactions were stopped with HCl and the $^{14}CO_2$ collected in CO_2-free NaOH and counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

The enzymes investigated were selected as representatives of various metabolic pathways associated with carbohydrate and fat metabolism and on the basis of previous reports that their activities were affected by diet composition (1–7). Glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase and pentose phosphate-metabolizing activity were selected as indexes of the potential activity of the hexose monophosphate pathway. Hexokinase, glucose-6-P isomerase, glucose-P mutase and glucose-6-phosphatase were selected because of their relationships to the metabolism of glucose-6-phosphate. Fructose-1,6-diP aldolase and glyceraldehyde-3-P dehydrogenase were selected because they function in the Embden-Meyerhof glycolytic pathway. UDPG pyrophosphorylase was determined because of its role in glycogen synthesis. Pyruvate dehydrogenase, α-glycerol-P dehydrogenase, glycerol kinase, aceto-CoA synthetase (extra-mitochondrial), malic enzyme, malate dehydrogenase (extra-mitochondrial), isocitrate dehydrogenase (extra-mitochondrial), and the citrate cleavage enzyme were selected because of their potential relationships to fat metabolism. Aspartate aminotransferase was studied as a general index. The ratio of $^{14}CO_2$ produced from glucose-6-^{14}C and glucose-1-^{14}C by tissue slices was selected as a general index of the relative activities of the alternate glycolytic pathways.

The activities of the enzymes observed in liver samples obtained from rats, guinea piglets, piglets and calves fed the experimental diets are presented in tables 2 to 5, respectively. With rats, marked decreases in the activities of a number of the enzymes studied were observed in response to decreases in the ratio of calories from carbohydrate to calories from carbohydrate plus fat in the diets (table 2). The activity of glucose-6-P dehydrogenase in livers of rats fed diet A (high carbohydrate) was 24 times greater than in livers of rats fed diet D (high fat). The activities of the malic enzyme, citrate cleavage enzyme, aceto-CoA synthetase, glucose-P mutase, hexokinase, 6-P-gluconate dehydrogenase and pyruvate dehydrogenase were 9.1, 7.0, 5.5, 4.8, 3.8, 2.6 and 2.0 times greater, respectively, with the high carbohydrate diet (A) than with the high fat diet (D). The activities of most of these enzymes decreased logarithmically as the ratio of calories from carbohydrate to calories from carbohydrate plus fat in the diet decreased (fig. 1), apparently an indication that the enzymatic changes were related to the caloric contributions rather than the amounts, of carbohydrate and fat per se in the diets. The general nature of the enzymatic changes and the ratios of $^{14}CO_2$ produced from glucose-6-^{14}C and glucose-1-^{14}C were consistent with previous reports which indicated that as the

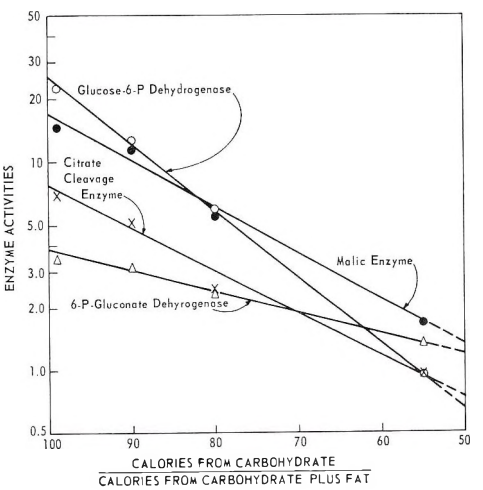


Fig. 1 Effect of ratio of calories of carbohydrate to calories of carbohydrate plus fat in the diet upon the activities of several enzymes in rat livers.

TABLE 2

Effect of varying levels of carbohydrate and fat in the diet upon the activities of several enzymes in rat livers

Enzyme	Diets							
	A		B		C		D	
	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
Glucose-6-P dehydrogenase (EC1.1.1.49)	22.4 [1]	4.67 [2]	12.7 [1]	2.20 [2]	5.92 [1]	1.11 [2]	0.94 [1]	0.13 [2]
6-P-Gluconate dehydrogenase (EC1.1.1.44)	3.42	0.91	3.17	0.69	2.28	0.60	1.33	0.36
PPMA [3]	1.42	0.47	2.60	0.92	2.53	0.88	2.23	0.96
Hexokinase (EC2.7.11)	1.89	0.10	0.93	0.28	0.58	0.16	0.39	0.13
Glucose-6-P isomerase (EC5.3.1.9)	48.8	4.71	56.2	6.95	53.0	8.90	42.0	5.33
Glucose-P mutase (EC2.7.5.1)	7.77	1.03	7.02	2.93	3.31	0.88	2.06	0.76
Fructose-1,6-diP aldolase (EC4.1.2.7)	4.41	0.62	5.31	0.99	5.70	0.40	5.92	1.02
Glyceraldehyde-3-P dehydrogenase (EC1.2.1.12)	28.2	4.22	16.8	4.22	18.8	4.24	18.3	5.66
Pyruvate dehydrogenase (EC1.2.2.2)	12.6	1.69	11.2	0.70	7.94	0.77	6.50	0.48
α-Glycerol-P dehydrogenase (EC1.1.1.8)	9.21	2.64	9.37	2.17	9.31	1.50	6.19	1.67
Glycerol kinase (EC2.7.1.30)	0.78	0.13	0.88	0.22	0.54	0.11	0.67	0.15
Aceto-CoA synthetase (EC6.2.1.1)	1.11	0.11	0.54	0.05	0.42	0.14	0.17	0.09
Citrate cleavage enzyme (EC4.1.3.7)	6.98	0.78	5.09	0.62	2.48	0.44	0.95	0.11
Malic enzyme (EC1.1.1.38)	14.6	2.24	12.2	2.68	5.69	1.72	1.64	0.48
UDPG pyrophosphorylase (EC2.7.7.9)	2.65	0.38	2.98	0.38	2.40	0.53	2.53	0.43
Isocitrate dehydrogenase (EC1.1.1.42)	24.5	3.71	23.8	2.49	19.5	3.53	20.7	3.50
Glucose-6-^{14}C/Glucose-1-^{14}C	0.30 [4]	0.06	0.36 [4]	0.04	0.45 [4]	0.05	0.98 [4]	0.11

[1] Units of enzyme/gram of tissue. One unit of enzyme is defined as the amount required to convert one μmole of substrate/minute at 25° except glucose-6-P isomerase and pyruvate dehydrogenase where one unit causes a change of 1.0 absorbancy unit/minute.

[2] Stanard error (N = 6).

[3] Pentose phosphate-metabolizing activity.

[4] Ratio of $^{14}CO_2$ produced from respective substrates.

fat content of the diet decreased, activity of the hexosmonophosphate pathway, rates of fatty acid synthesis and the activities of enzymes involved in these functions increased (1–6).

The results of the study with guinea piglets (table 3) were somewhat different from those observed in the rat study. Only one enzyme, glucose-6-P dehydrogenase, decreased in activity as the ratio of calories from carbohydrate to calories from carbohydrate plus fat decreased and this change was about two-fold as compared with 24-fold in rats. The decrease appeared to represent a logarithmic response similar to that observed in the rats (fig. 2). The activities of several enzymes tended to increase in the guinea piglet livers as the fat content of the diet increased. The activities of α-glycerol-P dehydrogenase, glycerol kinase, isocitrate dehydrogenase and aldolase were 2.8, 2.6, 2.2 and 2.1 times greater, respectively, with diet D than with diet A. The changes in the activity of α-glycerol-P dehydrogenase appeared to increase logarithmically as the ratio of carbohydrate calories to carbohydrate and fat calories in the diet decreased (fig. 2). The activity of glycerol kinase increased in a similar fashion until the carbohydrate-to-carbohydrate plus fat ratio reached 0.80 (diet C) and then no further increase was noted (fig. 2). The activities of aldolase and isocitrate dehydrogenase did not increase until the carbohydrate-to-carbohydrate plus fat ratio had decreased to 0.90 and thereafter increased logarithmically as the ratio decreased (fig. 2). These latter observations might suggest that there are limits to the extent to which adaptive changes can occur despite further changes in diet. The enzymatic changes observed were consistent with the tissue slice data obtained with glucose-1-^{14}C and glucose-6-^{14}C, in that both indicated very little change in the relative activities of the hexose monophosphate and Embden-Meyerhof pathways. The changes in the activities of α-glycerophosphate dehydrogenase and glycerol kinase with increasing fat intakes appeared to represent an increased capacity of the liver for triglyceride synthesis (7). It is interesting that α-glycerol-P dehydrogenase, glycerol kinase and isocitrate dehydrogenase, the enzymes most affected by the dietary variations in the guinea piglets were three of the enzymes affected least in the rat.

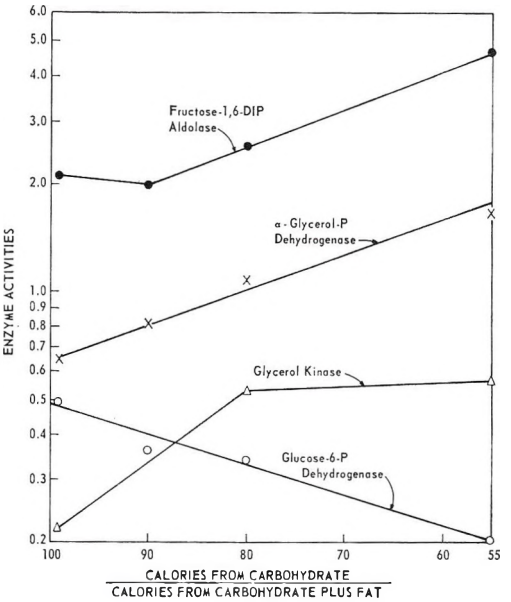


Fig. 2 Effect of ratio of calories of carbohydrate to calories of carbohydrate plus fat in the diet upon the activities of several enzymes in guinea piglet livers.

The results obtained in the calf study are presented in table 4. The most notable characteristic of these data was the lack of differences in the activities of liver enzymes between diets. Glucose-6-P dehydrogenase activity and citrate cleavage enzyme activities appeared to be lower and the activity of α-glycerol-P dehydrogenase appeared to be higher when the fat-containing diet was fed. The activities of the remaining enzymes did not appear to be affected significantly. The activities of aceto-CoA synthetase and malic enzyme were below the effective sensitivities of the assays used. The ratio of $^{14}CO_2$ produced by tissue slices incubated with glucose-6-^{14}C to that from glucose-1-^{14}C was slightly higher when diet D was fed and may have indicated that a slight shift in pathways of carbohydrate metabolism had occurred.

The data obtained in the piglet study are presented in table 5. There were fewer animals in this study than in the others

TABLE 3

Effect of varying levels of carbohydrate and fat in the diet upon the activities of several enzymes in guinea piglet livers

Enzyme	Diets							
	A		B		C		D	
	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
Glucose-6-P dehydrogenase	0.49 [1]	0.19 [2]	0.36 [1]	0.14 [2]	0.34 [1]	0.23 [2]	0.20 [1]	0.04 [2]
6-P-Gluconate dehydrogenase	0.57	0.32	0.54	0.26	0.33	0.03	0.63	0.25
PPMA [3]	0.26	0.14	0.24	0.11	0.28	0.11	0.23	0.08
Glucose-6-P isomerase	47.8	12.6	54.4	27.2	33.5	10.2	40.8	19.0
Glucose-P mutase	28.5	3.86	23.9	7.98	27.5	10.8	27.3	14.9
Fructose-1,6-diP aldolase	2.10	0.32	1.96	0.94	2.51	0.89	4.53	0.52
Glyceraldehyde-3-P dehydrogenase	11.2	4.50	9.00	3.61	14.1	3.43	9.86	3.11
Pyruvate dehydrogenase	8.10	1.24	7.58	2.81	5.71	2.47	6.03	0.87
α-Glycerol-P dehydrogenase	0.65	0.36	0.81	0.45	1.15	0.66	1.79	0.38
Glycerol kinase	0.22	0.05	0.35	0.16	0.53	0.07	0.57	0.20
Malate dehydrogenase (EC1.1.1.37)	28.6	4.21	31.7	4.31	28.3	6.71	29.9	2.73
UDPG Pyrophosphorylase	1.19	0.15	1.36	0.64	1.26	0.41	1.14	0.20
Glucose-6-phosphatase	0.29	0.08	0.25	0.02	0.39	0.07	0.30	0.07
Isocitrate dehydrogenase	26.7	3.32	25.6	7.64	36.2	8.38	59.5	9.29
Aspartate aminotransferase (EC2.6.1.1)	21.8	4.50	19.5	6.35	20.2	2.65	17.8	5.37
Glucose-6-^{14}C/glucose-1-^{14}C	0.65 [4]	0.02	0.76 [4]	0.12	0.79 [4]	0.09	0.75 [4]	0.07

[1] Units of enzyme/gram of tissue. One unit of enzyme is defined as the amount required to convert one μmole of substrate/minute at 25° except glucose-6-P isomerase and pyruvate dehydrogenase where one unit causes a change of 1.0 absorbancy unit/minute.

[2] Standard error (N = 6).

[3] Pentose phosphate-metabolizing activity.

[4] Ratio of $^{14}CO_2$ produced from respective substrates.

TABLE 4

Effect of varying levels of carbohydrate and fat in the diet upon the activities of several enzymes in calf livers

Enzyme	Diets			
	A		D	
	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
Glucose-6-P dehydrogenase	2.00 [1]	0.43 [2]	1.30 [1]	0.21 [2]
6-P-Gluconate dehydrogenase	1.00	0.17	1.11	0.23
PPMA [3]	0.18	0.06	0.20	0.06
Hexokinase	—	—	0.23	0.08
Glucose-P mutase	73.7	31.1	99.5	21.2
Fructose-1,6-diP aldolase	3.48	0.62	2.75	0.64
Glyceraldehyde-3-P dehydrogenase	14.9	4.40	19.4	8.81
Pyruvate dehydrogenase	0.12	0.04	0.08	0.01
α-Glycerol-P dehydrogenase	0.51	0.21	1.09	0.34
Glycerol kinase	0.14	0.07	0.09	0.03
Citrate cleavage enzyme	0.22	0.09	0.07	0.03
Isocitrate dehydrogenase	12.1	4.47	13.5	3.08
Glucose-6-^{14}C/glucose-1-^{14}C	0.36 [4]	0.07	0.46 [4]	0.05

[1] Units of enzyme/gram of tissue. One unit of enzyme is defined as the amount required to convert one μmole of substrate/ minute at 25° except pyruvate dehydrogenase where one unit causes a change of 1.0 absorbancy unit/minute.
[2] Standard error (N = 6 for diet A and N = 9 for diet D).
[3] Pentose phosphate-metabolizing activity.
[4] Ratio of $^{14}CO_2$ produced from respective substrates.

TABLE 5

Effect of varying levels of carbohydrate and fat in the diet upon the activities of several enzymes in piglet livers

Enzyme	Diets			
	A		D	
	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
Glucose-6-P dehydrogenase	1.94 [1]	0.45 [2]	1.64 [1]	0.60 [2]
6-P-Gluconate dehydrogenase	4.49	2.05	4.09	0.48
PPMA [3]	1.43	1.20	0.69	0.15
Hexokinase	2.85	0.80	2.91	1.44
Glucose-6-P isomerase	45.2	16.2	31.9	4.96
Glucose-P mutase	3.79	1.45	6.90	3.91
Fructose-1,6-diP aldolase	8.04	4.45	5.28	1.65
Glyceraldehyde-3-P dehydrogenase	34.9	22.9	19.0	8.25
Pyruvate dehydrogenase	3.62	0.36	1.61	1.37
α-Glycerol-P dehydrogenase	2.36	0.82	1.98	0.69
Aceto-CoA synthetase	4.84	1.10	2.76	0.81
Citrate cleavage enzyme	0.39	0.54	0.25	0.09
UDPG pyrophosphorylase	164	44.1	111	29.8
Glucose-6-phosphatase	0.12	0.01	0.12	0.04
Isocitrate dehydrogenase	26.2	10.0	21.4	3.22
Aspartate aminotransferase	4.73	0.81	9.59	1.40
Glucose-6-^{14}C/glucose-1-^{14}C	0.54 [4]	0.08	0.56 [4]	0.07

[1] Units of enzyme/gram of tissue. One unit of enzyme is defined as the amount required to convert one μmole of substrate/minute at 25° except glucose-6-P isomerase and pyruvate dehydrogenase where one unit causes a change of 1.0 absorbancy unit/minute.
[2] Stanard error (N = 3 for diet A and N = 5 for diet D).
[3] Pentose phosphate-metabolizing activity.
[4] Ratio of $^{14}CO_2$ produced from respective substrates.

discussed above, because of the difficulties referred to in the methods and materials section. The standard errors ($s_{\bar{x}}$) of many of the estimates were fairly large. In view of the difficulties involved in the piglet study, the data must be considered with caution. They appear to indicate, however, that responses of piglets to high carbohydrate diets were not large.

The present study was undertaken in an attempt to determine whether the apparent lack of enzymatic responses to changes in carbohydrate and fat intakes observed in preliminary studies with calves

was unique to young calves. Further studies were also required to determine whether the apparent lack of enzymatic response by calves to changes in diet was related to previous difficulties encountered in attempts to develop high carbohydrate, fat-free diets for calves (9, 10). In guinea piglets and piglets as well as calves the responses to changes in carbohydrate and fat intakes appeared to be much more limited than those which occurred in rats. These differences may reflect either a lack of ability of neonatal guinea piglets, piglets and calves to adapt metabolically to changes in diet, the existence of adaptive mechanisms that do not involve changes in the activities of liver enzymes, or lack of capacity, in these 3 species, for adaptation to high carbohydrate diets at any age. There are data that indicate that the capacity for adaptation to changing physiological conditions develops slowly in the livers of neonatal animals (14). It is possible that the results of the present experiment can be explained by suggesting that the rats were more mature than the other species studied and thus were capable of greater responses to changes in diet. However, guinea piglets, piglets and calves are more fully developed at birth than rats and there is evidence that guinea piglets form some adaptive enzymes at an earlier age than rats (14, 15). The possibility of alternate adaptive mechanisms in calves, guinea piglets and piglets cannot be excluded. For example, a greater participation of adipose tissue in fat synthesis in these species might explain the results obtained. However, no data are available at present to support this possibility.

The considerable difficulty that has been encountered in attempts to develop high carbohydrate, fat-free diets for calves, the high B-vitamin and mineral requirements of calves fed such diets, and clinical data (9, 10), suggested that metabolic disorders occurred in calves fed high carbohydrate diets. The results of the present study indicated that the difficulties encountered might have been due to a lack of adaptive enzymatic changes required for the conversion of increased amounts of carbohydrate to fat in the liver. Guinea piglets and piglets also appeared to lack adaptive capacity as compared with the rat.

The activities of a number of enzymes were similar, whereas others were considerably lower in the livers of calves fed the high fat diet (D) than in the livers of rats fed the same diet. Many of the enzymes whose activities were lower appeared, according to present concepts (1), to be related to the capacity for synthesis of fatty acids in liver. The activities of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were similar in the 2 species; however, the activities of the pentose phosphate-metabolizing enzymes were 10 times lower in calves than in rats, suggesting that the potential for pentose cycle activity in calf livers was lower than in rat livers. Such a deficiency might be expected to affect the potential availability of NADPH for fatty acid synthesis. The activities of pyruvate dehydrogenase, citrate cleavage enzyme and acetyl CoA synthetase were much lower in calf livers than in rat livers. The low levels of these enzymes in calf livers might be expected to affect the availability of acetyl-CoA for fat synthesis. The activity of malic enzyme was very low in calf livers (12) and the activity of α-glycerol dehydrogenase was 5 times lower in calf livers than in rat livers. The activities of these enzymes have also been related to fat synthesis (1). These apparent limitations in the potential capacity for fat synthesis in calf livers as compared with rat livers became much more prominent when animals fed the high carbohydrate diets (A) were compared. Differences in the absolute activities of enzymes involved in fat synthesis were not as great when guinea piglets and piglets were compared to rats.

LITERATURE CITED

1. Tepperman, J., and H. M. Tepperman 1965 Adaptive hyperlipogenesis-late 1964 model. Ann. N. Y. Acad. Sci., *131:* 404.
2. Hill, R., W. W. Webster, N. M. Linazasoro and I. L. Chaikoff 1960 Time of occurrence of changes in the livers capacity to utilize acetate for fatty acid and cholesterol synthesis after fat feeding. J. Lipid Res., *1:* 150.
3. Kornacker, M. S., and J. M. Lowenstein 1964 Citrate cleavage enzyme in livers of obese and nonobese mice. Science, *144:* 1027.

4. Tepperman, H. M., and J. Tepperman 1963 On the response of hepatic glucose-6-phosphate dehydrogenase activity to changes in diet composition and food intake pattern. In: Advances in Enzyme Regulation, vol. 1, ed., G. Weber. The Macmillan Company, New York, p. 121.
5. Masoro, E. J. 1962 Biochemical mechanisms related to the homeostatic regulation of lipogenesis in animals. J. Lipid Res., 3: 149.
6. Allman, D. W., D. D. Hubbard and D. M. Gibson 1965 Fatty acid synthesis during fat-free refeeding of starved rats. J. Lipid Res., 6: 63.
7. Howard, C. F., Jr., and J. M. Lowenstein 1965 The effect of glycerol-3-phosphate on fatty acid synthesis. J. Biol. Chem., 240: 4170.
8. Gershoff, S. N. 1964 Effects of dietary levels of macronutrients on vitamin requirements. Federation Proc., 23: 1077.
9. Benevenga, N. J., and M. Ronning 1965 The effect of certain B-vitamin supplements on the survival and performance of calves fed a high carbohydrate low fat diet. Hilgardia, 36: 333.
10. Ronning, M., R. L. Baldwin and B. C. Tennant 1966 Study of nutritional defects of a nonfat milk solid and glucose diet for young calves. J. Dairy Sci., 49: 986.
11. Baldwin, R. L., and M. Ronning 1966 Effects of several constituents of semi-purified diets upon carbohydrate and fat metabolism in calves. J. Dairy Sci., 49: 688.
12. Benevenga, N. J., R. L. Baldwin and M. Ronning 1966 Alterations in liver enzyme activities and blood metabolite levels during the onset of thiamine deficiency in the dairy calf. J. Nutr., in press.
13. Baldwin, R. L., and L. P. Milligan 1966 Enzymatic changes associated with the initiation and maintenance of lactation in the rat. J. Biol. Chem., 241: 2058.
14. Walker, D. G., and G. Holland 1965 The development of hepatic glucokinase in the neonatal rat. Biochem. J., 97: 845.
15. Dawkins, M. J. R. 1966 Biochemical aspects of developing function in newborn mammalian liver. Brit. Med. Bull., 22: 27.

Dietary Metal-complexing Agents and Zinc Availability in the Rat [1,2]

DONALD OBERLEAS,[3] MERLE E. MUHRER AND BOYD L. O'DELL
Department of Agricultural Chemistry, University of Missouri, Columbia, Missouri

ABSTRACT The growth rate of weanling rats was used to determine the effects of phytate, calcium and ethylenediaminetetraacetate (EDTA) upon the physiological availability of zinc. Phytate decreased availability and the effect was augmented by excess dietary calcium. Calcium had no effect in the absence of phytate so that its effect must be mediated through an interaction with phytate. EDTA increased zinc availability when the diet contained phytate but had no significant effect upon the growth rate in the absence of phytate. In vitro experiments showed that zinc phytate is highly insoluble at the pH range encountered in the small intestine. Addition of calcium to the medium produced an even more insoluble complex containing zinc, calcium and phytate. The results suggest that the formation of such complexes with phytate is the mechanism whereby zinc is made less available and the more complete precipitation of zinc in the presence of calcium explains the effect of excess calcium. In vitro studies with intestinal strips and ^{65}Zn showed that zinc uptake was progressively decreased as the ratio of calcium to phytate was increased. This effect was counteracted in part by the addition of EDTA. It appears that EDTA increases zinc availability by competing with phytate and forming a soluble complex which allows absorption across membranes.

The indispensability of zinc in the nutrition of the rat was demonstrated some 30 years ago, but the requirement was extremely low and great care was necessary in order to produce deficiency symptoms (1). The significance of zinc in practical diets was not realized until Tucker and Salmon (2) showed that zinc would prevent or cure parakeratosis in swine fed diets composed of natural feedstuffs. The fact that zinc deficiency develops in animals fed diets based on soybean protein and corn whereas it does not occur when the diets are based on animal protein containing the same amount of zinc has led to the hypothesis that the zinc in certain plant proteins is not readily available to animals.

The addition of phytic acid to diets based on casein or free amino acids has been shown to reduce the availability of zinc to the growing chick (3–5) the pig (6) and the rat (7). Under some conditions excess calcium decreases the availability of zinc and this is related to the source of protein (8). By the use of diets based on casein with and without added phytic acid it has been shown that calcium exerts a deleterious effect only in the presence of phytic acid (7, 9). The addition of ethylenediaminetetraacetate (EDTA) to soybean protein increases the availability of zinc to the turkey poult (10) and the rat (11) and counteracts the effect of phytate in the diet of chicks (9).

In the experiments described here, the rat was used to determine the dietary interrelationships of calcium, phytate and EDTA with respect to zinc availability. In vitro studies were also performed to investigate the mechanism by which these factors affect zinc availability.

EXPERIMENTAL

Weanling male albino rats were maintained in stainless steel cages and allowed to consume their respective diets ad libitum. Distilled water was supplied in glass bottles. The animals were weighed weekly for 4 weeks.

Received for publication March 28, 1966.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series no. 3081.

[2] Taken from a thesis by D. Oberleas submitted to the Graduate School, University of Missouri, in partial fulfillment of the requirements for the Ph.D. degree. A preliminary account has been presented: Oberleas, D., M. E. Muhrer and B. L. O'Dell 1965 Federation Proc., 24: 170.

[3] Present address: Department of Medicine, Wayne State University College of Medicine, Detroit, Michigan, 48207.

The basal diet contained: (g/kg) glucose hydrate, 700; soybean oil, 100; protein source,[4] 150; minerals,[5] 50; methionine,[6] and vitamin supplement.[7] This diet [8] was calculated to contain approximately 12% of crude protein, 0.8% of calcium, and 0.6% of phosphorus. The zinc content as determined by atomic absorption spectrophotometry ranged from 6 to 8 mg/kg. Phytate phosphorus was determined by a modification of the method of Earley (12) and by this method the purified soybean protein contained the equivalent of 2.5% of inositol hexaphosphate. Commercial phytic acid was used to supplement the casein diets and was added on the basis of analysis to supply the equivalent of 1% of inositol hexaphosphate. All supplements were added in lieu of glucose, calcium and zinc as the carbonates and EDTA as the disodium salt.

For the solubility studies, solutions of sodium phytate (7.85×10^{-3} M), calcium chloride (0.05 M) and zinc sulfate (0.1 M) were used. Aliquots were added to a beaker to give a minimum of 10^{-4} mole of each component in a total volume of 30 ml. In some cases the calcium chloride concentration was 2×10^{-4} mole. After all additions were made, the pH was adjusted upwards to provide a series from pH 3 to 9. After standing 24 hours the samples were centrifuged, the precipitate washed with 5 ml of water dried at 60° in vacuo, weighed, wet-ashed and analyzed for phosphate, calcium and zinc. Phosphate was determined by the method of Fiske and Subbarow (13), calcium according to the AOAC oxalate-permanganate procedure (14) and zinc by the Zincon method (15).

Initially, a modification of the everted gut sac technique (16) was used to determine the effect of calcium, phytate and EDTA on zinc absorption in vitro. The buffer had the following composition: (g/liter) NaCl, 8.54; KCl, 0.30; fructose, 3.60; 2 - amino - 2 - hydroxymethyl - 1,3-propanediol (Tris), 0.485; adjusted to pH 6.0 with HCl. The final concentrations of the additives were: phytate, 10^{-3} M; EDTA, 1.3×10^{-6} M, and zinc, 1.3×10^{-6} M. Calcium was added to give calcium-to-phytate ratios ranging from 1:1 to 16:1. Each incubation vessel contained 0.05 μCi of ^{65}Zn. At least 2.0 ml of buffer medium were used in each vessel and the total volume was 2.5 ml. After preliminary study of this system the everted sac technique was discontinued because active transport was not observed. Zinc uptake by intestinal tissue was determined under the same conditions. A normal animal was killed with chloroform and a portion of the small intestine about 30 cm in length, starting with the jejunum was removed to a cold porcelain plate and into a pool of cold isotonic saline. The intestine was cut into segments approximately 3 cm in length, freed of adhering tissue, split longitudinally, weighed and placed in the incubation vessel. During this time the buffer and tissues were maintained near 2°. After all additions were made the vessels were oxygenated for 15 seconds, stoppered and then incubated in a Dubnoff water bath for one hour at 37° with shaking at the rate of 80 oscillations per minute. Following incubation the tissues were immediately removed, rinsed with a stream of cold saline, placed in a tube with 2 ml of concentrated sulfuric acid and heated in boiling water for 15 minutes. The hydrolysate was counted in a well-type scintillation counter which gave a counting efficiency of 2.69%. The samples were then analyzed for nitrogen by the micro-Kjeldahl method.

RESULTS AND DISCUSSION

Growth rate and interaction of calcium, zinc and phytate. The results of experi-

[4] Reprecipitated and purified casein or purified soybean protein (Promine, Central Soya Company, Chicago).

[5] The following minerals were supplied: (g/kg diet) $CaCO_3$, 10.74; $CaHPO_4 \cdot 2H_2O$, 16.73; $MgCO_3$, 1.00; $MgSO_4 \cdot 7H_2O$, 1.21; NaCl, 5.00; KCl, 0.84; $FePO_4$ (soluble), 1.60; KH_2PO_4, 12.67; $MnSO_4 \cdot H_2O$, 0.76; $CuSO_4 \cdot 5H_2O$, 0.064; $AlK(SO_4)_2 \cdot 12H_2O$, 0.0096; KI, 0.024; $CoCl_2 \cdot 6H_2O$, 0.0024; and NaF, 0.040.

[6] One gram of methionine for casein and 3 g for soybean protein diets.

[7] Vitamins were supplied at the following levels/kg of diet: vitamin A, 20,000 IU; vitamin D, 3,000 IU; menadione, 10; α-tocopheryl acetate, 30; thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; biotin, 0.2; folacin, 5; cyanocobalamin, 0.05; and choline chloride 1,000 mg. The antioxidant, ethoxyquin, was supplied at 100 mg/kg.

[8] The authors gratefully acknowledge gifts of dietary supplements from the following donors: biotin, Hoffmann-LaRoche, Inc., Nutley, New Jersey; folacin, American Cyanamid Company, Pearl River, New York; vitamin A, Distillation Products Industries, Rochester, New York; and other vitamins from Merck Sharp and Dohme, Rahway, New Jersey.

TABLE 1

Growth rate as affectd by calcium and phytate

Supplement	Casein basal		Basal + 1% phytate	
	0.8% Ca	1.6% Ca	0.8% Ca	1.6% Ca
	g	*g*	*g*	*g*
None (11) [1]	32 ± 1.3 [2]	33 ± 0.7	20 ± 1.5	7 ± 2.1
Zn, 55 ppm (7)	31 ± 1.8	32 ± 2.1	31 ± 1.8	33 ± 1.0

[1] Number of animals per treatment.
[2] Average weekly gains for 4 weeks ± SE of mean.

ments which involved 72 rats fed casein-base diets are shown in table 1. In the absence of phytate the calcium level had no effect upon growth rate with or without supplementary zinc. When the diet contained 1% phytate and no added zinc, the growth rate of animals fed 0.8% of calcium was about 60% of that of the zinc-supplemented controls, and those fed 1.6% of calcium gained at about 20% of the control rate. Analysis of variance of the groups without zinc showed a highly significant interaction between calcium and phytate ($P < 0.005$). The growth-depressing effects of calcium and phytate disappeared when a zinc supplement was supplied. Thus, the addition of phytate decreased the amount of zinc physiologically available and the results suggest that the detrimental effect of excess calcium is mediated through the phytate present in the diet. The results are in agreement with earlier observations made with the pig (6), the chick (9) and the rat (7).

Growth rate and interaction of EDTA, zinc and phytate. In the second experiment the basal diet was similar to the high calcium (1.6%) diet described above and a 2 × 2 factorial design was used. Phytate was added at the 1% and EDTA at the 0.1% level. The results, shown in table 2, not only confirm that phytate decreases growth when zinc is limiting ($P < 0.005$), but also show that EDTA markedly stimulates growth when added to low zinc diets containing phytate. EDTA slightly depressed the growth rate of rats fed the basal diet, but the effect was not statistically significant. The most noteworthy observation was the highly significant interaction ($P < 0.005$) between phytate and EDTA, confirming the results obtained with chicks (9).

Solubility studies. The solubility experiments were designed to investigate the effect of pH and the ratio of calcium and zinc to phytate or of zinc to phytate upon the quantity of precipitate formed. The amount of precipitate was calculated by the summation of inositol hexaphosphate (3.55 × phosphorus), calcium and zinc as determined analytically. No precipitate was visible below pH 3.7, but in the presence of zinc a precipitate formed at pH 3.8 and above.

As shown in figure 1 with calcium-to-phytate ratios of 1:1 there was only a trace of precipitate. Zinc phytate was much less soluble and at pH 6, 61% of the zinc was recovered in the precipitate. When equal molar concentrations of calcium, zinc and phytate were present, the quantity of precipitate formed was greater than the sum of the precipitates formed when the 2 cations were added to phytate separately. At pH 6 the precipitate formed by the 3 ions contained 77% of the zinc added.

When the calcium concentration was twice that of phytate a measurable amount of precipitate was formed but only 26% of the calcium was removed at the peak, pH 6. When the molar ratios of calcium, zinc and phytate were 2:1:1, the precipitate contained 97% of the zinc and 84% of

TABLE 2

Interaction of EDTA, zinc and phytate when added to a casein-based diet containing 1.6% calcium [1]

Without EDTA		Na_2 EDTA (0.1%)	
Basal	+ 1% Phytate	Basal	+ 1% Phytate
g	*g*	*g*	*g*
32 ± 2.7 [2]	8 ± 1.1	26 ± 2.2	23 ± 1.8

[1] Six animals per treatment.
[2] Average weekly gain for 4 weeks ± SE of mean.

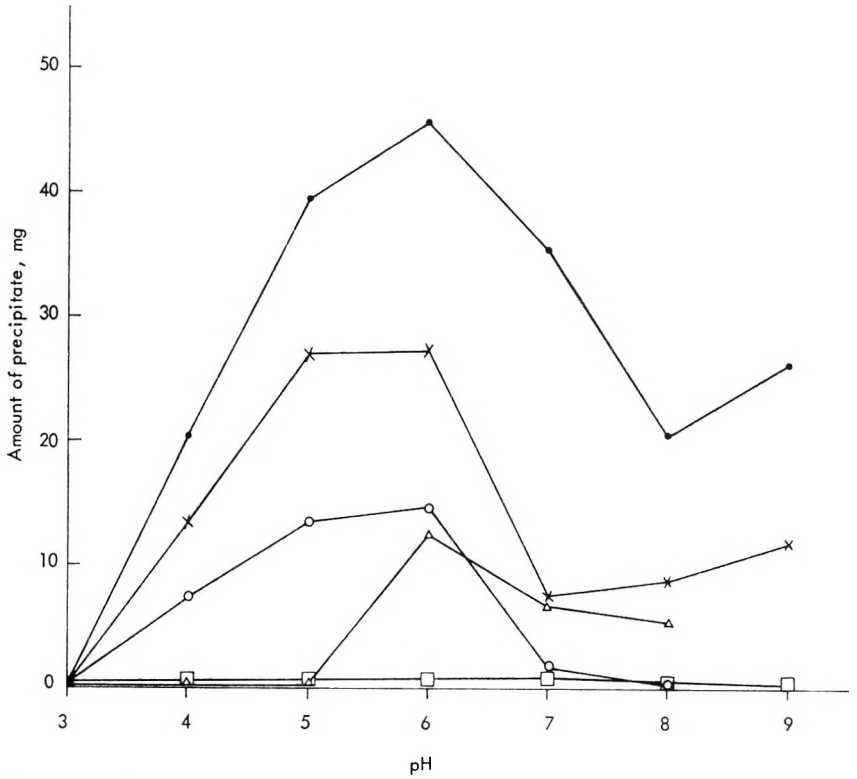


Fig. 1 Relation of solubility of metal phytate complexes to pH and ratios of calcium and zinc to phytate. □——□, Ca:phytate (1:1); △——△, Ca:phytate (2:1); ○——○, Zn:phytate (1:1); ×——×, Ca:Zn:phytate (1:1:1); ●——●, Ca:Zn:phytate (2:1:1).

the calcium originally present. Hoff-Jorgenson (17) has shown that the equilibrium between calcium and phytate is reached almost instantaneously, and that the solubility product of pentacalcium phytate is between 10^{-19} and 10^{-23}. Visual observations made in this study indicate that the zinc and zinc-calcium phytate equilibriums are also established rapidly.

If these results can be projected to a physiological situation, it would be expected that zinc salts of phytate in the food would be dissociated at the pH of gastric juice, and at the pH of the small intestine to exhibit minimum solubility. Because of the solubilizing effect in the stomach and the subsequent dilution of ions, it might be expected that the zinc would be nearly as available from zinc phytate as from other salts fed in low concentration. Green et al.[9] have demonstrated this fact in swine. In order for phytate to make zinc unavailable, the phytate must be present in sufficient quantity to overcome the effect of dissociation and dilution in the stomach and to assure formation of an insoluble compound in the intestine before absorption occurs.

In the in vitro experiments described here the ratio of zinc to calcium was extremely high for an animal diet. A conventional diet might contain 1% calcium, 30 mg/kg of zinc, and 1% of phytate, molar ratios of 540:1:33. Although the in vitro studies do not accurately represent a physiological situation, they clearly show that in the presence of excess calcium more zinc is precipitated by phytate. This offers an explanation for the observed effect that calcium decreases zinc availability when added to a diet contain-

[9] Green, J. D., J. T. McCall, V. C. Speer and V. W. Hays 1962 Effect of complexing agents on utilization of zinc by pigs. J. Animal Sci., 21: 997 (abstract).

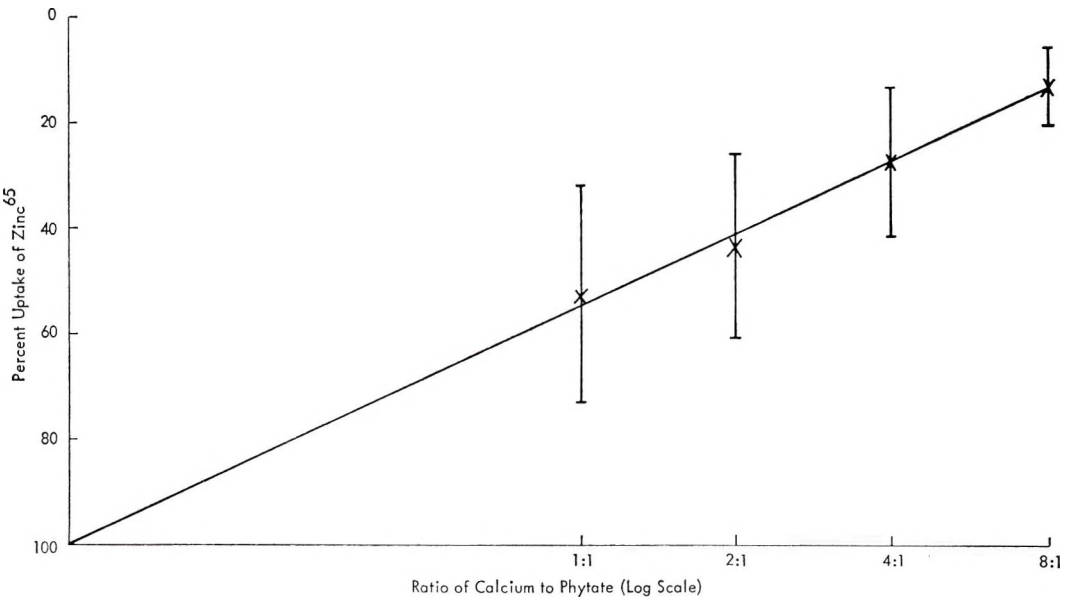


Fig. 2 Uptake of ^{65}Zn by rat jejunal tissue (per mg of N) as a percentage of the control in each replicate (7 replicates). The control contained no calcium or phytate. *X* represents mean at each ratio, and the vertical lines represent variation as determined by the least squares method.

ing phytate but fails to do so in the absence of phytate.

Tissue uptake of ^{65}Zn. The studies with intestinal strips were conducted in an attempt to more nearly duplicate physiological conditions. Preliminary work indicated that zinc was not actively absorbed by the everted sacs according to the criteria of Dowdle et al. (18). However, ^{65}Zn was taken up by the tissue in a manner similar to that reported for magnesium (19). The zinc accumulated was not easily removed by washing and thus it did not appear to be a simple adsorption phenomenon. The process was not affected by a nitrogen atmosphere, or by the addition of sodium cyanide, sodium iodoacetate, copper, cadmium, or ATP to the buffer medium. Neither was the rate of uptake affected by the zinc status of the animal. As used here the term uptake does not imply true absorption but suggests the movement of zinc into cells of the intestinal mucosa.

The effect of increasing the calcium-to-phytate ratio upon the uptake of ^{65}Zn by intestinal strips is shown in figure 2. These data include the results of 7 replicate experiments. Uptake was estimated by the ^{65}Zn activity per mg of tissue nitrogen and the values plotted are the averages of the percentage uptake based on the control in each replicate. The control vessels contained neither calcium nor phytate in the medium. Another series which contained graded levels of calcium but no phytate did not differ from the controls. A calcium-to-phytate ratio of 16:1 was included in most replicates but since it did not differ from the 8:1 ratio the values were not

TABLE 3

Effect of EDTA on the uptake of ^{65}Zn *by intestinal tissue in vitro* [1]

Ca:phytate ratio	Radioactivity [2]/mg N		Increase due to EDTA
	Without EDTA	With EDTA	
	10^4 μCi	10^4 μCi	%
Control	23.6	22.8	−3.5
1:1	9.8	10.3	5.1
2:1	6.4	7.6	18.8
4:1	3.6	4.1	13.9
8:1	2.7	3.5	29.6
16:1	3.5	4.1	17.1

[1] The medium contained 10^{-3} M phytate, 1.3×10^{-6} M zinc and, when added, 1.3×10^{-6} M EDTA. The molar concentration of calcium chloride varied as shown in the first column; the control contained neither calcium nor phytate.

[2] Average radioactivity of ^{65}Zn (3 trials), expressed as microcuries times 10^4.

plotted. The coefficient of correlation between zinc uptake and calcium-to-phytate ratios was -0.639, a value which is statistically highly significant ($P < 0.001$).

The effect of EDTA in the in vitro system is shown in table 3. EDTA was added in an equimolar ratio with zinc. In preliminary experiments it was shown that higher concentrations of EDTA decreased the uptake of ^{65}Zn by tissue in vitro, suggesting that excess EDTA may under some circumstances actually decrease zinc availability. When the calcium-to-phytate ratio in the medium was 2:1 or greater the uptake of zinc by the tissue was increased from 14 to 30% by addition of EDTA. An increase of this magnitude could make a significant contribution toward the zinc requirement of an animal fed a sub-optimal level of zinc in the presence of phytate.

The results presented here indicate that dietary phytate decreases the physiological availability of zinc to the rat. Excess calcium also decreases availability but only in the presence of phytate. There appears to be a three-way interaction among zinc, calcium and phytate. Similar results have been obtained by Byrd and Matrone (20). The chemical complexation of zinc by phytate, particularly in the presence of calcium to form an insoluble and non-absorbable compound is believed to be the mechanism by which zinc is made less available to animals. It appears likely that EDTA improves availability in competition with phytate by forming a soluble zinc-EDTA complex which when presented to the absorption sites in the intestine allows the absorption of zinc. The possibility that the total zinc-EDTA complex is absorbed cannot be eliminated. Results with chickens (21) show that EDTA can be absorbed, but work with rats suggests that very little is absorbed (22).

LITERATURE CITED

1. Todd, W. R., C. A. Elvehjem and E. B. Hart 1934 Zinc in the nutrition of the rat. Amer. J. Physiol., *107:* 146.
2. Tucker, H. F., and W. D. Salmon 1955 Parakeratosis or zinc deficiency disease in the pig. Proc. Soc. Exp. Biol. Med., *88:* 613.
3. O'Dell, B. L., and J. E. Savage 1960 Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med., *103:* 304.
4. Maddaiah, V. T., A. A. Kurnick and B. L. Reid 1964 Phytic acid studies. Proc. Soc. Exp. Biol. Med., *115:* 391.
5. Likuski, H. J. A., and R. M. Forbes 1964 Effect of phytic acid on the availability of zinc in amino acid and casein diets fed to chicks. J. Nutr., *84:* 145.
6. Oberleas, D., M. E. Muhrer and B. L. O'Dell 1962 Effects of phytic acid on zinc availability and parakeratosis in swine. J. Animal Sci., *21:* 57.
7. Likuski, H. J. A., and R. M. Forbes 1965 Mineral utilization in the rat. IV. Effect of calcium and phytic acid on the utilization of dietary zinc. J. Nutr., *85:* 230.
8. Forbes, R. M. 1960 Nutritional interactions of zinc and calcium. Federation Proc., *19:* 643.
9. O'Dell, B. L., J. M. Yohe and J. E. Savage 1964 Zinc availability in the chick as affected by phytate, calcium and ethylendiaminetetraacetate. Poultry Sci., *43:* 415.
10. Kratzer, F. H., J. B. Allred, P. N. Davis, B. J. Marshall and P. Vohra 1959 The effect of autoclaving soybean protein and the addition of ethylendiaminetetraacetate on the biological availability of dietary zinc for turkey poults. J. Nutr., *68:* 313.
11. Forbes, R. M. 1961 Excretory patterns and bone deposition of zinc, calcium and magnesium in the rat as influenced by zinc deficiency, EDTA and lactose. J. Nutr., *74:* 194.
12. Earley, E. B. 1944 Determining phytin phosphorus. Ind. Eng. Chem. (Anal. Ed.), *16:* 389.
13. Fiske, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.
14. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.
15. Rush, R. M., and J. H. Yoe 1954 Colorimetric determination of zinc and copper with 2 carboxy-2′-hydroxy-5′-sulfoformazylbenzene. Anal. Chem., *26:* 1345.
16. Wilson, T. H., and G. Wiseman 1954 The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol., *123:* 116.
17. Hoff-Jorgenson, E. 1944 Investigations on the solubility of calcium phytate. Danske Vidensk. Selsk., Mat-fys Medd., *21:* 1.
18. Dowdle, E. B., D. Schachter and H. Schenker 1960 Active transport of ^{59}Fe by everted segments of rat duodenum. Amer. J. Physiol., *198:* 609.
19. Schachter, D., C. B. Dowdle and H. Schenker 1960 Active transport of calcium by the small intestine of the rat. Amer. J. Physiol., *198:* 263.
20. Byrd, C. A., and G. Matrone 1965 Investigations of chemical basis of zinc-calcium-phytate interaction in biological systems. Proc. Soc. Exp. Biol. Med., *119:* 347.

21. Koike, T. I., F. H. Kratzer and P. Vohra 1964 Intestinal absorption of zinc or calcium-ethylenediaminetetraacetic acid complexes in chickens. Proc. Soc. Exp. Biol. Med., *117*: 483.

22. Foreman, H. 1960 Metal Binding in Medicine, eds., M. J. Seven and L. S. Johnson. J. B. Lippincott Company, Philadelphia, p. 82.

Carbohydrate Metabolism and Physical Activity in Rats Fed Diets Containing Purified Casein Versus a Mixture of Amino Acids Simulating Casein [1]

RICHARD A. AHRENS AND JAMES E. WILSON, JR.
Human Nutrition Research Division, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland

ABSTRACT The effects of varying energy intake levels on physical activity and glucose metabolism were studied in young growing rats. Male rats 28 days of age were fed for 31 days diets containing either casein or an amino acid mixture simulating casein at 2 levels of calorie intake. Physical activity was measured and ^{14}C recovery from injected glucose-1-^{14}C, -6-^{14}C, and -U-^{14}C was determined as percentage of dose in expired CO_2, feces, and urine, from one to 24 hours after giving each rat his daily ration. The average revolutions per day run was higher for calorie-restricted animals, but there was no significant difference in activity due to substituting the mixture of amino acids for casein, although casein-fed rats tended to be more active. This increase in physical activity caused by calorie restriction was due to a significant difference in daytime activity, as all rats were equally active in the dark. There were no significant differences due to calorie level or nitrogen source in $^{14}CO_2$ recovery from glucose-6-^{14}C and glucose-U-^{14}C, but amino acid-fed rats converted more glucose-1-^{14}C to $^{14}CO_2$ as measured cumulatively 6 and 23 hours after injection. Calorie-restricted rats expired less glucose-1-^{14}C as $^{14}CO_2$ during the first 6 hours after injection, but this effect was not evident after 23 hours. There was a trend toward lower ^{14}C recovery from glucose-U-^{14}C in urinary citrate of amino acid-fed rats, although urinary citrate excretion was unaltered by diet. These data indicate a greater utilization of alternative pathways to the glycolytic scheme and tricarboxylic acid cycle for metabolism of glucose in amino acid-fed rats and several possible explanations are discussed.

Work reported from this laboratory in a previous paper (1) showed that for young rats fed 2 levels of nitrogen and at 2 levels of calorie intake, there were higher nitrogen gains when rats in the high calorie group received the nitrogen as casein rather than as a mixture of amino acids simulating casein. Rats of the same age fed diets providing similar nitrogen intakes at a lower calorie level showed no significant advantage in nitrogen storage for casein over amino acids. The dependence of nitrogen storage on calorie intake raised a question as to the effect the substitution of casein for amino acids might have on energy metabolism.

Calorie intake level has been reported to affect the physical activity of the rat, but the nature of this effect is uncertain. Hughes (2) reported increased physical activity in food-deprived animals, but Fabry et al. (3) interpreted their oxygen-consumption data as indicating reduced activity in animals that were intermittently starved. Caloric restriction has been implicated in the activity of pathways of carbohydrate metabolism. Lee and Lucia (4) reported a decrease in the direct oxidation of glucose with caloric restriction to the extent that severe restriction caused the hexose monophosphate pathway to disappear irreversibly. Benevenga et al. (5) noted that a starvation-refeeding regimen produced significant increases in pentose phosphate-metabolizing enzyme activity.

The present paper reports the effect in rats of substituting casein for a mixture of amino acids simulating casein as measured by ^{14}C-labeled glucose metabolism at either of 2 calorie intake levels. To study the effect, if any, of the dietary regimens on voluntary activity the rats were allowed access to activity cages.

Received for publication March 16, 1966.

[1] Preliminary results of the investigation were reported to the American Institute of Nutrition at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1966.

EXPERIMENTAL

Specific-pathogen-free male rats [2] obtained at 21 days of age were housed individually and fed a stock diet [3] for one week before being fed their particular experimental regimen. A control group of 6 rats was killed to determine initial carcass content of calories and nitrogen and to furnish data for correcting initial weight of the experimental animals to the ingesta-free basis. On the basis of these data, initial live weight was multiplied by 91.9% to obtain ingesta-free carcass weight. Contents of the gastrointestinal tract were removed and the carcasses homogenized in a Waring Blendor before analysis (6).

Four diets were used in this study: 1) high calorie level with casein as the nitrogen source; 2) high calorie level with an L-amino acid mixture simulating casein as the nitrogen source; 3) restricted calories with nitrogen provided as casein; and 4) restricted calories with nitrogen provided as amino acids. Diets at the high calorie level contained 3% nitrogen. Restricted-calorie diets were formulated with increases in proportion of protein or amino acids, vitamins, salts, and roughage so that, when calorie intake was reduced from the high calorie level by approximately one-third, intakes of those nutrients were the same as the intakes at the higher calorie level. The diets and care of the animals have been described earlier (1).

The casein and amino acids were assayed for purity as reported earlier (1). The rats, 6 per group, were housed in a well-ventilated room at 28°, 50% relative humidity, and 12 hours per day of darkness and light. After being fed the experimental diets for 2 weeks all rats were assigned at random to rotating-treadmill activity cages for 3 weeks and reading of revolutions turned were taken at 12-hour intervals coinciding with the darkness/light changeover. During this 3-week period 1-μCi doses of high specific activity glucose-1-^{14}C, glucose-6-^{14}C, and glucose-U-^{14}C were injected intraperitoneally at weekly intervals into each rat in a pattern of reversal (i.e., 2 rats in each group received glucose-1-^{14}C the first week, 2 rats received glucose-1-^{14}C the second week, etc.). Each 1-μCi dose involved the injection of 0.036 to 0.060 mg of glucose. All rats were placed in the respiration apparatus with their daily ration one hour before ^{14}C injection and remained there for 23 hours after injection where all ^{14}C lost in CO_2, feces, and urine was recovered. All solid samples were dried, ignited in an O_2 bomb, and $^{14}CO_2$ was trapped and counted as $Ba^{14}CO_3$. Respired $^{14}CO_2$ was collected at 30-minute intervals for the first 6 hours, and again determined at 11 and 23 hours. All samples were counted at infinite thickness in a thin-window gas-flow counter with an anticoincidence correction.[4] Total CO_2 recovery was determined and all specific activities were converted to total activity and expressed as percentage of dose injected as suggested by Wang (7). This avoids the dilution effects of endogenous materials on specific activity pointed out by Wood (8).

Aliquots of the ^{14}C-labeled glucose samples used were ignited in an O_2 bomb and $Ba^{14}CO_3$ was recovered and counted. The counts recovered in 1 μCi were 92% of the theoretical amount (i.e., 2,042,810 dpm vs. 2,220,000 dpm), close enough so that the difference could be explained by small errors in measuring initial volume, determining counter efficiency,[5] converting infinite to zero thickness values, etc. Eight of the experimental animals were killed within minutes of removal from the respiration apparatus. In these 8 animals the carcass ^{14}C as actually determined was 27.9% ± 3.9 [6] of the injected dose, whereas the ^{14}C recovered in respiratory CO_2, feces, and urine was 68.4% ± 5.4 of the total indicating that about 96% of injected ^{14}C could be accounted for by actual measurement.

Urine was fractionated into a variety of components prior to counting for ^{14}C. Ether and chloroform extracts were made to determine radioactivity present as lactic acid or ketone bodies. Urea CO_2 was released and ^{14}C was counted following treatment of the urine with urease (9). Oxalate was precipitated as calcium oxalate (10) and sugars were precipitated as the osazones (11). Amino acids were absorbed and separated into acidic, neutral, and basic frac-

[2] Lew strain from Microbiological Associates, Bethesda, Maryland.
[3] D & G Research Animal Laboratory Diet, Price-Wilhoite Company, Frederick, Maryland.
[4] All samples were counted for long periods of time on a Sharp "Wide-beta" gas-flow counter having a low background.
[5] See footnote 4.
[6] Standard error of the mean.

tions by the use of Amberlite IR-4 (12) following hydrolysis of the urine with HCl. Creatinine was isolated by the method of Owen et al. (13). Non-amino organic acids were determined with paper chromatography after first passing the sample through a Dowex 50 column to remove amino acids and salts. The strips were developed in a 4:1:5 (v/v) upper phase N-butanol:acetic acid: water solvent system and were sprayed with 0.04% bromophenol blue in 95% ethanol. Spots were identified from standards, cut from the paper, ignited, and $Ba^{14}CO_3$ was determined. The amount of citric acid present was determined by titration of the eluted material with a dilute base.

This study was conducted according to a randomized complete block design where blocks were composed of those 4 animals, one per group, which received the 3 labeled glucose moieties in the same order of injection. Analyses of variance were conducted treating the investigation as a 2 × 2 factorial.

RESULTS AND DISCUSSION

Physical activity. The effect of the dietary treatments on physical activity is shown in table 1. Diets were given once daily just after the darkness to light changeover. Rats receiving the restricted-calorie intake level consumed all of their diet during the first hour after feeding. Rats fed the casein diet at the high calorie level ate all of their diet during the first 8 hours after feeding. Rats fed the amino acid diet at the high calorie level ate all of the diet given them, but took 12 hours or more to do so. At either level of calorie intake there was no significant effect on physical activity due to substituting casein for amino acids as the nitrogen source, but there was a consistent tendency for the casein-fed rats to be more active, which bordered on significance. An analysis of variance, however, showed a highly significant effect ($P < 0.01$) of calorie intake on voluntary physical activity. This increase in activity due to caloric restriction appears to reflect an abolition of quiescence rather than an elevation of peak activity. During the 12 hours of darkness when the rat is normally most active (3), there was no difference in physical activity between groups. The 12 hours of light caused no appreciable change in the activity of the calorie-restricted animals, but the illumination caused a significant reduction in activity for rats fed at the high calorie level ($P < 0.01$). Thus, rats receiving the high calorie intake had about 70% of their activity at night, whereas calorie-restricted animals had nearly the same activity, day or night. Food deprivation appears to influence voluntary activity of the rat in a manner which overrides normal diurnal variation. The pattern of physical activity and the eating pattern appear to be related.

Carcass gains. The calorie, nitrogen, and ingesta-free weight gains are given in table 2. Calorie, nitrogen, and weight gains were significantly reduced ($P < 0.01$) by calorie restriction. Rats receiving casein at the higher calorie level gained more weight ($P < 0.05$) than amino acid-fed rats; although the change in nitrogen source had no statistically significant effect on calorie gain due to greater variability in the calorie gain data, there was a trend toward greater calorie gain in casein-fed rats, similar to the trend in our earlier report (1). An

TABLE 1

Diurnal variation in revolutions turned in activity cages over a 3-week period by rats fed nitrogen either as amino acids or as casein, at 2 levels of calorie intake

N source [1]	Intake [2]		Mean voluntary physical activity		
	N	Calories	12-hr light	12-hr dark	24-hr total
	g	*kcal*	*rev*	*rev*	*rev*
AA	8.59	1400	1379 ± 456 [3]	4143 ± 701	5523 ± 838
Casein	8.59	1425	1981 ± 465	4230 ± 736	6211 ± 997
AA	8.93	982	4584 ± 1023	3961 ± 417	8544 ± 1153
Casein	8.94	1040	6074 ± 1169	4964 ± 1050	11,038 ± 1158

[1] AA = amino acid mixture simulating casein.
[2] Mean dietary intake/rat over the entire 31-day experimental period.
[3] SE of mean; 6 rats/group.

TABLE 2

Weight gains, calorie and nitrogen storage of rats fed casein or a mixture of amino acids simulating casein at 2 calorie intake levels

N source [1]	Intake [2]		Weight gains [3]	Nitrogen stored	Calories stored
	N	Calories			
	g	*kcal*	*g*	*g*	*kcal*
AA	8.59	1400	78 ± 3.2 [4] *	3.35 ± 0.21	113.7 ± 11.4
Casein	8.59	1425	87 ± 1.6 *	3.59 ± 0.07	129.7 ± 9.4
AA	8.93	982	25 ± 3.5	1.59 ± 0.15	14.8 ± 5.5
Casein	8.94	1040	33 ± 2.3	1.87 ± 0.11	26.3 ± 3.3

[1] AA = amino acid mixture simulating casein.
[2] Mean dietary intake/rat over the entire 31-day experimental period.
[3] Gain in ingesta-free carcass weight over the 31-day period.
[4] SE of mean; 6 rats/group.
* Adjoining means significantly different ($P < 0.05$).

analysis of variance showed a significant difference between casein and amino acid-fed rats in nitrogen gain per gram of digestible nitrogen intake ($P < 0.05$), with casein-fed rats being more efficient, and there was a trend toward greater nitrogen storage per gram of gross nitrogen intake in these rats. With large groups in the earlier study, differences in gross nitrogen storage were found to be significant.

Radiorespirometry studies. There was no significant effect of energy level or nitrogen source on the percentage of injected dose recovered in expired $^{14}CO_2$, feces, or urine during the 23 hours following injection of glucose-6-^{14}C and glucose-U-^{14}C (table 3). An analysis of variance shows that the nitrogen source in the diet had a significant effect ($P < 0.05$) on the percentage of injected glucose-1-^{14}C recovered as $^{14}CO_2$ (table 4). This is in agreement with our earlier report of differences in nitrogen storage at the high calorie intake level, since a change in the activity of the pathways of carbohydrate metabolism would be expected to alter protein metabolism as well. The significantly greater recovery of $^{14}CO_2$ from glucose-1-^{14}C in the amino acid-fed rats indicates greater use of alternative pathways of carbohydrate metabolism in these animals (8). The time the diet was fed since the last injection did not appear to affect the metabolism of the labeled glucose, as block effects were not significant. Despite reports of changes in the activity of the hexose monophosphate shunt due to calorie restriction (4, 5) our $^{14}CO_2$ recovery totals indicated no difference due to varying the calorie level (tables 3, 4). The explanation for this difference in results can best be explained by examining the pattern of $^{14}CO_2$ recovery plotted against time. Figure 1 shows the $^{14}CO_2$ recovery from glucose-1-^{14}C from the second to the twenty-fourth hour after feeding. An analysis of variance (table 4) of the percentage of injected glucose-1-^{14}C recovered as $^{14}CO_2$ after 6 hours shows a significant effect of calorie level ($P < 0.05$). This agrees with the earlier report of Lee and Lucia (4), who used a 3.5-hour collection period, that calorie-restricted animals have a less active hexose monophosphate shunt. This measurement of activity of the hexose monophosphate shunt does not appear to reflect accurately the total amount of glucose metabolized by this route, at least for the casein-fed rats, since rats fed casein at the high calorie intake showed a reduced $^{14}CO_2$ release from the sixth to twenty-third hour after injection, whereas calorie-restricted animals fed casein maintained a steady rate of $^{14}CO_2$ production. As a result, the percentage of injected glucose-1-^{14}C metabolized to $^{14}CO_2$ after 23 hours was unaffected by calorie intake. The effect of changing nitrogen source in the diet on $^{14}CO_2$ production was significant at 6 hours after injection ($P < 0.01$) as well as after 23 hours ($P < 0.05$). An examination of the mean squares gives an indication of the greater magnitude of the effect of source of dietary nitrogen on glucose-1-^{14}C metabolism as compared with the effect of calorie restriction (table 4). An analysis of variance of $^{14}CO_2$ recovery after 6 hours from glucose-6-^{14}C and glucose-U-^{14}C showed no effect due to calorie level or nitrogen source, similar to the total 23-hour observations.

TABLE 3

Percentage of injected ^{14}C from radioactive glucose recovered in expired $^{14}CO_2$, feces, and urine during the first 23 hours following injection

N source [1]	Intake [2]		Glucose-1-^{14}C			Glucose-6-^{14}C			Glucose-U-^{14}C			
	N	Calories	$^{14}CO_2$	Feces	Urine	$^{14}CO_2$	Feces	Urine	$^{14}CO_2$	Feces	Urine	Urinary citrate [4]
	g	*kcal*	*% injected ^{14}C*			*% injected ^{14}C*			*% injected ^{14}C*			
AA	8.59	1400	67.7 ± 4.4 [3]	1.1 ± 0.3	1.9 ± 0.4	38.0 ± 4.0	2.9 ± 0.5	12.8 ± 4.1	53.7 ± 6.3	2.8 ± 0.7	14.6 ± 4.4	3.7 ± 1.2
Casein	8.59	1425	53.1 ± 4.8 [3] *	0.6 ± 0.2	1.9 ± 0.3	37.4 ± 7.4	3.4 ± 1.3	12.5 ± 2.7	52.8 ± 6.3	2.0 ± 0.4	14.3 ± 3.5	5.5 ± 2.4
AA	8.93	982	63.4 ± 3.9	0.9 ± 0.4	3.1 ± 0.4	46.7 ± 10.2	3.0 ± 1.1	16.6 ± 5.5	63.4 ± 8.4	2.1 ± 0.7	16.6 ± 4.9	2.6 ± 1.3
Casein	8.94	1040	56.6 ± 4.9	0.4 ± 0.1	2.0 ± 0.2	33.0 ± 3.5	2.6 ± 0.9	18.9 ± 3.9	46.2 ± 5.3	2.6 ± 0.7	19.3 ± 5.3	5.7 ± 2.1

[1] AA = amino acid mixture simulating casein.

[2] Mean dietary intake/rat over the entire 31-day experimental period.

[3] SE of mean; 6 rats/group.

[4] Specific activities (μCi/mEq) were: AA, 1400 kcal = 0.037 ±0.012; casein, 1425 kcal = 0.057 ± 0.025; AA, 982 kcal = 0.024 ± 0.012; casein, 1040 kcal = 0.052 ± 0.019; none of these values proved to be significantly different from the others.

* Designated means significantly different ($P < 0.05$).

The differences in physical activity between day and night in rats receiving the high calorie intake might have been altered if the rats had been fed at night rather than the early morning, and this might have had an effect on metabolism. However, all rats in the present study were fed in the same manner and according to the same schedule so that the differences observed are valid within the confines of this particular schedule of care and feeding, which is that most commonly followed for laboratory rats.

We observed earlier that rats fed casein diets at a high calorie level consumed their diets more rapidly than rats fed a comparable amino acid diet (1). Tepperman and Tepperman (14) and Hollifield and Parson (15) reported evidence for greater activity of the hexose monophosphate shunt and greater fat deposition in rats trained to eat their food in a shorter period of time. This does not appear to be the reason for the greater use of the hexose monophosphate shunt in our amino acid-fed rats, therefore, since faster eating by the casein-fed rats would be expected to increase hexose monophosphate shunt activity in these animals. In addition, the $^{14}CO_2$ recovery data indicated a similar difference between casein- and amino acid-fed rats at a restricted level of intake (tables 3 and 4) where all rats consumed their diets in one hour or less.

These data are not consistent with the concept that voluntary activity reflects the state of energy metabolism in the animal. Although calorie restriction increased voluntary activity ($P < 0.01$), it had little effect on $^{14}CO_2$ recovery from injected glucose-1-^{14}C over a 23-hours period. Furthermore, glucose-1-^{14}C metabolism was affected most by substituting for casein in the diet a mixture of amino acids simulating casein. This change in nitrogen source had no significant effect on voluntary activity.

Urinary ^{14}C excretion. Table 3 lists the percentage of injected ^{14}C recovered in the urine during the 23 hours following administration of glucose-1-^{14}C, -6-^{14}C and -U-^{14}C. Although dietary treatment had no effect on urinary ^{14}C excretion, a greater percentage of the injected dose ($P < 0.01$) was excreted in urine when glucose-6-^{14}C or

TABLE 4

Mean squares of the analyses of variance for $^{14}CO_2$ recovery and urinary N

Source of variation	$^{14}CO_2$ recovered				Urinary N excretion
	Labeled carbohydrate administered				
	Glucose-U-^{14}C	Glucose-6-^{14}C	Glucose-1-^{14}C		
	(% /23 hr) [2]	(% /23 hr) [2]	(% /6hr) [2]		(mg/24 hr) [3]
Blocks	354.17	514.33	233.32	148.00	479
E = energy level	13.78	29.10	0.95	425.97*	16,970**
S = nitrogen source	485.55	308.24	688.33*	1,226.23**	1,883*
ES	397.97	256.04	89.12	10.28	170
Error	238.60	199.87	84.08	74.67	446

** Significant ($P < 0.01$).
* Significant ($P < 0.05$).

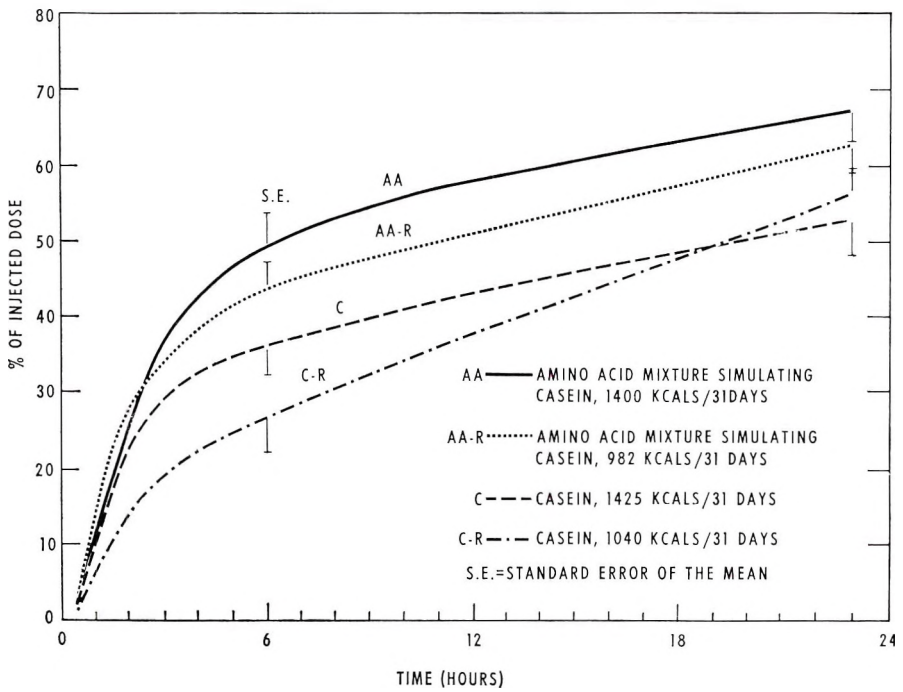


Fig. 1. Effect of calorie intake and nitrogen source on percentage of ^{14}C from intraperitoneally injected glucose-1-^{14}C recovered as $^{14}CO_2$.

glucose-U-^{14}C was given than when glucose-1 ^{14}C was the injected material. Urea ^{14}C accounted for only about 2% of urinary ^{14}C from glucose-6-^{14}C or glucose-U-^{14}C. Urease released about 20% of the urinary ^{14}C contributed by glucose-1-^{14}C, indicating that the bulk of the ^{14}C excreted was in other end products of metabolism.

Urinary ^{14}C excretion from glucose-6-^{14}C or glucose-U-^{14}C was similar, indicating that the terminal 5 carbons of glucose were metabolized by the same route. In fractionating the urinary ^{14}C activity, less than 5% was found in oxalic acid and between 5 and 10% was found in the ether extract, creatinine, osazone, and dicarboxylic amino acid fractions. The quantitatively greatest source of urinary ^{14}C was found to be citrate which accounted for about 30% of the urinary label and the percentage of injected glucose-U-^{14}C that was recovered as urinary citrate is reported in table 3.

Although no significant differences could be established due to the high variability in citrate-^{14}C excretion, a clear trend was evident indicating greater ^{14}C recovery in urinary citrate when casein was the nitrogen source rather than a mixture of amino acids simulating casein. The mean citrate excretion in the urine of rats consuming the 4 diets was nearly the same, ranging from 0.96 ± 0.11 mEq/day with the high calorie diet containing casein to 1.10 ± 0.12 mEq/day with low calorie diet containing casein. Bellin and Steenbock (16) earlier reported wide variation in citraturia regardless of dietary changes.

The specific activity of urinary citrate following glucose-U-^{14}C administration tended to be greater from rats fed casein than from those fed amino acids (table 3). If this trend is assumed to be real and urinary citrate is assumed to be representative of tricarboxylic acid cycle intermediates throughout the body, it appears that more of the ^{14}C from glucose is passing through the glycolytic scheme and into the tricarboxylic acid cycle in the casein-fed rats. This is consistent with the conclusion from the radiorespirometry data that rats fed amino acid diets derived a significantly larger portion of their energy from direct oxidation of glucose than casein-fed rats did. However, the possibility exists that the diammonium citrate in the amino acid diets diluted the ^{14}C activity in body citrate pools since the dietary intake of citrate was about 2 mEq/day and urinary output was about 1 mEq/day. The explanation for apparently greater use of the glycolytic scheme and tricarboxylic acid cycle in glucose metabolism of casein-fed rats is not clear. There are several possible explanations, including the following:

1) A dilution of the tricarboxylic acid cycle intermediates with intermediates from rapid amino acid breakdown might cause increased gluconeogenesis and a resulting inhibition of glycolysis causing glucose to be diverted to alternative pathways of metabolism. The normal route of glutamic acid breakdown involves the formation of α-ketoglutarate (17), whereas propionic acid formed in the degradation of methionine and threonine (18), serine, alanine, valine, and isoleucine (19) can be converted to succinate (20) in rather large quantities (21). Succinate and α-ketoglutarate thus formed could be metabolized via the tricarboxylic acid cycle. Gupta et al. (22), however, observed that free amino acids did not disappear from the digestive tract more rapidly than intact protein unless the protein was relatively insoluble (zein). This would indicate that amino acid absorption in rats fed casein or amino acids is not different.

2) The NH_4 citrate supplement added to amino acid diets to increase their N content might be metabolized by the tricarboxylic acid cycle and utilized for gluconeogenesis, resulting in inhibition of glycolysis and causing glucose to be diverted to alternative pathways of metabolism. A quantitatively great effect would not be expected, however, since the daily intake of NH_4 citrate by amino acid fed rats was about 0.16 g.

3) There are a number of recent reports in the literature that would suggest that the amino acid mixture used in this study, despite the fact that it simulates casein, does not contain adequate glutamic acid, glutamine, asparagine and arginine to support maximal weight gains of rats (23–26). Most workers who have fed "adequate levels" of dispensable and indispensable amino acids still obtain growth rates somewhat less than they obtain with protein-containing diets and thus the possibility of deficiencies of unknown factors such as strepogenin, postulated by Woolley (27), cannot be eliminated. Schwartz et al.[7] have indicated preliminary evidence for the existence of a previously unrecognized growth factor in 15% casein diets. The metabolic adaptation in direct oxidation of glucose observed in this study may be a consequence of a deficiency of either known or unknown substances.

The trend toward increased non-protein calorie storage (presumably fat) in young rats fed amino acid rather than casein diets reported in an earlier paper (1), might be due to the increased direct oxidation of glucose on amino acid diets and might be an adaptation to a deficiency.

---

[7] Schwarz, K., J. C. Smith and T. A. Oda 1966 Factor G, an agent promoting growth of animals on amino acid diets. Federation Proc., 25: 542 (abstract).

LITERATURE CITED

1. Ahrens, R. A., J. E. Wilson, Jr. and M. Womack 1966 Calorie and nitrogen storage from diets containing purified casein versus a mixture of amino acids simulating casein. J. Nutr., *88:* 219.
2. Hughes, R. N. 1965 Food deprivation and locomotor exploration in the white rat. Animal Behav., *13:* 30.
3. Fabry, P., R. Petrasek, E. Horakova, E. Konopasek and T. Braun 1963 Energy metabolism and growth in rats adapted to intermittent starvation. Brit. J. Nutr., *17:* 295.
4. Lee, M., and S. P. Lucia 1961 Some relationships between caloric restriction and body weight in the rat. II. The metabolism of radioactive glucose and the activity of some TPN-linked enzymes in the liver. J. Nutr., *74:* 249.
5. Benevenga, N. J., W. J. Stielau and R. A. Freedland 1964 Factors affecting the activity of pentose phosphate-metabolizing enzymes in rat liver. J. Nutr., *84:* 345.
6. Womack, M., M. W. Marshall and H. E. Hildebrand 1964 Utilization of wheat gluten by adult rats of two ages. J. Gerontol., *19:* 45.
7. Wang, C. H. 1961 Metabolism studies by radiorespirometry. In: Advances in Tracer Methodology, vol. 1, ed., S. Rothchild. Plenum Press, New York, pp. 274–290.
8. Wood, H. G. 1955 Significance of alternate pathways in the metabolism of glucose. Physiol. Rev., *35:* 841.
9. Sumner, J. B. 1926 The isolation and crystallization of the enzyme urease. J. Biol. Chem., *69:* 435.
10. Harrison, H. E., and H. C. Harrison 1955 A micromethod for determination of serum calcium. J. Lab. Clin. Med., *46:* 662.
11. Noggle, C. R. 1957 The identification and the quantitative determination of carbohydrates. In: The Carbohydrates, ed., W. Pigman. Academic Press, New York, pp. 608–610.
12. Cannan, R. K. 1944 The estimation of the dicarboxylic amino acids in protein hydrolysates. J. Biol. Chem., *152:* 401.
13. Owen, J. B., B. Iggo, F. J. Scandrett and C. P. Stewart 1954 The determination of creatinine in plasma or serum, and in urine; a critical examination. Biochem. J., *58:* 426.
14. Tepperman, J., and H. M. Tepperman 1958 Effects of antecedent food intake pattern on hepatic lipogenesis. Amer. J. Physiol., *193:* 55.
15. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 245.
16. Bellin, S. A., and H. Steenbock 1952 Vitamin D and citraturia. J. Biol. Chem., *194:* 311.
17. Dianzani, M. U. 1955 Content and distribution of pyridine nucleotides in fatty livers. Biochim. Biophys. Acta, *17:* 391.
18. Fruton, J. S., and S. Simmonds 1958 Special aspects of amino acid metabolism. In: General Biochemistry, ed. 2. John Wiley and Sons, New York, pp. 790–795.
19. Feller, D. D., and E. Feist 1962 Metabolism of alanine, serine, and valine in adipose tissue. Metabolism, *11:* 448.
20. Lardy, H. A., and J. Adler 1956 Synthesis of succinate from propionate and bicarbonate by soluble enzymes from liver mitochondria. J. Biol. Chem., *219:* 933.
21. Friedberg, F., J. Adler and H. A. Lardy 1956 The carboxylation of propionic acid by liver mitochondria. J. Biol. Chem., *219:* 943.
22. Gupta, J. D., A. M. Dakrowry and A. E. Harper 1958 Observations on protein digestion in vivo. I. Rate of disappearance of ingested protein from the gastrointestinal tract. J. Nutr., *64:* 447.
23. Rogers, Q. R., and A. E. Harper 1965 Amino acid diets and maximal growth in the rat. J. Nutr., *87:* 267.
24. Breuer, L. H., Jr., W. G. Pond, R. G. Warner and J. K. Loosli 1964 The role of dispensable amino acids in the nutrition of the rat. J. Nutr., *82:* 499.
25. Ranhotra, G. S., and B. C. Johnson 1965 Effect of feeding different amino acid diets on growth rate and nitrogen retention of weanling rats. Proc. Soc. Exp. Biol. Med., *118:* 1197.
26. Hepburn, F. N., and W. B. Bradley 1964 The glutamic acid and arginine requirement for high growth rate of rats fed amino acid diets. J. Nutr., *84:* 305.
27. Woolley, D. W. 1945 Observations on the growth-stimulating action of certain proteins added to protein-free diets compounded with amino acids. J. Biol. Chem., *159:* 753.

Diurnal Rhythms of Tissue Components Related to Protein Metabolism in Normal and Virus-infected Chicks [1]

ROBERT L. SQUIBB

Laboratories of Disease and Environmental Stress, Bureau of Biological Research, Rutgers, The State University, New Brunswick, New Jersey

ABSTRACT Diurnal changes in protein metabolism were studied over a 72-hour incubation period in Newcastle disease virus-infected chicks. Protein, DNA, RNA and free amino acid levels were determined, where applicable, in liver, muscle and serum at 4 daily intervals. The data, which included non-infected controls, were plotted in relation to clock hours. Significant diurnal rhythms in all 3 tissues indicated the presence of periodicity. In the controls, liver DNA was highest in the evening but maximal values of RNA, protein and the free amino acids occurred during the day. While the free amino acid pool in the liver was significantly depressed by the Newcastle disease virus (NDV), in the serum there was an increase in values. The NDV also caused a significant desynchronization in relation to clock hours of the rhythmic patterns in the liver and to a lesser extent in muscle. The effect in the liver was noted within 12 hours post-inoculation. Serum patterns in the NDV chicks, however, remained synchronized during the incubation period of the disease cycle. The effect of these diurnal rhythms on experimental error was discussed.

An earlier report from these laboratories (1) described significant diurnal oscillations of total protein and the nucleic and free amino acids in avian liver during the active involvement stage of a Newcastle disease virus (NDV) infection. The patterns of the oscillations differed in control and infected tissues, but in both cases the patterns suggested a relationship to clock hours. The fact that diurnal oscillations of tissue components were observed in both normal and NDV-infected animals raised the question whether there would be a similar effect during the incubation stage of NDV.

The phenomenon of diurnal rhythms has been known for many years. Halberg (2), Simmonet (3) and Bünning (4) have made extensive reviews of the literature. However, as far as is known, none of the research has been directed toward a simultaneous comparison of diurnal changes in protein metabolism, in the normal as well as the diseased states, in several tissues that reflect the body's major protein stores.

The present paper reports the results of an experiment undertaken to study diurnal changes in several parameters of protein metabolism in liver, blood and muscle in 4-week-old chicks during the first 72 hours (incubation stage) of an NDV infection.

PROCEDURES

A standard reference diet (5) was fed ad libitum to approximately 400 one-day-old White Leghorn cockerels of known breeding for a 4-week period. At the end of that time average weekly body weights, compared with a reference curve, indicated that the chicks had met their genetic potential for growth with this particular reference diet. Thus standardized, the birds were divided into 2 groups: Group 1 served as non-infected controls; those in group 2 were injected in the leg muscle with NDV (0.1 ml/bird of a 10^{-3} concentration of virus). In our laboratories 50% mortality in 8 days can be expected from this virus concentration. The groups, housed in separate isolated rooms, were continued on the same management procedures that prevailed prior to this time, namely, constant artificial light and temperature, and the same caretaker who entered the rooms at the same time each day. The standard

Received for publication March 3, 1966.

[1] Supported in part by Contract no. DA-49-193-MD-2694, U. S. Army Medical Research and Development Command.

reference diet and water were continued ad libitum.

Starting at time of NDV inoculation (0800), 8 birds were selected at random from each treatment group and killed at 0800, 1500, 2000 and 2400 hours for 3 consecutive days, the incubation period of the NDV (5). The same personnel participated in the tissue sampling throughout the experiment. At each killing period body and liver weights were recorded and individual samples of serum, liver and pectoral muscle were obtained and stored in glass containers under deep refrigeration. Depending on the tissue, the samples were analyzed individually for DNA, RNA, total protein and 7 free amino acids (the leucines were not separated). To equalize possible analytical errors, each period and treatment were equally represented in any one series of biochemical analyses; standardized reference tissues were also included. Total protein was determined by the biuret method and was standardized by the Kjeldahl procedure. DNA and RNA were determined by the modified method described by Wannemacher et al. (6) and the free amino acids by the procedure of Squibb (7).

The data for the muscle and liver were first calculated in terms of milligrams per gram of fresh tissue and then in terms of DNA; the serum was expressed as milligrams per 100 milliliters. All values were then plotted for each sampling period as a percentage of initial control values. The oscillations thus represent deviations from the initial period. These were statistically treated according to Snedecor (8).

RESULTS

Figures 1 and 2 show that in the control groups increments in body and liver weight met normal expectations, as determined from a standard reference curve, during the 3-day observation period. In muscle the DNA did not change; RNA and protein fluctuated significantly ($< 5\%$) the first 36 hours, whereas the free amino acids oscillated significantly ($< 5\%$) the entire trial period. The diurnal fluctuations in serum free amino acids were significant ($< 1\%$), as were those of the total protein ($< 5\%$). In the liver there were significant daily fluctuations of DNA

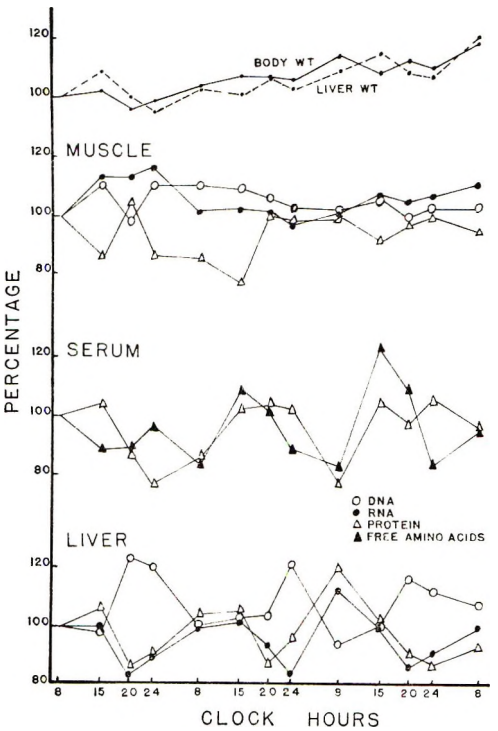


Fig. 1 Body and liver weights of normal chicks and diurnal changes in protein and nucleic and free amino acids of several tissues.

($< 3\%$) and protein ($< 5\%$) and the free amino acid pool ($< 5\%$).

The diurnal changes of individual free amino acids in muscle, serum and liver of the controls are shown in figure 2. In some cases the magnitude of change exceeded 40%. Significance levels ranged from $< 5\%$ to $< 1\%$ but changes in alanine and the leucines in the liver were not significant.

The effects of NDV on the various parameters are shown in figures 3 and 4. The body weight increase was similar to that of the controls during the first 60 hours but in the final 12 hours, just prior to active involvement of the NDV (5), there was a depression of weight gains. However, there was a significant ($< 1\%$) increase in liver weight and a concomitant significant ($<1\%$) increase in liver as percentage of body weight. Oscillations of serum protein and free amino acids, as in the controls, were significant ($< 1\%$) but there was no significant difference between

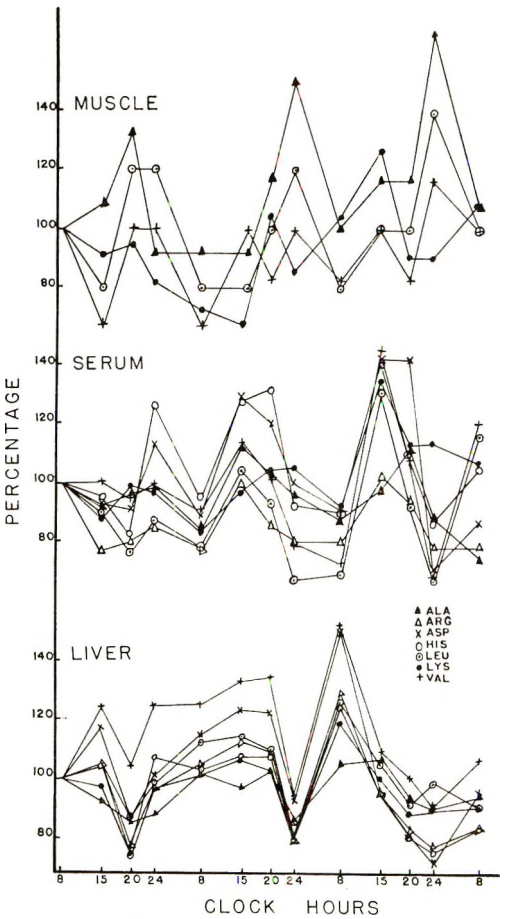


Fig. 2 Diurnal changes in several free amino acids in normal chick tissues.

control and NDV values. Except for RNA (< 1%), lysine (< 5%) and histidine (< 1%), fluctuations in the liver no longer were significant. In comparison with controls, the NDV caused a significant (< 1%) linear decline in all free amino acid levels in the liver.

Most of the free amino acids in NDV-infected muscle, serum and liver showed significant (< 1%) diurnal changes, but in the liver and muscle the rhythms differed from those in the controls. The NDV caused a desynchronization of patterns (defined as a statistically significant divergence from control values at specific sampling hours). In muscle the RNA and free amino acid patterns were also desynchronized. However, no desynchronization was evident in the serum free amino acids or protein. Although the data were calculated wherever possible in terms of DNA, the same rhythm patterns were observed when values were plotted as milligrams per gram of tissue.

DISCUSSION

This study throws light on the phenomenon of periodicity and its effect on parameters representative of a highly dynamic process — protein metabolism — in normal, rapidly growing animals as well as in those under disease stress. The subject is one that has been ignored by many disciplines.

Significant diurnal fluctuations, some of more magnitude than others, occurred in all 3 tissues examined. That dietary intake is not involved is evidenced by an earlier report from our laboratories (1)

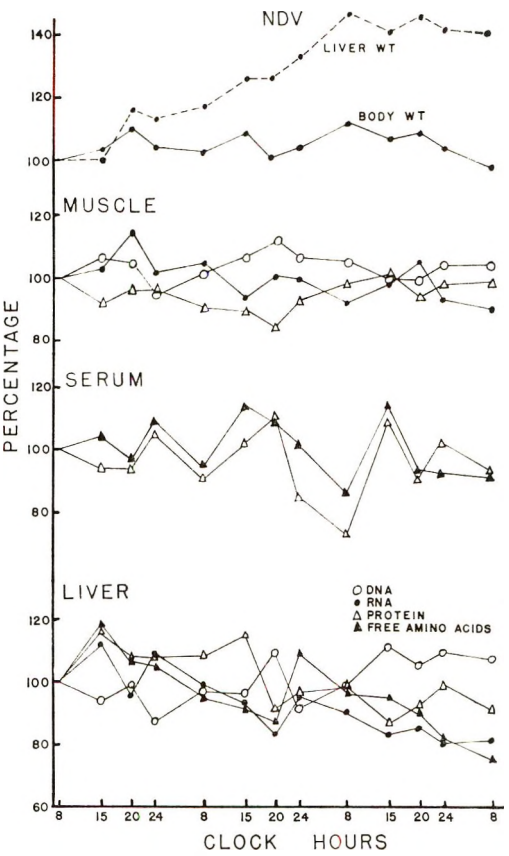


Fig. 3 Body and liver weights of NDV-infected chicks and diurnal changes in protein and nucleic and free amino acids of several tissues.

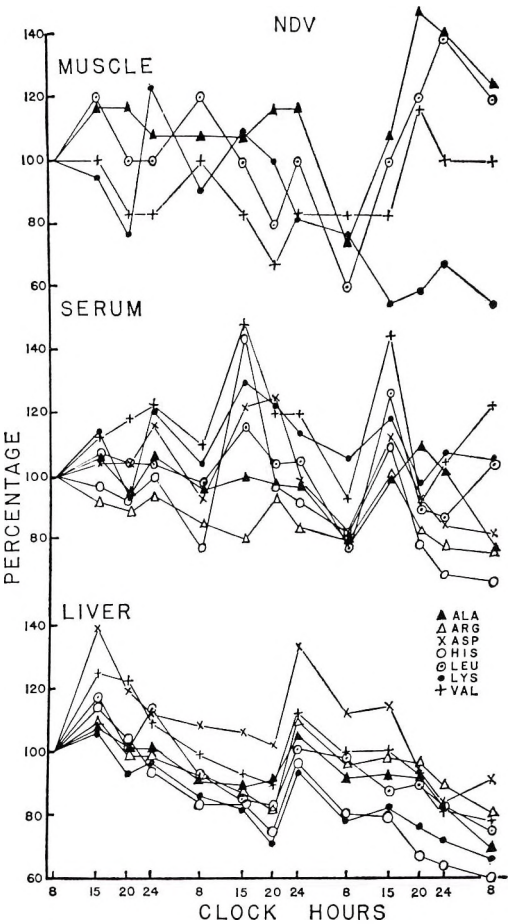


Fig. 4 Diurnal changes in several free amino acids in NDV-infected chick tissues.

that showed rhythms also occurred during the active involvement stage of NDV, a time when inanition is acute. Halberg et al. (9) also have reported that rhythms of several metabolic processes in mice persisted even though the animals were starved.

Of particular interest were the diurnal changes observed in liver nucleic acids and protein. Highest levels of DNA in the non-infected birds occurred in the evening and lowest in the daytime; similar observations have been reported for various tissues of a number of species (1, 10–14). RNA and protein synthesis in the liver, however, were highest during daylight hours, which is in line with Halberg's observations (12) in mouse liver wherein the peak for RNA preceded that of DNA.

Using the amplitudes of the oscillations as indicators, DNA, RNA and protein synthesis were more dynamic in liver than in muscle. The free amino acids, however, were equally dynamic in all 3 tissues.

When values for control and NDV chicks were compared with respect to clock hours, it was apparent that disease stress brought about a desynchronization of all parameters in the liver within 12 hours post-inoculation. Not only were the rhythms desynchronized; by the end of the 72-hour incubation period the free amino acid levels were also linearly depressed. The free amino acid pattern in the muscle was also desynchronized but not to the same extent as in the liver. However, the rhythms in the sera of control and infected groups continued in synchronization, with the curve for the NDV chicks slightly higher than that for the controls.

Other analyses of the data (15) revealed that despite the significant diurnal changes in the size of the free amino acid pool in both control and infected birds, the ratios of the individual free amino acids within the pool to total pool size remained remarkably constant.

Examination of data for individual birds indicated that for the specific parameters under study, less within-group variability occurred in the evening hours than in the daytime. This low variability, reported previously for the chick (1), may also be observed when data reported by Halberg et al. (8) and Frei and Ritchie (11) are examined for this phenomenon. This suggests that variables associated with genetic differentiation are more manifest during daylight hours.

Although body weights fluctuated slightly, the net gain in the controls during the 72-hour period was significantly higher, which would be expected. As body weights changed in these groups, so did liver weights, thus maintaining a constant ratio expected for the age period studied. The increase in liver weight of the NDV chicks confirms an earlier observation of our laboratories (5) and also correlates with a previously observed increase in nitrogen retention during the incubation period of the NDV cycle (16). The latter is believed to be a part of the defense

mechanism, which includes antibody production.

The data in the present study indicate that diurnal rhythms of various tissue constituents can occur with considerable magnitude even though all conditions of environment, genetics, feeding and management are carefully controlled. Periodicity phenomena therefore must be recognized and considered in the design and interpretation of experiments with intact systems. When periodicity is suspected, then treatment results must be re-examined for their patterns since desynchronization may be a major or a contributing effect. As demonstrated here, it is possible to obtain a linear depression of values and desynchronization from the same treatment. Research toward an understanding of the biological significance of desynchronization is indicated. Certainly this phenomenon changes biological relationships.

ACKNOWLEDGMENTS

The nucleic and free amino acid analyses were carried out by Marie Utzinger and Carol Mundy.

LITERATURE CITED

1. Squibb, R. L. 1964 Nutrition and biochemistry of survival during Newcastle disease virus infection. IV. Diurnal changes in protein, nucleic and free amino acids of avian liver. Nature, 202: 1138.
2. Halberg, F. 1959 Physiologic 24-hour periodicity: General and procedural considerations with reference to the adrenal cycle. Z. Vitamin-Hormon Fermentforsch., *10:* 225.
3. Simonnet, H. 1964 Rythmes et cycles biologiques chez les organismes animeux. Ext. Biol. Med., 53: 266.
4. Bünning, E. 1964 The Physiological Clock. Academic Press, New York.
5. Squibb, R. L. 1963 Nutrition and biochemistry of survival during Newcastle disease virus infection. I. Liver nucleic acid, protein and lipid patterns in chicks. J. Nutr., *81:* 48.
6. Wannemacher, R. W., Jr., W. L. Banks, Jr. and W. H. Wunner 1965 Use of a single tissue extract to determine cellular protein and nucleic acid concentrations and rate of amino acid incorporation. Anal. Biochem., *11:* 320.
7. Squibb, R. L. 1963 Thin-layer chromatographic separation and quantitative determination of several free amino-acids of avian liver. Nature, *199:* 1216.
8. Snedecor, G. W. 1957 Statistical Methods. Iowa State College Press, Ames.
9. Halberg, F., J. H. Galicich, F. Ungar and L. A. French 1965 Circadian rhythmic pituitary adrenocorticotropic activity, rectal temperature and pinnal mitosis of starving, dehydrated C mice. Proc. Soc. Exp. Biol. Med., *118:* 414.
10. Dzickanowski, D., and A. Nowak 1962 Studies on the control of the mitotic activity in white mice. II. Diurnal variations in the mitotic activity of epidermis and intestinal glands and in the level of liver glycogen. Act. Physiol. Polon., *13:* 700.
11. Frei, J. V., and A. C. Ritchie 1964 Diurnal variation in the susceptibility of mouse epidermis to carcinogen and its relation to DNA synthesis. J. Nat. Cancer Inst., 32: 1213.
12. Halberg, F. 1960 The 24-hour scale: A time dimension of adaptive functional organization. Persp. Biol. Med., 3: 491.
13. Ritchie, A. C., J. V. Frei and H. Shinozuka 1963 The duplication of deoxyribonucleic acid and epidermal carcinogenesis. Acta Union Int. Centre Cancer, 29: 579.
14. Horvath, G. 1963 Natural occurring variations in rat liver DNA content. Nature, 200: 26.
15. Squibb, R. L. 1966 The nature of the free amino-acid pool in avian tissues. Nature, 209: 710.
16. Sanslone, W. R., and R. L. Squibb 1962 Avian disease virus and nutrition relationships. III. Effect of Newcastle disease virus on nitrogen retention in the immature fowl. J. Nutr., 76: 86.

Copper, Sulfate and Molybdenum Interrelationships in Sheep

R. D. GOODRICH [1] AND A. D. TILLMAN
Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma [2,3]

ABSTRACT The experiment was designed to study the interrelationship of copper, molybdenum and sulfur in ruminant nutrition. Eighty lambs were used in the replicated, factorially arranged experiment, which involved 2 levels of copper (10 and 40 ppm), molybdenum (2 and 8 ppm), and sulfate-sulfur (0.10 and 0.40%). Response criteria were growth rate, hematology, and blood and liver mineral levels. Gain and efficiency were reduced by increasing the sulfur level in diets containing 2 ppm of molybdenum; however, this was not true when the diet contained 8 ppm of molybdenum, indicating that molybdenum alleviated the detrimental effects of sulfate. Hemoglobin concentration, erythrocyte counts and plasma protein, copper and calcium were not affected by treatments. Three-way interactions existed for hematocrit, plasma phosphorus and liver molybdenum. Hematocrit was lowered when sulfate-sulfur was increased from 0.10 to 0.40% except in the diet containing 10 ppm of copper and 8 ppm of molybdenum. Plasma phosphorus was also lowered by feeding 0.40% sulfate-sulfur except when 40 ppm of copper and 8 ppm of molybdenum were included in the diet. The three-way interaction for liver molybdenum was caused by decreases in liver molybdenum when 2 ppm of molybdenum and 10 or 40 ppm of copper were fed; feeding of 0.40% sulfate-sulfur, 8 ppm of molybdenum and 40 ppm of copper resulted in slightly higher final liver values, whereas high values were obtained for sheep fed 0.40% sulfur, 8 ppm of molybdenum and 10 ppm of copper. Liver copper was decreased by feeding 0.40% sulfate-sulfur or 8 ppm of molybdenum and increased by feeding 40 ppm of copper. Liver iron was increased as the sulfate-sulfur level was increased from 0.10 to 0.40%. Correlations among the plasma and liver values were also discussed.

Interrelationships among copper, sulfate and molybdenum have been demonstrated many times since Dick (1) reported that the limiting effect of molybdenum on the copper nutrition of sheep was dependent on the sulfate level of the ration. Other results have shown that excess levels of copper, molybdenum or sulfate also exert independent effects (2). As only a few experiments have been conducted for the specific purpose of studying the effects of improper ratios of the 3 minerals, the study reported herein was conducted to study the interrelationships of copper, sulfate and molybdenum when 2 levels of each in all possible combinations were fed to sheep.

EXPERIMENTAL PROCEDURE

A replicated, randomized block design with a 2^3 factorial arrangement of treatments was used so that all possible combinations of 2 levels of copper, molybdenum and sulfur were fed to growing lambs. Levels were 10 and 40 ppm of copper, 2 and 8 ppm of molybdenum and 0.10 and 0.40% of sulfur. The first trial was initiated in the late autumn of 1964 with 40 lambs, which were grade Rambouillets and averaged 27.1 kg, and the second trial was initiated in the early spring of 1965 with 40 Rambouillet × Suffolk crossbred lambs averaging 33.9 kg. Lambs in the first trial were fed for 66 days and in the second for 60 days. Blocking on location in the barn was done in both treatments.

The lambs, which were wormed with a phenothiazine-lead arsenate bolus 14 days prior to the start of the experiments and placed in individual pens with slatted floors, were fed the basal ration during this adjustment period. At the end of the adjustment period, initial weights were taken after feed and water had been removed for 17 hours. All animals then were fed their

Received for publication February 21, 1966.

[1] Present address: Department of Animal Husbandry, University of Minnesota, St. Paul, Minnesota.
[2] Department of Animal Science.
[3] Approved by the Director.

TABLE 1

Composition of the basal purified diet

	g/100 g diet
Cornstarch [1]	34.40
Dextrose	24.40
Cellulose [2]	30.00
Urea [3]	4.20
Corn oil [4]	1.00
Polyethylene resin [5]	1.00
Choline chloride	0.10
Vitamins A and D [6]	0.02
K_2CO_3	2.22
$CaHPO_4$	1.32
$MgSO_4$	0.12
$MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$	0.27
Na_2SO_4	0.25
NaCl	0.62
	mg/100 g diet
$FeSO_4$	42.50
$MnSO_4 \cdot H_2O$	11.50
$Na_2B_4O_7$	12.50
$ZnSO_4 \cdot 7H_2O$	15.00
$CuCO_3 \cdot Cu(OH)_2$	1.75
$Na_2MoO_4 \cdot 2H_2O$	0.50
CaF_2	0.20
	μg/100 g diet
KI	15.00
$Cr_2(SO_4)_3$	40.00
$CoCl_2 \cdot 6H_2O$	45.00
Na_2SeO_4	25.00

[1] This diet contained 0.10% sulfur, 10 ppm of copper and 2 ppm of molybdenum. All modifications to obtain the experimental diets were made by reducing starch in accord with an increase in Na_2SO_4, $CuCO_3 \cdot Cu(OH)_2$ or $Na_2moO_4 \cdot 2H_2O$.

[2] Solka-Floc (B-W 20), Brown Company, Berlin, New Hampshire.

[3] Crystalline urea, courtesy of John Deere Chemical Company, Pryor, Oklahoma.

[4] Mazola, Corn Products Company, Santoquin (Monsanto Company, St. Louis) added to give 0.0125% in total ration.

[5] Alathon, E. I. DuPont de Nemours, Inc., Wilmington, Delaware.

[6] 20,000 IU and 2,500 USP units of vitamins/g.

appropriate experimental rations, compositions of which are shown in table 1. Feed and water were provided free-choice. Final weights were also preceded by a period of 17 hours without food and water.

Blood samples, taken by jugular puncture, were obtained at the beginning and end of the growth trial. Hemoglobin values were determined on the citrated blood by the method of Sheard and Sanford (3). Erythrocytes and the percentage of packed cells were measured by the method of Schalm (4) and the microhematocrit method, respectively. All analyses involving whole blood were completed soon after bleeding, but the plasma was frozen until analysis was performed. Plasma copper was determined by the method of Cartwright et al. (5) and plasma calcium by the method of Kramer and Tisdal (6) with modifications for citrated plasma as described by Harrison (7) and with a Perkin-Elmer Atomic Absorption Spectrophotometer, Model 303, using methods suggested by the manufacturer. Other procedures included plasma phosphorus (8), plasma protein (9) and plasma molybdenum and liver copper, iron and molybdenum (10). The data were subjected to analysis of variance.

RESULTS AND DISCUSSION

As the replication × treatment and block-within-replication × treatment interactions were insignificant ($P > 0.05$), the results of the 2 trials were combined and are shown in table 2. Main effects which are also a part of a significant interaction are presented in the footnotes to table 2, but are discussed only in relation to the second factor. Sulfur level × molybdenum level interactions were found for gains ($P < 0.05$) and feed efficiency ($P < 0.01$): When the sulfur level was increased from 0.10 to 0.40% in diets containing 2 ppm of molybdenum both of these response criteria were reduced ($P < 0.01$); however, no such reduction was obtained when the sulfur level was increased in diets containing 8 ppm of molybdenum, indicating that 8 ppm of molybdenum partly overcame the depression caused by feeding 0.40% sulfur This interaction did not exist for feed consumption, however; the higher sulfur level reduced ($P < 0.05$) feed consumption regardless of level of molybdenum. Other workers (11, 12) have shown that sulfate exerts a protective effect against molybdenum toxicity because it reduced absorption and increased urinary excretion of this element. Results of the present experiment indicate that the poor performance caused by a high level of sulfate was partially alleviated by feeding additional molybdenum, a relationship not found by previous workers.

Hemoglobin concentration and erythrocyte counts were not significantly affected by treatments. A three-way interaction existed for hematocrit; the higher sulfur level lowered hematocrit values except in sheep receiving the diet containing 10 ppm of copper and 8 ppm of molybdenum.

TABLE 2

Effects of sulfur, molybdenum and copper levels on sheep

Sulfur level, %	0.10				0.40				
Molybdenum level, ppm	2		8		2		8		SE
Copper level, ppm	10	40	10	40	10	40	10	40	
No. of lambs [1]	10	10	10	10	10	10	10	10	
Avg daily gain,[2] g	102.7	98.6	87.5	78.2	50.1	39.9	94.7	60.5	15.40
Avg daily feed,[3] kg	1.12	1.19	1.15	1.09	0.94	0.86	1.12	0.92	0.08
Gain/100 g feed,[4] g	8.86	7.61	6.53	7.01	4.34	3.58	8.03	5.99	1.20
Hemoglobin, g/100 ml blood	13.1	13.5	12.4	13.0	12.2	12.9	13.2	12.6	0.34
Hematocrit,[5] %	41.3	41.4	38.9	40.4	37.7	40.7	41.2	38.7	1.21
Erythrocytes, 10^6/mm^3	12.8	12.1	12.7	12.2	11.7	12.3	11.9	12.1	2.91
Plasma protein, g/100 ml	6.47	6.66	6.61	6.92	6.47	6.22	6.34	6.30	0.09
Plasma copper, μg/100 ml	111.1	112.2	104.4	107.2	99.8	105.4	110.9	116.2	7.44
Plasma calcium, mg/100 ml	10.9	10.9	11.8	11.5	11.5	11.6	11.8	11.6	0.18
Plasma phosphorus,[8] mg/100 ml	7.27	8.66	7.28	7.14	6.54	6.65	6.66	7.20	0.10
Plasma molybdenum,[9] μg/100 ml	10.7	10.5	29.2	20.3	10.2	7.9	41.0	30.4	4.68
Liver copper,[10] μg/g dry liver	153.9	267.7	118.4	179.0	117.0	120.1	67.2	113.8	23.70
Liver iron,[9] μg/g dry liver	497	456	431	466	502	551	576	659	70.17
Liver molybdenum,[10] μg/g dry liver	3.83	3.78	4.57	4.33	3.65	3.70	9.38	5.72	0.17

[1] Five lambs per treatment for erythrocyte counts; all other values are an average obtained from 10 lambs.

[2] Gain, g/day:
1) 0.10% S(91.8) > 0.40% S(61.3)(**). Main effects which were found to be different and the level of significance are enclosed in parentheses.
2) Significant S × Mo level interaction (*).

	0.10% S		0.40% S
2 ppm Mo	100.6	**	45.0
8 ppm Mo	82.8		77.6

[3] Feed, kg/day: 0.10% S(1.14) > 0.40% S(0.96)(**).

[4] Efficiency, g gain/100 g feed:
1) 0.10% S(7.50) > 0.40% S(5.48)(*).
2) S × Mo level interaction (**).

	0.10% S		0.40% S
2 ppm Mo	8.24	**	3.96
8 ppm Mo	6.77		7.01

[5] Liver iron, μg/g dry liver:
1) 0.40% S(572) > 0.10% S(462)(*).

[6] Liver molybdenum, μg/g dry liver:
1) 0.40% S(5.61) > 0.10% S(4.13)(**).
2) 8 ppm Mo(6.00) > 2 ppm Mo(3.74)(**).
3) 10 ppm Cu(5.36) > 40 ppm Cu(4.38)(*).
4) S × Mo level interaction (**).

	0.10% S		0.40% S
2 ppm Mo	3.80		3.68
	4.45	**	7.55

5) S × Cu level interaction (*).

	0.10% S		0.40% S
10 ppm Cu	4.20	**	6.52
40 ppm Cu	4.06		4.71

6) Mo × Cu interaction (*).

	2 ppm Mo		8 ppm Mo
10 ppm Cu	3.74	**	6.98
40 ppm Cu	3.74	*	5.02

7) S × Mo × Cu level interaction (*).

* $P < 0.05$.

** $P < 0.01$.

[7] Hematocrit: S × Mo × Cu level interaction (*).

[8] Plasma phosphorus, mg/100 ml:
1) 0.10% S(7.59) > 0.40% S(6.76)(**).
2) 40 ppm Cu(7.41) > 10 ppm Cu(6.94)(*).
3) S × Mo level interaction (*).

	0.10% S		0.40% S
2 ppm Mo	7.96	**	6.60
8 ppm Mo	7.21		6.93

4) S × Mo × Cu level interaction (*).

[9] Plasma molybdenum, μg/100 ml:
1) 8 ppm Mo(30.2) > 2 ppm Mo(9.8)(**).

[10] Liver copper, μg/g dry liver:
1) 0.10% S(179.8) > 0.40% S(104.5)(**).
2) 2 ppm Mo(164.7) > 8 ppm Mo(119.6)(**).
3) 40 ppm Cu(170.2) > 10 ppm Cu(114.1)(**).

Protein, copper and calcium levels in blood plasma were not affected by treatments. Plasma phosphorus was affected ($P < 0.05$) by both copper and sulfur levels; the higher level of sulfur decreased ($P < 0.05$), whereas the higher level of copper increased ($P < 0.05$) plasma phosphorus levels. Also, there was a significant interaction between sulfur and molybdenum levels on plasma phosphorus level: Increasing the molybdenum level from 2 to 8 ppm caused reduced ($P < 0.05$) plasma phosphorus levels when 0.10% sulfur was fed but had no effect when the diet contained 0.40% sulfur. Also, when the level of sulfur was increased from 0.10 to 0.40% in diets containing 2 ppm of molybdenum plasma phosphorus was reduced ($P < 0.01$) but the addition had no effect when the diet contained 8 ppm of molybdenum.

There was a copper × sulfur × molybdenum level interaction ($P < 0.05$) in plasma phosphorus level: Sheep fed 0.40% sulfur had a lower level of plasma phosphorus except when the diet contained 40 ppm of copper and 8 ppm of molybdenum. increased dietary levels of both copper and molybdenum were required to return plasma phosphorus to normal. Shirley et al. (13, 14) reported losses of phosphorus from the bodies of steers or rats to be two to three times normal when the diet contained high levels of molybdenum and low levels of copper. As increased levels of sulfate lowers the retention of copper in sheep (15) and adequate copper levels are required for proper phosphorus metabolism (14), it appears that molybdenum acts to correct the effect of a high level of sulfate and that additional copper was required because the molybdenum level did not completely counteract the effect of sulfate. Growth results of the present experiment, in which sheep fed 0.40% sulfur and 8 ppm of molybdenum gained much faster than those fed 0.40% sulfur and 2 ppm of molybdenum, support this idea.

Plasma molybdenum levels increased as the dietary levels increased and these results agree with those of Cox and Harris (16) and Gray and Daniel (17).

The higher level of sulfur or molybdenum reduced ($P < 0.01$) copper storage in the liver and increased dietary copper caused increased storage of the element.

Goodrich and Tillman (15) observed that dietary sulfate in comparison with elemental sulfur lowered liver copper even in the presence of low levels of molybdenum. As Dick (18) reported that ferrous sulfide reduced copper absorption, it appears that insoluble copper sulfide may be formed from either sulfate or sulfide, resulting in reduced absorption of copper from the intestinal tract of ruminants. Molybdenum also reduces copper stores (19, 20). Dick (18) suggested that high intakes of molybdenum reduce copper absorption and increase copper excretion only in the presence of adequate endogenous or exogenous sulfate. Results of the present experiment indicate that 0.10% sulfur, as sulfate, was adequate for molybdenum to exert this effect. As the effect of elemental sulfur upon copper storage appears to be different (15), further work in this area is indicated.

Liver iron levels were significantly increased as the sulfur level of the ration was increased and are interpreted to be a reflection of the influence of sulfate on copper metabolism since Matrone (21) observed that during copper deficiency iron can enter liver tissue.

Liver molybdenum levels were affected ($P < 0.05$) by all treatments, and all interactions were significant. The copper × sulfur × molybdenum interaction was caused by liver molybdenum being lower when the diet contained 0.40% sulfur plus 2 ppm of molybdenum and 10 or 40 ppm of copper, in contrast with the small increase obtained when the diet contained 0.40% sulfur, 8 ppm of molybdenum and 40 ppm of copper and a large increase when the diet contained 0.40% sulfur, 8 ppm of molybdenum and 10 ppm of copper. These data are in agreement with results of other workers (2), but the reason for the increase in liver molybdenum when 0.40% sulfur, 8 ppm of molybdenum and 10 or 40 ppm of copper were fed remains obscure.

Correlations of various plasma and liver mineral levels are shown in table 3. The correlations represent within-treatment and within-replication calculations and thus are unbiased by treatment means. Only the plasma molybdenum level and liver molybdenum coefficient was significant ($P < 0.05$); however, the data tend to sup-

TABLE 3

Within treatment, within replication correlation coefficients among some plasma and liver values

Variables	Correlation Coefficients [1]
Liver Cu and liver Fe	0.11
Liver Cu and liver Mo	−0.19
Liver Cu and plasma Cu	0.04
Liver Cu and plasma Mo	−0.03
Liver Fe and liver Mo	−0.08
Liver Mo and plasma Mo	0.49 [2]
Plasma Cu and plasma Mo	0.26

[1] Degrees of freedom are 48.
[2] $P < 0.01$.

port the idea (2) that lambs with high molybdenum levels have low liver copper and high plasma molybdenum levels and those with high plasma copper levels also tend to have high plasma molybdenum levels.

LITERATURE CITED

1. Dick, A. T. 1952 The effect of diet and of molybdenum on copper metabolism in sheep. Australian Vet., J., *28:* 30.
2. Underwood, E. J. 1962 Trace minerals in human and animal nutrition, ed. 2. Academic Press, New York.
3. Sheard, C., and A. H. Sanford 1929 A photo-electric hemoglobinometer. J. Lab. Clini. Med., *14:* 558.
4. Schalm, O. W. 1961 Veterinary Hematology. Lea and Febiger, Philadelphia.
5. Cartwright, G. E., P. J. Jones and M. M. Wintrobe 1945 A method for the determination of copper in blood serum. J. Biol. Chem., *160:* 593.
6. Kramer, B., and F. F. Tisdall 1921 A simple method for the determination of calcium and magnesium in small amounts of serum. J. Biol. Chem., *47:* 475.
7. Harrison, G. A. 1957 Chemical Methods in Clinical Medicine. Their Application and Interpretation with Techniques of Simple Tests. J. and A. Churchill Ltd. London, p. 366.
8. Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, ed. 13. The Blakiston Company, Philadelphia.
9. Stadtman, E. R., G. D. Novelli and F. Lipmann 1951 Alimentary excretion of phosphorus-32 in steers on high molybdenum and copper diets. J. Animal Sci., *9:* 552.
10. Sandell, E. B. 1959 Colorimetric Determination of Trace of Metals, ed. 3. Interscience Publishers, New York.
11. Van Reen, R., and M. A. Williams 1956 Studies on the influence of sulfur compounds on molybdenum toxicity in rats. Arch. Biochem. Biophys., *63:* 1.
12. Mills, C. F., K. J. Monty, A. Ichihara and P. B. Pearson 1958 Metabolic effects of molybdenum toxicity in the rat. J. Nutr., *65:* 129.
13. Shirley, R. L., R. D. Owens and G. K. Davis 1950 Deposition and alimentary excretion of phosphorus-32 in steers on high molybdenum and copper diets. J. Animal Sci., *9:* 552.
14. Shirley, R. L., R. D. Owens and G. K. Davis 1951 Alimentary excretion of phosphorus-32 in rats on high molybdenum and copper diets. J. Nutr., *44:* 595.
15. Goodrich, R. D., and A. D. Tillman 1966 Effects of sulfur and nitrogen sources and copper levels on the metabolism of certain minerals by sheep. J. Animal Sci., *25:* in press.
16. Cox, D. H., and D. L. Harris 1960 Effect of excess dietary zinc on iron and copper in the rat. J. Nutr., *70:* 514.
17. Gray, L. F., and L. J. Daniel 1954 Some effects of excess molybdenum on the nutrition of the rat. J. Nutr., *53:* 43.
18. Dick, A. T. 1956 Molybdenum in animal nutrition. Soil Sci., *81:* 229.
19. Cunningham, I. J., K. G. Hogan and B. M. Lawson 1959 The effect of sulfate and molybdenum on copper metabolism in cattle. New Zealand J. Agr. Res., *2:* 145.
20. Dick, A. T. 1954 Studies on the assimilation and storage of copper in crossbred sheep. Australian J. Agr. Res., *5:* 511.
21. Matrone G. 1960 Interrelationships of iron and copper in the nutrition and metabolism of animals. Federation Proc., *19:* 659.

Correlation of Liver Cytochrome Oxidase Activity with Mitochondrial Cytochrome Oxidase and Phospholipid Concentrations in Protein-deficient Rats

J. N. WILLIAMS, JR., R. M. JACOBS AND ALICE J. HURLEBAUS
Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

ABSTRACT The object of the present study was two-fold: To observe to what extent cytochrome oxidase activity as measured manometrically is correlated with cytochrome oxidase concentration as measured spectrophotometrically and with mitochondrial phospholipid concentration during progressive protein depletion followed by repletion; and to study the influence of methionine fed in a protein-free ration on the maintenance of liver cytochrome oxidase activity. Phospholipids have been shown by others to be necessary for the normal functioning of cytochrome oxidase. Methionine fed in a protein-free ration has been shown to enable the maintenance of certain other liver enzyme and lipid components at higher levels than if methionine is omitted. In protein deficiency, rat liver cytochrome oxidase *activity* and cytochrome oxidase *concentration* as measured in isolated mitochondria followed almost identical patterns, both falling to 40 to 50% of normal after 8 weeks and remaining at that level until death of the animals at about 14 weeks. Upon repletion with protein, both activity and concentration of the enzymes returned to normal in a linear fashion within 7 days. Mitochondrial phospholipid concentration follows essentially the same pattern. It appears that, although phospholipid follows the same pattern and is essential for the activity of cytochrome oxidase, the changes in cytochrome oxidase activity are not a reflection of changes in mitochondrial phospholipid in protein deficiency, but more likely they represent a loss of enzyme protein. Addition of methionine to a protein-deficient diet does not protect against loss of cytochrome oxidase, in contrast with its protection of other components of the liver, such as succinic dehydrogenase, ubiquinone, and phospholipids.

In an earlier paper (1) it was shown that approximately 80% of the cytochrome oxidase activity of rat liver cells is lost after prolonged protein depletion. In those studies 0.30% DL-methionine was included in the protein-free ration. In subsequent studies it was found that methionine fed in such a ration protected against the loss of certain other enzymes in the electron transport system of liver mitochondria (2, 3). It also was found that liver phospholipid concentration was maintained at more nearly normal levels when methionine was fed in a protein-free ration (4). Brierley and Merola (5) observed that purified cytochrome oxidase loses almost all activity after phospholipids are removed by solvent extraction and that the activity is partially restored by addition of phospholipid. It was thus possible that omission of methionine from the protein-free ration would elicit a still further loss of cytochrome oxidase activity. Moreover, by using a method for measuring cytochrome oxidase concentration in whole liver mitochondria (6), both cytochrome oxidase activity as well as concentration could be followed. In this way it could be learned whether the losses of cytochrome oxidase activity (1) and phospholipid observed in protein deficiency (4) were related. In the spectrophotometric assay for cytochrome oxidase concentration the presence or lack of phospholipid in the preparation would not influence the results since the color produced is due to the cytochrome $a+a_3$ hemoprotein moieties of the mitochondria.

EXPERIMENTAL METHODS

Adult male rats of the Sprague-Dawley strain, previously adjusted for 3 weeks to a complete purified diet (7), were used throughout. They weighed approximately

Received for publication March 19, 1966.

300 g at the start of the experiment. Briefly, the complete ration (diet R1) consisted of: (in per cent) casein, 20; DL-methionine, 0.3; corn oil, 5; salts N plus molybdate (8), 6.5; glucose monohydrate, 63.5; choline chloride, 0.2; *i*-inositol, 0.02; and water-soluble vitamin mix in glucose monohydrate (9), 4.5. Fat-soluble vitamins (7) were given weekly to each rat in 2 drops of corn oil. Four groups of rats were usually used in the studies: Group 1 received the complete diet ad libitum. Group 2 received the same diet but was pair-fed with group 4 (average daily food consumption). Group 3 received diet R1 from which the casein was omitted. Group 4 received diet R1 from which both casein and methionine were omitted. The food consumption of groups 3 and 4 was almost identical.

The general plan of a portion of the studies is similar to that described in previous papers of this series (1–4, 7). Briefly groups 1 to 4 were fed their respective rations for 102 days, during which time 6 to 8 animals of each group were killed after 24, 56, and 102 days. At 102 days many of the animals in the deficient groups were moribund from the deficiency. Protein was then added to the diets of groups 3 and 4, and group 2 was still pair-fed with group 4. Rats from group 1 were killed after 22, 52, and 88 days post-repletion; and rats from groups 3 and 4 after 10, 52, and 88 days post-repletion. Mitochondrial isolations (10) and cytochrome oxidase assays (11) were performed immediately after removal of the livers, and portions of each liver were stored frozen for later DNA extractions (12) and analysis (13). Portions of the mitochondria were mixed with 20 volumes of 2:1 chloroform-methanol and stored at $-15°$ for later extraction of lipids by the method of Folch et al. (14). Phosphorus in aliquots of the lipid extracts was analyzed by the method of Fiske and Subbarow (15).

In some of the studies the cytochrome oxidase activity of the whole liver homogenate in 0.25 M sucrose was compared with that of mitochondria suspended in 0.25 M sucrose. In these experiments aliquots of the mitochondrial suspensions were used which were equivalent to the amount of liver in the flasks containing the whole homogenates. In other experiments mixtures of whole homogenates and mitochondrial suspensions from control and deficient rats were studied to determine whether there was an inhibitor of cytochrome oxidase in the deficient liver which might account for the lowered cytochrome oxidase activity. Also such studies would indicate whether normal liver could supply a substance such as phospholipid, necessary for the normal operation of cytochrome oxidase in liver from protein deficient animals. Again, amounts of mitochondrial suspensions equivalent to the amount of liver in the whole homogenates were used.

Finally, a series of studies was performed in which rats receiving diet R1 or diet R1 less casein and methionine (similar to groups 1 and 4 above, respectively) were used. Groups of animals were killed after 56 and 100 days of protein deficiency followed by 1, 2, 3, 4, and 7 days of protein repletion. In these studies cytochrome oxidase activity, cytochrome oxidase concentration, and phospholipid concentration, in the liver mitochondria only, were followed.

RESULTS

In figure 1 are shown the results of the first phase of these studies. The food intakes of groups 3 and 4 and those of group 2 (pair-fed controls) were considerably lower than normal, reaching about one-third of normal after 80 to 100 days of protein depletion. The food intakes of groups 3 and 4 were almost identical. Lowered food intake (group 2) did not influence liver cytochrome oxidase activity. (In other studies, we have occasionally noticed a decrease in this group maximally to 60 to 70% of normal. The reason for this variation from one group of rats to another or from one study to another is not clear. However, in the present studies no significant change from normal was observed.) The cytochrome oxidase activity of both groups 3 and 4 fell to approximately the same levels, there being no effect upon this enzyme of methionine added to the protein-free ration.

In table 1 a comparison of cytochrome oxidase activity from whole homogenates and isolated mitochondria is presented for each of the groups after 56 days of feeding the experimental diets. These results indi-

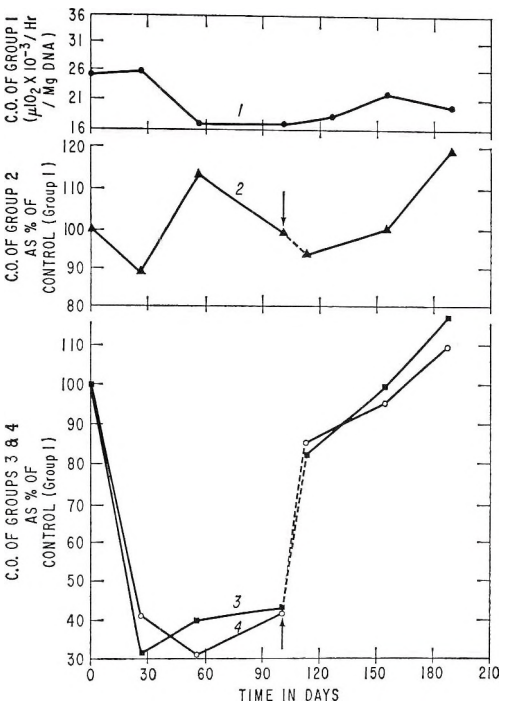


Fig. 1 Response of cytochrome oxidase (C.O.) activity of liver homogenates to protein depletion with and without 0.3% DL-methionine, and repletion. Protein repletion was begun at the arrows. ● = group 1 (ad libitum-fed, normal controls); △ = group 2 (pair-fed controls); ■ = group 3 (protein-deficient rats fed 0.3% DL-methionine); ○ = group 4 (protein-deficient rats). Statistical analysis using Student's *t* test indicated the following significant differences ($P < 0.01$) among the points in the figure:

24 days: 1 versus 3, 1 versus 4, 2 versus 3, 2 versus 4; 56 days: 1 versus 3, 1 versus 4, 2 versus 3, 2 versus 4; 102 days: 1 versus 3, 1 versus 4, 2 versus 3, 2 versus 4.

cate that there is no difference in activity between the 2 types of enzyme preparations in any of the groups. This indicates that the lowered activity in the protein-deficient groups is not due to the presence of an inhibitor in extramitochondrial liver fractions but is more than likely due simply to a lowered concentration of the enzyme. This is further substantiated by the results (table 2) which show that mixing of homogenates or mitochondria from normal and deficient rat livers had no effect on the activity of the opposite group.

Cytochrome oxidase activity as measured manometrically and cytochrome oxidase concentration as measured spectrophotometrically in mitochondria gave almost identical results throughout the entire course of protein depletion and repletion (fig. 2). Variation between the 2 sets of results was not significant. In the same figure it can be seen that the results for phospholipid changes were also similar to the cytochrome oxidase results. However, as will be pointed out later, this similarity is fortuitous and unless a comparison had been made between cytochrome oxidase activity and cytochrome oxidase concentration, it might have been concluded that the loss in cytochrome oxidase activity was a direct effect of loss of mitochondrial phospholipid.

DISCUSSION

Although the phospholipid results were very similar in general to those for cytochrome oxidase, evidence that the loss of cytochrome oxidase activity in protein deficiency is due to loss of enzyme protein rather than phospholipid associated with the protein is that the response of cyto-

TABLE 1

Comparison of homogenate and mitochondrial cytochrome oxidase activities after 56 days of depletion

Group	Homogenate cytochrome oxidase activity	Mitochondrial cytochrome oxidase activity
	μliters O_2/hr/mg liver DNA	*μliters O_2/hr/mg liver DNA*
1 (ad libitum-fed normal controls)	27,900 ± 2,950 [1]	24,200 ± 2,050
2 (pair-fed controls)	27,800 ± 2,100	23,400 ± 4,100
3 (protein-deficient rats fed 0.3% DL-methionine)	8,200 ± 2.050	6,970 ± 1,640
4 (protein-deficient rats)	9,840 ± 820	10,250 ± 2,460

[1] SEM, 4 rats/group.

TABLE 2

Cytochrome oxidase activity of homogenates and mitochondria of controls mixed in equal amounts with those from experimental groups

Groups mixed	Homogenate cytochrome oxidase activity		Mitochondrial cytochrome oxidase activity	
	Mixed homogenates	Avg of individual groups alone	Mixed mitochondria	Avg of individual groups alone
	µliters O_2/hr/mg liver		*µliters O_2/hr/mg liver*	
1+4	48.0±5.4 [1]	48.0±3.8	49.3±5.1	41.1±6.9
2+3	39.8±3.9	39.6±5.8	43.7±3.4	35.2±3.2

[1] SEM, 4 rats/group.

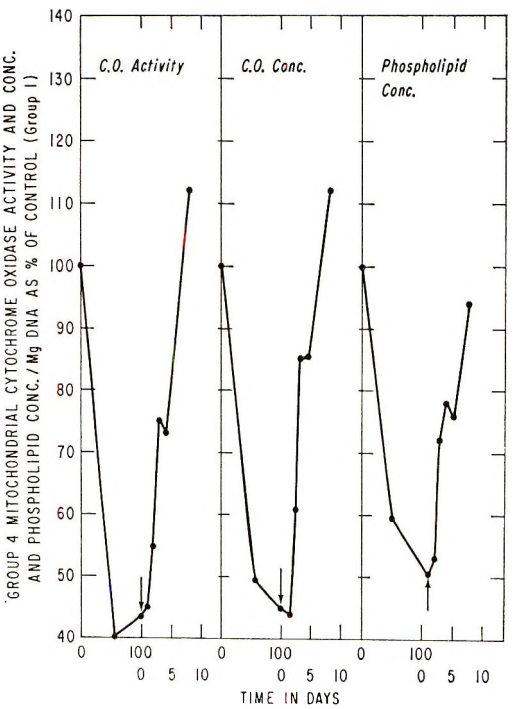


Fig. 2 Response of mitochondrial cytochrome oxidase activity, cytochrome oxidase concentration and phospholipid concentration to protein depletion followed by repletion. Protein repletion was begun at the arrows. Statistical analysis using Student's *t* test indicated the following significant differences ($P < 0.01$) between the protein-deficient group (group 4) and the ad libitum-fed normal controls (group 1): day 56, day 100 and day 1 post-repletion.

chrome oxidase *concentration* was almost identical to that of the enzyme *activity*. In the spectrophotometric assay for mitochondrial cytochrome oxidase concentration the presence or absence of phospholipid would make little difference. It has been shown by Brierley and Merola (5) that cytochrome oxidase from which phospholipids have been extracted gives almost the same spectrum as unextracted oxidase, the only difference being a shift in the 605 mµ peak to 603 mµ. This slight shift would have negligible influence on the results in the spectrophotometric assay for mitochondrial cytochrome oxidase since the 605 mµ peak is fairly broad in these preparations (6). Further evidence that the loss of cytochrome oxidase activity in protein deficiency is due to loss of enzyme protein rather than phospholipid associated with the protein is as follows: Since methionine was observed earlier to protect against loss of total phospholipids, it would have been expected that, if the loss of cytochrome oxidase activity was due to loss of phospholipid (5), it should have protected against loss of the enzyme activity. Furthermore, in the experiments in which control enzyme preparations were mixed with deficient preparations, some equilibration of phospholipids from the normal to the deficient should have occurred. Thus if the lower enzyme activity in the deficient preparations were due to lowered phospholipid, the activity of the mixed preparations should have been higher than the sum of the activities of the individual preparations. That the lower enzyme activity of the protein-deficient group was not due to production of inhibitors by the protein deficiency is also indicated by the fact that the enzyme preparations from the deficient groups had no influence on the activity of those from the control groups. Thus it appears that the similarities in the loss of phospholipids and cytochrome oxi-

dase activity in protein deficiency are probably coincidental.

The responses of cytochrome oxidase as observed in the present paper and of succinic dehydrogenase (3) to uncomplicated protein deficiency are almost identical, pointing to perhaps a common regulatory mechanism controlling the maintenance of levels of these 2 mitochondrial enzymes under the stress of protein deficiency. However, the fact that methionine protected against the loss of succinic dehydrogenase but had no effect on cytochrome oxidase indicates that such a common regulatory mechanism, if it exists, is more complex than might be suspected at first glance. Further studies are being carried out at present concerning such a possible common regulatory mechanism.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Esther Hurley and Woodrow Duvall for care and feeding of the animals in these studies.

LITERATURE CITED

1. Williams, J. N., Jr. 1961 Response of the liver to prolonged protein depletion. II. The succinic oxidase system and its component enzymes. J. Nutr., *73:* 210.
2. Williams, J. N., Jr. 1963 Response of the liver to prolonged protein depletion. III. Coenzyme Q. Arch. Biochem. Biophys., *101:* 512.
3. Williams, J. N., Jr. 1964 Response of the liver to prolonged protein depletion. IV. Protection of succinic oxidase and succinic dehydrogenase by dietary methionine and cystine in a protein-free ration. J. Nutr., *82:* 51.
4. Williams, J. N., Jr., and A. J. Hurlebaus 1965 Response of the liver to prolonged protein depletion. VI. Total phospholipids and plasmalogens, and protection of phospholipids by methionine and cystine. J. Nutr., *85:* 82.
5. Brierley, G. P., and A. J. Merola 1962 Studies of the electron-transfer system. XLVIII. Phospholipid requirements in cytochrome oxidase. Biochim. Biophys. Acta, *64:* 205.
6. Williams, J. N., Jr. 1964 A method for the simultaneous quantitative estimation of cytochromes a, b, c_1, and c in mitochondria. Arch. Biochem. and Biophys., *107:* 537.
7. Williams, J. N., Jr. 1961 Response of the liver to prolonged protein depletion. I. Liver weight, nitrogen, and deoxyribonucleic acid. J. Nutr., *73:* 199.
8. Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutr., *72:* 243.
9. Fox, M. R. S., L. O. Ortiz and G. M. Briggs 1955 Toxicity of ethionine in the young chick. Agr. Food Chem., *3:* 436.
10. Schneider, W. C., and G. H. Hogeboom 1950 V. Further studies on the distribution of cytochrome c in rat liver homogenates. J. Biol. Chem., *183:* 123.
11. Umbreit, W. W., R. H. Burris and J. F. Stauffer 1959 Manometric Techniques. Burgess Publishing Company, Minneapolis, p. 173.
12. Schneider, W. C. 1945 Phosphorus compounds in animal tissue. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.
13. Davidson, J. N., and C. Waymouth 1944 Tissue nucleic acids. 3. The nucleic acid and nucleotide content of liver tissue. Biochem. J., *38:* 379.
14. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipides from animal tissue. J. Biol. Chem., *226:* 497.
15. Fiske, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J Biol. Chem., *66:* 375.

Effect of Iron on the Growth Rate of Fishes [1,2]

MARTIN ROEDER AND RACHEL H. ROEDER
Department of Biological Science, Florida State University, Tallahassee, Florida

ABSTRACT To determine the nutritional requirment for iron in fish growth, the effect of daily addition of supplemental ferrous sulfate on the growth of *Xiphophorus helleri* and *Xiphophorus maculatus* (the swordtail and the platyfish) was measured by determination of weight gain and hematocrit levels. Both variables increased as a result of the added iron. The effectiveness of the treatment diminished as sexual maturity was approached. Treatment with ferrrous sulfate led to an increase in growth rate even in fish fed maximally with live brine shrimp. Ferric salt was not judged effective. Addition of ferrous iron also significantly decreased the mortality from hatching to maturity.

Little is known of the mineral requirements, or of the mineral metabolism of fishes. Lovelace and Podoliak (1) showed that calcium is absorbed through the gill of the brook trout, and Phillips and his co-workers (2) have reported dietary requirements for growth in trout, although their main concern was with the organic constitutents of prepared hatchery feeds. They determined calcium, phosphorus, and magnesium levels in their diets, but did not systematically vary these. Cheprakova (3) reported some effects of iron salts on developing eggs of the loach, the sig, and the perch, but the data reported in that paper are not at all conclusive.

In connection with studies in our laboratory concerned with respiratory rate and other factors in the swordtail (*Xiphophorus helleri*), the platyfish (*Xiphophorus maculatus*) and the hybrid *helleri* × *maculatus* (4), we noted that the addition of ferrous sulfate to water in which the fishes were raised appeared to have a stimulatory effect on the growth of these fishes, as compared with untreated controls. Accordingly studies were made in which we systematically varied the amount and kind of iron available to newly hatched fry and to older fishes, and compared weight changes and changes in hematocrit values of treated to untreated animals.

METHODS

Fish were maintained in 13.5-liter aquariums, filled with water withdrawn from the Wakulla River. The pH of the water was adjusted to between 7 and 8, and Anacharis (Elodea) and calcium carbonate blocks were added to the tanks. Snails were introduced and allowed to grow. As soon as possible after swimming fry appeared in a tank, they were removed, and fry of a single brood were separated into groups of equal size, and placed into newly established aquariums. Fry were fed an average of 90 mg of dry food/day/tank, with a supplement of 300 mg of liquid food, containing 50 mg dry weight.[3] The dried food contained 45% crude protein and 3% crude fat, with a maximal fiber content of 4%, and the analysis of the liquid food showed 5% crude protein, 1% crude fat with a maximal crude fiber content of 1.5%. The iron content of these feeds, of the aquarium water, and of live brine shrimp was measured, using a modification of the technique of Sherman et al. (5), in which alpha-alpha' dipyridyl is used as the color reagent, and which is reported to measure biologically

Received for publication March 24, 1966.
[1] Supported by grant GB 2664 from the National Science Foundation.
[2] We wish to thank Wardley Products, Inc., Long Island City, New York, for their courtesy in supplying dried food diets and numerous other aquarium supplies.
[3] Dried food was prepared from: animal liver meal, whale meal, meat meal, menhaden fish meal, crab meal, shrimp meat, salmon egg meal, wheatgerm meal, dried *Daphnia*, kelp meal, oat meal, wheat flour, corn meal, soya flour, 3% whole egg solids, 3% dried skim milk, 3% salt, 2% mosquito larvae, 2% ground aniseed, 1% calcium triphosphate, 1% barley malt and 1% primary dried yeast. Liquid food supplement contained: whole eggs, yeast hydrolysate, 3% cod liver oil, 2.5% kelp meal, 2% animal liver meal, 0.5% spinach powder, 0.5% gum acacia, 0.5% gum tragacanth, 1% dextrin, water and 0.25% sodium bisulphite.

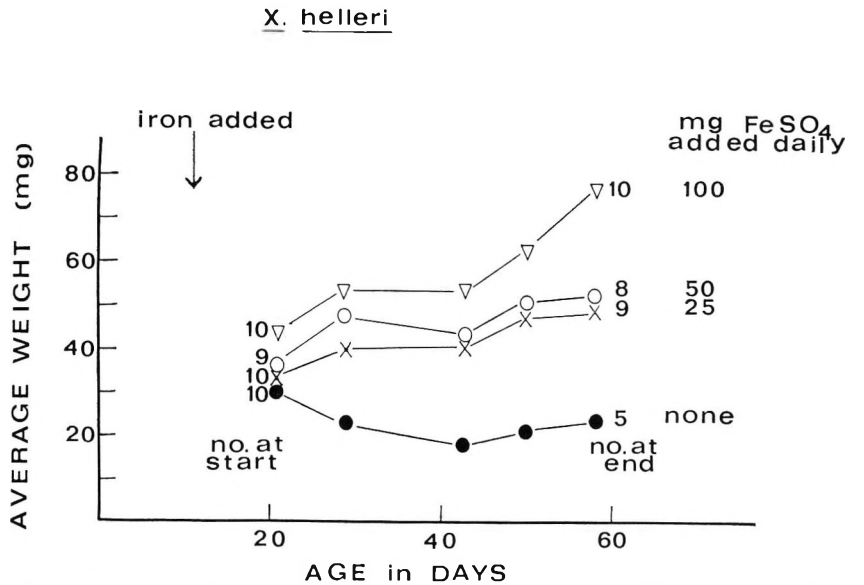


Fig. 1 Effect of varying concentrations of ferrous sulfate on the growth of the green swordtail, *Xiphosphorus helleri*.

available iron, as measured by growth responses, rather than total iron, which may be bound and not available for absorption into the blood of the organism. The aquarium water contained less than one part per million of iron, making the total content of an untreated tank less than 0.2 mg. Daily rations of food contained less than 0.07 mg available iron, while an average feeding of live brine shrimp (*Artemia* sp.) contained only 5×10^{-4} mg. In comparison with the amount of added iron these are considered to be negligible levels. Supplemental iron salts were added daily at the indicated levels, as analyses for soluble iron showed complete disappearance of the dissolved salt after 4 hours. Copious precipitates of ferrous hydroxide were observed in all treated tanks. Hematocrit levels were measured using a modification of the orbital bleeding technique of Riley (6) in heparinized micro-hematocrit tubes.

EXPERIMENTAL RESULTS

Initially, 40 fish from 2 broods of swordtails were combined, and then separated into 4 tanks, each containing 10 fish. Three groups were treated by daily addition of varying amounts of ferrous sulfate, and one was kept as a control group. The results of this initial experiment are shown in figure 1. After 60 days, the differences in weight and in survival were striking, especially as a higher growth rate in the tanks containing fewer fish would be expected (7). After 100 days the groups which had received 50 and 100 mg of ferrous sulfate were divided, and the treatment continued for half, and discontinued in the other half. The data are presented in figure 2, and indicate that continued treatment with the salt loses its effectiveness after 100 days. The apparent depressing effect of the iron treatment on the fish in the group to which 100 mg had been continued may be explained by noting that in this group no evidence of sexual maturity was noted, whereas in the group which had been removed from treatment with 100 mg two of five had already shown marked sexual differentiation. In swordtails, the striking sexual dimorphism leads to both size and weight differences between the sexes, females being larger and heavier than males. When secondary sex differentiation occurs, then, the data can no longer be treated as coming from a common pool, but would have to be paired for sex. The indication that iron treatments lose effectiveness was confirmed by several attempts to change growth rates in adult fish after they had been

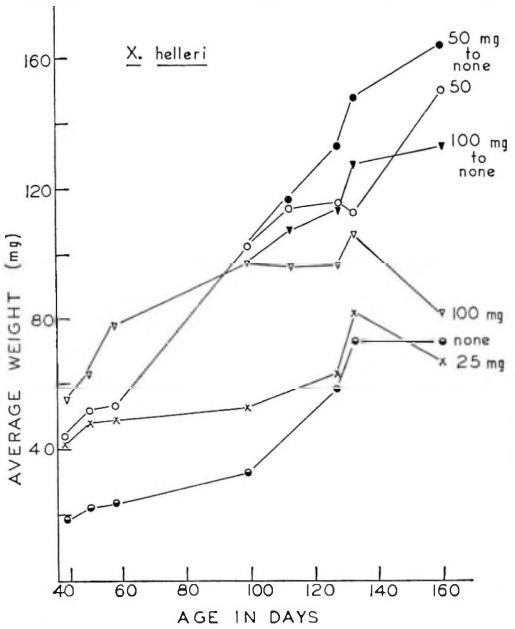


Fig. 2 Decreasing effectiveness of ferrous sulfate treatment after 100 days from hatching. The 50- and 100-mg groups were split at 100 days, and treatment was discontinued in half of each group.

raised with no iron present; no changes in growth rate or in hematocrit values were observed in any of these experiments.

The data from 2 experiments utilizing the platyfish are presented in figure 3. In the lower portion of the figure the curves indicate that after 75 days the growth rate of the control group exceeded that of the 50-mg group, and that the growth rate before this time, as indicated by the slope of the curves, is approximately the same for the control, 25-mg, and 50-mg groups. These data must be treated with caution, as by the 44th day, two of the 25-mg group and three of the control group had died, whereas all of the treated fish still survived, and did so to the end of the experiment. On the upper portion of the figure, 2 groups in which the survival rate was similar are compared. With similar mortalities the growth rate of the treated fish exceeds that of the control group by 65%.

Efforts were made in subsequent experiments to balance any changes which might have resulted from crowding ef-

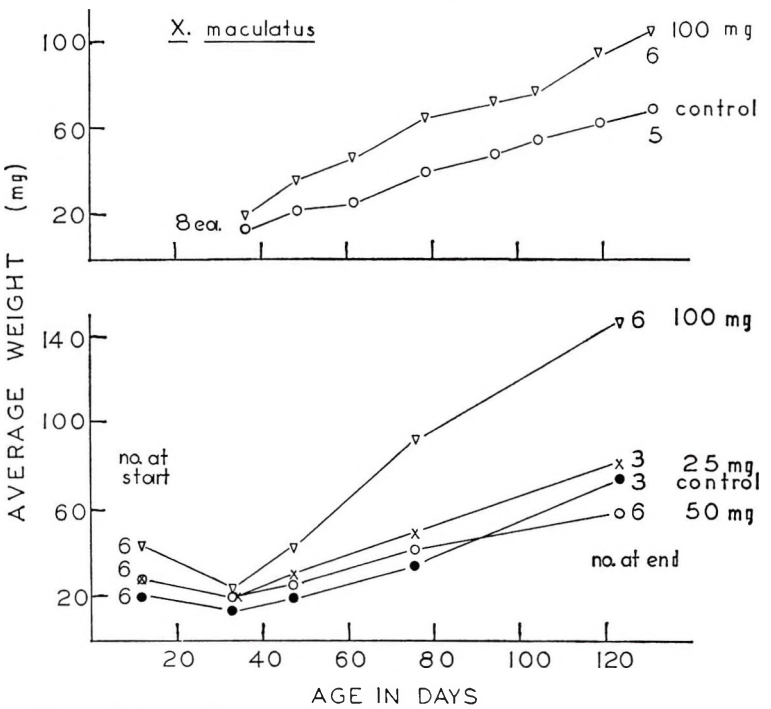


Fig. 3 Effect of daily addition of ferrous sulfate on the growth of the platyfish, *Xiphophorus maculatus*. After the 44th day only 3 control fish were left, and only four in the 25-mg treated group, in the lower portion of the figure.

fects by removing fish from the various groups in which little or no mortality occurred, so that the size of the control and experimental groups remained similar throughout the experiment. Difficulties of this sort led us to examine our data in terms of increased survival due to supplemental iron treatments. These data are presented in table 1, in which all experiments using both platyfish and swordtails are combined. Utilizing a contingency chi-square test the differences in survival rates of the groups treated with 50 mg and 100 mg are significant, P being less than 0.01, whereas the 25-mg groups gave a P value between 0.1 and 0.2. Accordingly the use of the 25-mg supplement was discontinued as being, at best, submaximal.

The addition of live brine shrimp (*Artemia*) is often used to supplement fish feeding regimens, with excellent results reported in terms of fish growth (8). Accordingly a brood of fry was separated into 2 equal groups and one, used as a control, was fed maximally a diet supplemented daily with brine shrimp; the other was fed in the same way but daily additions of 100 mg of ferrous sulfate were also made. Figure 4 shows that until 66 days after hatching a stimulation due to added iron was still present.[4] Both groups grew faster on this regimen than with a diet with only occasional brine shrimp supplements, but it appears that brine shrimp do not supply enough iron, at least in the early growth stages.

Together with increased growth of the treated fish we observed an increase in hematocrit levels, when compared with control groups. In analyzing these data it became necessary to pool the results of many experiments, as the value of the hematocrit, from control group to control

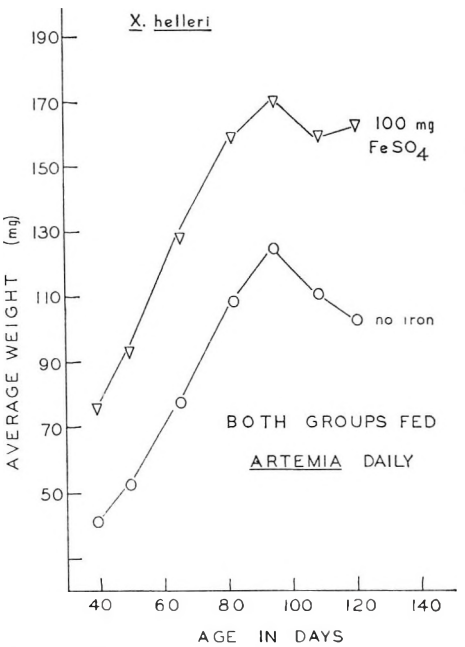


Fig. 4 Effect of the addition of ferrous sulfate to swordtails fed live brine shrimp daily.

group, was highly variable, for reasons not clear. These data are presented in table 2. In no case did the hematocrit value of a control fish exceed the value for any in the treated group matched with that control, although on occasion one control group might have a higher value than that observed in a different experimental group. Despite the overlap in standard deviations, the differences, when subjected to an analysis of variance, are significant at the 5% level of confidence.

Similar experiments were made using ferric nitrate as the iron source, and add-

[4] The ratio of the slope of the treated to the untreated group is: days 40 to 50, 1.7/1.1; days 50 to 66, 2.15/1.56.

TABLE 1

Effect of treatment with ferrous sulfate on survival of platyfish and swordtails from hatching to 120 days

Treatment	Initial no.	Final no.	Mortality
			%
None (control)	59	24	58
25 mg $FeSO_4$ added daily	16	10	38
50 mg $FeSO_4$ added daily	15	14	8
100 mg $FeSO_4$ added daily	59	45	24
Combined treated group	90	69	23

TABLE 2

Hematocrit levels of fishes with various treatments [1]

Type of fish	Daily treatment		
	100 mg $FeSO_4$	50 mg $FeSO_4$	Control
Xiphophorus helleri (green swordtail)	38.0 ± 6.6 [2] (14) [3]	40.0 ± 4.0 (22)	37.2 ± 4.9 (34)
Xiphophorus maculatus (platyfish)	38.7 ± 6.8 (15)		32.1 ± 4.3 (16)
Hybrid (*helleri* × *maculatus*)		40.22 ± 1.4 (8)	31.7 ± 0.04 (5)

[1] We are indebted to Miss Patricia Hopper for the data on the hybrid fishes, and for much of the data on swordtails.
[2] Mean packed cell volume/100 ml ± SD.
[3] Numbers in parentheses indicate number of fish.

ing the salt at the level of 100 mg/day to tanks containing new-hatched swordtail fry. At the end of an 84-day period the average weight and the rate of growth of the control group and the treated fish were essentially the same, 2 groups of treated fish had average weights of 145 mg, and 2 control groups 130 mg, (144 treated vs. 144 control; 146 treated vs. 117 control). We conclude that this treatment does not appear effective in stimulating growth; hematocrit values in both groups were not statistically different (35.5 treated vs. 34.9 control).

DISCUSSION

The observed increases in growth rate and in hematocrit levels indicate that, as with chicks, iron is an essential nutrient for these fish during the period immediately following hatching. As sexual maturity is approached the stimulation due to iron addition to the diet disappears, and it is absent in adult fish. Absorption probably takes place across the gill membrane, and is evidently accomplished rapidly, as the disappearance of soluble iron from solution limits the time of exposure. Since ferric iron does not appear to be utilized, it is possible that the mechanics of transport bear some relationship to the absorption of iron through the intestinal mucosa in mammalian systems (9). The rise in hematocrit levels indicates that the iron is both absorbed and utilized. Further experiments with chelated iron and with possible synergistic effects of other minerals are in progress.

LITERATURE CITED

1. Lovelace, F. E., and H. A. Podoliak 1952 Absorption of radioactive calcium by brook trout. Progr. Fish Cult., *14:* 154.
2. Phillips, A. M. Jr., F. E. Lovelace, D. R. Brockway, G. C. Balzer et al. 1954 The nutrition of trout. Bur. Fisheries. N.Y. Fisheries Res. Bull. *17*, Cortland Hatchery Rep. 22. N.Y. State Dept. of Conservation, Cortland, New York.
3. Cheprakova, U. I. 1960 Some facts about the influence of iron salts concentration on the development and survival of fish eggs. Vop. Ikhtiologii, *14: 110.*
4. Roeder, M., and R. H. Roeder 1964 The respiration of a graded series of two species of small whole Xiphophorin fishes. J. Cell. Comp. Physiol., *63:* 115.
5. Sherman, W. C., C. A. Elvehjem and E. B. Hart 1934 Further studies on the availability of iron in biological materials. J. Biol. Chem., *107:* 383.
6. Riley, V. 1960 Adaptation of orbital bleeding technic to rapid serial blood studies. Proc. Soc. Exp. Biol. Med., *104:* 751.
7. Breder, C. M., Jr. 1935 The aquarium and research. Bull. N.Y. Zool. Soc., 38 (4): 110.
8. Dempster, P. P. 1953 The use of larval and adult brine shrimp in Aquarium fish culture. Calif. Fish Game, *39:* 355.
9. Ruch, T. C. and F. D. Patton 1965 Physiology and Biophysics, ed. 19. W. B. Saunders Company, Philadelphia, p. 1003.

Effect of α Methionine-deficient Diet on Amino Acid Incorporation in Rat Liver Cell-fractions [1]

E. A. KEAN
Biochemistry Department, University of the West Indies, Kingston, Jamaica

ABSTRACT An investigation was made into the relationship between nutritive value of a protein-rich food and amino acid incorporation into cell-free preparations from rat liver. A low protein efficiency ratio due in part to a methionine deficiency was associated with increased levels of protein labeling. The effect was observed with methionine-^{35}S but not with leucine-^{14}C, and was apparently mediated through alterations occurring predominantly in the pH 5-enzyme fraction. The observations indicated that an increase in the activity of amino acid-activating enzymes may be induced by dietary deficiency of the corresponding amino acids.

The amounts and activity of liver components which are involved in protein synthesis may vary according to the amounts and relative proportions of dietary amino acids. Thus, there are reports (1, 2) that amino acid-activating enzymes tested in vitro showed higher activity than normal in liver cell-sap from protein-depleted rats; this contrasted with reduced activity in heart and gastrocnemius muscle. These investigators pointed out that the differential response was in keeping with the changes in the levels of amino acid incorporation which they observed in the respective tissues in vivo.

The effects of omitting a single amino acid from the diet have been explored by measuring amino acid incorporation in preparations of liver microsomes and ribosomes (3, 4). Fleck et al. (3) concluded that absence of tryptophan from an amino acid mixture administered to rats one hour before killing led to a reduction in the capacity of microsomes for amino acid incorporation. On the other hand, Sidransky et al. (4) force-fed diets devoid of threonine to rats for 3 or 7 days, and observed that this led to increased leucine incorporation. They concluded that the ribosomes were predominantly responsible for the observed effect.

The present work was based on feeding trials with rats, which showed that the growth-promoting value of a protein-rich product from peanut was greatly enhanced by supplementation with methionine; this implied that there was a relative deficiency of methionine in the protein. Experiments were carried out to determine which, if any, of the components participating in protein biosynthesis was affected by methionine deficiency. These components were isolated from livers of animals used in the feeding trials. By varying the source of these components in protein biosynthetic systems in vitro, it was found that methionine deficiency led to increased incorporation of methionine into protein, and furthermore that a fraction containing amino acid-activating enzymes was responsible.

EXPERIMENTAL

A depletion-repletion technique (5, 6) was adopted for the determination of protein efficiency ratios (PER) of 3 protein-rich supplements. The depletion period during which rats were fed a protein-free diet lasted 10 days; thereafter the supplement was fed along with the protein-free diet and weight gains were measured after 7 days.

Animals and diets. Male albino rats 4 to 5 weeks old were used. They had been fed a stock diet [2] for 1 to 2 weeks after weaning. Each animal was housed in its own metabolism cage and fed ad libitum a diet (protein-free diet) of the following composition: (in per cent) sucrose, 8.8;

Received for publication February 21, 1966.
[1] This work was supported by Public Health Service Research Grant no. AM 07623, from the National Institute of Arthritis and Metabolic Diseases.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

cornstarch, 75; butter, 10; salt mixture, USP XIV, 4; and vitamin fortification mixture,[3] 2.2. The vitamin mixture contained per g: vitamin A, 9,000 USP units; vitamin D, 18,000 USP units; (in milligrams) α-tocopherol, 5; ascorbic acid, 45; inositol, 5; choline chloride, 75; menadione, 2.25; *p*-aminobenzoic acid, 5; nicotinic acid, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; Ca pantothenate, 3; and (in micrograms) biotin, 20; folic acid, 90; vitamin B_{12}, 1.35.

The supplements for which determinations of PER were made are as follows, with the amount fed in the daily ration: 1) peanut protein,[4] 1.9 g; 2) peanut protein, 1.9 g + L-methionine, 0.05 g; 3) egg albumin,[5] 1.27 g + hydrogenated coconut oil,[6] 0.60 g. These supplements were prepared in bulk, as liquid suspensions blended into 4% sucrose solution for palatability. They were compounded to supply as nearly as possible an equal amount of nitrogen and of lipid to each animal in a test, and were fed as a measured volume (usually 10 ml/rat) daily. Each rat soon learned to consume its daily ration within 3 hours. The N intake varied in different tests between 0.16 and 0.18 g/day/rat. After the protein supplements had been fed for 7 days, gain in body weight was recorded. PER was calculated as gain in weight (g) per N eaten (g).

The feeding regimen was continued for a second week, and then the animals were killed for preparation of liver cell-fractions.

Preparation and incubation of liver-cell fractions. Rats were killed by cervical fracture and decapitated. Livers were removed and chilled on cracked ice. The subsequent procedures were varied to suit different experiments in which the following fractions were tested either alone or in combination: 1) a 15,000 × *g* supernatant; 2) a 105,000 × *g* supernatant — referred to as cell-sap, and a pH 5-enzyme preparation derived from it; 3) washed microsomes; and 4) ribosomes. All isolation procedures were performed at zero to 2°. For tests on fraction 1, liver was minced with scissors and homogenized in 2 volumes of 0.25 M sucrose. The homogenate was centrifuged at 15,000 × *g* for ten minutes and the supernatant containing microsomes and cell-sap was used as such (15,000 × *g* supernatant). This preparation contained 30 to 40 mg protein/ml. For tests of amino acid incorporation into protein of this fraction, 0.15 ml was incubated in 1.0 ml medium containing: (μmoles) Tris buffer, pH 7.5, 30; KCl, 80; NaCl, 50; $MgCl_2$, 5; ATP, 1.0; creatine phosphate, 20; GTP, 0.60; 30 μg creatine kinase; and radioactive amino acid, 0.2 μmoles.

For isolation of the cell-sap, pH 5 fraction and washed microsomes, the method was essentially that of Stone and Joshi (7). Pooled livers from 2 or more animals in the same dietary group were used for these preparations. Cell-sap so prepared contained about 25 to 30 mg protein/ml. It was used either as such, or as a source of pH 5-enzyme. The latter was prepared by isoelectric precipitation at pH 5.2. After redissolving in buffered salt medium, 1.0 ml for each gram of liver homogenized, the pH 5-enzyme preparations contained about 10 to 15 mg protein/ml.

Preparations of washed microsomes were derived from the pellet precipitated by centrifuging the 15,000 × *g* supernatant at 105,000 × *g* for one hour. After dispersal in buffered salt medium 0.5 ml/g liver homogenized, these preparations contained about 20 to 25 mg protein/ml.

Ribosomes were isolated by the second method of Korner (8). Livers from 6 to 8 animals were used for each preparation. Ribosomal pellets were resuspended for use in 0.25 ml buffered salt medium/g of liver homogenized. These preparations contained about 10 mg protein/ml.

Incubation of recombined fractions. Microsome preparation, 0.5 ml was incubated with 0.5 ml cell-sap or pH 5-enzyme preparation in a total volume of 2.0 ml, containing: (μmoles/ml) Tris buffer, pH 7.5, 162.5; sucrose, 62.5; KCl, 25; $MgCl_2$, 5; ATP, 4; GTP, 0.5; creatine phosphate, 20; 25 μg creatine kinase; and 0.4 μmoles radioactive amino acid.

Ribosome preparations, 0.4 ml, were incubated either with cell-sap or pH 5-en-

[3] Obtained from Nutritional Biochemicals Corporation, Cleveland.

[4] Lypro, a spray-dried powder containing about 65% protein and 32% fat. Obtained through the courtesy of International Protein Products Ltd., London, England.

[5] See footnote 3.

[6] Puritan cooking oil, Seprod Ltd., Kingston, Jamaica.

zyme preparations, 0.2 ml, in a total volume of 1 ml medium of composition similar to that used for the 15,000 × *g* supernatant, with the addition of glutathione, 2.5 μmoles.

All samples were incubated in duplicate, for 30 minutes with shaking at 38° in air. Each set included a blank which was kept at 0° during addition of fractions and deproteinized at zero time. Incubations were terminated by adding an equal volume of 10% trichloroacetic acid containing 100 mg of the appropriate nonradioactive amino acid.

Isolation of protein and measurement of radioactivity. Samples were washed twice with 5% trichloroacetic acid, heated at 90° for 10 minutes with the same reagent, washed once again with cold 5% trichloroacetic acid and once each with ethanol-ether (3:1) mixture, and with ether. The residue was dissolved in the minimum volume of 1.0 N NaOH (about 0.1 ml/mg protein), and reprecipitated with ethanol (final concentration > 99%). The precipitate was washed once each with ethanol, ethanol-ether (3:1) mixture and finally with ether. For determination of radioactivity, a weighed amount (1–3 mg) of the air-dried residue was transferred to glass-counting vials, 1.0 ml of 1M *p*-(diisobutyl-cresoxy-ethoxy ethyl) dimethyl benzyl ammonium hydroxide [7] in methanol was added and the protein dissolved by warming at 60° for 3 hours. Scintillator solution was then added and counts determined with a Packard Tri-Carb liquid scintillation spectrometer to a probable error of < 2%. Appropriate standards prepared from the stock solutions of radioactive amino acids used (L-leucine-^{14}C,[8] L-lysine-^{14}C, and L-methionine-^{35}S) were always counted along with experimental samples; levels of incorporation could thus be calculated as micromoles of amino acid per milligram of protein counted. Since analyses of protein were made on all cell fractions used for incubations, calculations could be made, when required, of total uptake into protein of incubation mixtures and then expressed as millimicromoles per milligram of microsomal or ribosomal protein.

Analyses. Protein was determined by the biuret method of Gornall et al. (9). Ribonucleic acid was determined by the method of Fleck and Munro (10).

RESULTS

Values are given as means for appropriate groups ± standard error. Significance of difference between groups was determined by a standard *t* test and was accepted at a level of $P = 0.05$ or less.

Nutritive value of protein supplements. Representative values for PER are shown in table 1. Each of the 3 values is significantly different from the others. Addition of methionine improved the growth-promoting value of peanut protein; this implies that the protein was deficient in methionine.[9] However, the supplementation with methionine did not improve peanut protein to the level of egg albumin.

Experiments with 15,000 × g supertant. Table 2 shows results of tests for uptake of leucine-^{14}C with this fraction. There was no difference between the groups on the basis of these results. It was thought that a relative deficiency of

TABLE 1

PER [1] of protein-rich supplements fed to rats

Protein supplement fed	PER
Peanut protein	10.1 ± 0.31 [2] (16) [3]
Peanut protein + L-methionine	16.1 ± 0.43 (12)
Egg albumin	21.2 ± 0.77 (14)

[1] $\text{PER} = \dfrac{\text{gain in weight (g)}}{\text{N intake (g)}}$.

[2] Mean ± SE. Each mean is significantly different ($P < 0.05$) from the other two.

[3] Numbers in parentheses represent number of animals contributing to each value.

TABLE 2

Effect of dietary protein on incorporation of leucine into protein of a 15,000 × g supernatant of liver

Protein supplement fed	Uptake of leucine-^{14}C
	mμmoles/mg protein
Peanut protein	0.141 ± 0.014 [1] (5) [2]
Egg albumin	0.149 ± 0.011 (5)

[1] Mean ± SE. The 2 means are not significantly different ($P > 0.05$).

[2] Numbers in parentheses represent number of animals contributing to each value.

[7] Hydroxide of Hyamine 10-X, Packard Instrument Company, La Grange, Illinois.

[8] Labeled amino acids were purchased from Schwartz Bio-Research Inc., Orangeburg, New York.

[9] Analytical data suggested a possible deficiency of lysine, but addition of lysine did not alter the PER (unpublished observations).

methionine in the diet might specifically affect levels of incorporation of this amino acid; hence in another series of experiments, tests were repeated with this fraction using methionine-^{35}S instead of leucine-^{14}C. In this case, a group fed peanut protein showed significantly higher levels of incorporation than a group fed egg albumin (table 3).

To determine whether microsomes contributed to the observed differences in levels of incorporation, preparations of washed microsomes were made from dietary groups fed either peanut protein or egg albumin. These were incubated with a common pH 5-enzyme preparation derived from animals fed the stock diet. Results are shown in table 4. These data show that there is a slight advantage to the group fed peanut protein but this proved to be not significant on the basis of either microsomal protein or RNA. On this basis, it seemed likely that changes in levels of incorporation reflected changes of activity in cell-sap. To test this, a common ribosome preparation obtained from rats fed the stock diet was incubated with cell-sap prepared from rats fed either peanut protein or peanut protein + methionine. Table 5 shows that the cell-sap from the former group produced higher levels of incorporation. The difference persisted, although to a less marked degree, when pH 5-enzyme instead of cell-sap from these 2 dietary groups was tested with a common ribosomal preparation (table 5). It is clear that at least a part of the dietary influence on incorporating activity takes effect on the pH 5-enzyme fraction.

Amino acid-activating enzymes occur in the fraction designated pH 5-enzyme (11, 12). Enhanced activity of these enzymes, such as has been reported for liver cell-sap from protein-depleted animals (1, 2) might result in increased incorporation. Furthermore, it might be expected that the effect would be non-specific, applying to other amino acids besides methionine, if the pH 5-enzyme from protein-depleted animals were tested by the criteria used

TABLE 3

Effect of dietary protein on incorporation of methionine into protein of a 15,000 × g supernatant fraction of liver

Protein supplement fed	Uptake of methionine-^{35}S
	mμmoles/mg protein
Peanut protein	0.134 ± 0.009 [1] (10) [2]
Egg albumin	0.096 ± 0.007 (10)

[1] Mean ± SE. The 2 means are significantly different ($P < 0.05$).
[2] Numbers in parentheses represent number of animals contributing to each value.

TABLE 4

Effect of dietary protein on incorporation of methionine into protein, using microsomes from 2 dietary groups with a common pH 5-enzyme preparation [1]

Protein supplement fed	Uptake of methionine-^{35}S	
	mμmoles/mg microsomal protein	*mμmoles/mg microsomal RNA*
Peanut protein	0.167 ± 0.014 [2] (10) [3]	1.15 ± 0.11 (10)
Egg albumin	0.142 ± 0.026 (10)	1.01 ± 0.08 (10)

[1] A preparation derived from animals fed the stock diet.
[2] Mean ± SE. The difference between the 2 means in each column is not significant ($P > 0.05$).
[3] Numbers in parentheses represent numbers of animals contributing to each value.

TABLE 5

Effect of dietary protein on incorporation of methionine into protein, using cell-sap or pH 5-enzyme from 2 dietary groups with a common ribosome preparation [1]

Protein supplement fed	Uptake of methionine-^{35}S	
	With cell-sap	With pH 5-enzyme
	mμmoles/mg ribosome protein	
Peanut protein	0.216,[2] 0.239	0.173, 0.172
Peanut protein + methionine	0.162, 0.170	0.151, 0.137

[1] A preparation derived from animals fed the stock diet.
[2] Each entry is a result for cell-sap or pH 5-enzyme derived from pooled livers of 2 animals.

TABLE 6

Effect of diet on incorporation of lysine into protein using a common ribosome preparation [1] with pH 5-enzyme from 2 dietary groups

Diet	Uptake of lysine-^{14}C
	mμmoles/mg ribosome protein
Protein-free	0.092,[2] 0.095
Stock	0.073, 0.070

[1] A preparation derived from animals fed the stock diet.

[2] Each entry is a result for pH 5-enzyme derived from pooled livers of 2 animals.

in the present work. This proved to be the case when the pH 5-enzyme was prepared from groups of rats fed either the protein-free diet or the stock diet for 2 weeks after weaning and was incubated with a common ribosome preparation from rats fed the stock diet. Table 6 shows that the pH 5-enzyme from protein depleted rats was more active in promoting uptake of lysine.

DISCUSSION

The protein-rich peanut product gave a much lower PER than the reference protein egg albumin. To explain this, the amino acid balance was considered to be of primary importance; digestibility studies were not attempted. A dramatic improvement in PER was achieved by adding to the peanut protein enough methionine to equalize the sulphur amino acid content, per 100 g N, of the test and reference proteins. Hence it appeared very likely that a relative deficiency of methionine existed in the peanut protein, although the possibility has to be borne in mind that the content of other amino acids also may have been suboptimal.

The results of this investigation indicate that components of cell-sap were responsible for differences in levels of amino acid uptake between liver cell preparations from different dietary groups. It appears that an initial step of protein biosynthesis was more active when there was a relative methionine deficiency in the diet. This step requires the activation of amino acids by enzymes of narrow specificity; amino acyl adenylates are formed and linked to appropriate acceptor chains of soluble RNA (sRNA). The necessary factors occur in cell-sap and are part of the pH 5-enzyme complex (13, 14).

Dietary influence on incorporation was demonstrated when the 15,000 × *g* supernant was used with labeled methionine, but not wtih labeled leucine. A possible interpretation is that there was an increase in the activity of the enzyme methionyl-sRNA synthetase selectively among the other amino acid-activating enzymes in liver cell-sap of animals fed peanut protein.

Mentioned earlier was an apparent conflict between previous observations (3, 4) concerning the effects of diets lacking a single amino acid on incorporation levels. The present study did not resolve these differences, since no significant change in the incorporating capacity of microsomes was noted. The duration of the dietary treatment, as well as the labeled amino acid used, may be important in this connection. Sidransky et al. (4) did find that cell-sap from threonine-deprived rats was about 21% more active than that from controls in stimulating uptake of leucine by ribosomes; in view of the present observations, it is interesting to speculate what result would have emerged if uptake of threonine had been measured in their experiments.

LITERATURE CITED

1. Mariani, A., M. A. Spadoni and G. Tomassi 1963 Effect of protein depletion on amino acid activating enzymes of rat liver. Nature, *199:* 378.
2. Gaetani, S., A. M. Paolucci, M. A. Spadoni and G. Tomassi 1964 Activity of amino acid-activating enzymes in tissues from protein-depleted rats. J. Nutr., *84:* 173.
3 Fleck, A., J. Sheperd and H. N. Munro 1965 Protein synthesis in rat liver: influence of amino acids in diet on microsomes and polysomes. Science, *150:* 628.
4. Sidransky, H., T. Staehelin and E. Verney 1964 Protein synthesis enhanced in the livers of rats force-fed a threonine-devoid diet. Science, *146:* 766.
5. Frost, D. V., and H. R. Sandy 1949 Assay of dry proteins by rat repletion method. J. Nutr., *39:* 427.
6. Rippon, W. P. 1959 A comparison of several methods for estimating the nutritive value of proteins. Brit. J. Nutr., *13:* 243.
7. Stone, D., and S. Joshi 1962 Some evidence for a pathway of amino acid incorporation, in rat-liver microsomes, which does not require transfer ribonucleic acid. Biochim. Biophys. Acta, *55:* 335.

8. Korner, A. 1961 Amino acid incorporation into ribosomes. Biochem. J., *81:* 168.
9. Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum protein by means of the biuret reaction. J. Biol. Chem., *177:* 751.
10. Fleck, A., and H. N. Munro 1962 The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid determination. Biochim. Biophys. Acta, *55:* 571.
11. Hoagland, M. B. 1955 An enzymatic mechanism for amino acid activation in animal tissues. Biochim. Biophys. Acta, *16:* 288.
12. Hoagland, M. B., E. B. Keller and P. C. Zamecnik 1956 Enzymatic carboxyl activation of amino acids. J. Biol. Chem., *218:* 345.
13. Allen, E. H., E. Glassman and R. S. Schweet 1960 Incorporation of amino acids into ribonucleic acid. 1. The role of activating enzymes. J. Biol. Chem., *235:* 1061.
14. Allen, E. H., E. Glassman and R. S. Schweet 1960 Incorporation of amino acids into ribonucleic acid. II. Amino acid transfer ribonucleic acid. J. Biol. Chem., *235:* 1068.

Bioassay of Vitamin K in Chicks [1]

JOHN T. MATSCHINER AND E. A. DOISY, JR.
Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri

ABSTRACT A convenient, precise bioassay for vitamin K is reported based on the analysis of chick plasma with Russell's viper venom. Data obtained by this procedure were normally distributed for statistical evaluation. The relative molar activities of several forms of vitamin K and the concentration of vitamin K in a number of animal tissues are given.

Bioassay remains an indispensible technique in studies on the occurrence and metabolism of vitamin K. Despite a number of available procedures, assay in chicks has been used most often because these animals are sensitive to deprivation of the vitamin and respond in a reliable manner to prophylactic or curvative treatment. During recent years we have adopted a modified chick assay which appears to have considerable advantage over published methods.

MATERIALS AND METHODS

Chicks. Day-old White Rock chicks of mixed sex were otbained from a local hatchery. Preliminary studies with sexed chicks indicated no difference between male and female in response to deficient diets or to repletion with the vitamin. The chicks were housed in wire-floor brooders with contact heat.[2] Room temperature was maintained at 24° and a roosting period was provided between midnight and 6 AM by timed overhead lights. Fresh food and water were provided daily.

Diet. Chicks were grown for 10 days with the vitamin K-deficient diet shown in table 1. After 10 days they were separated into groups of ten and fed the same diet containing controlled amounts of vitamin K for 4 days. Lipids were conveniently assayed by mixing them in place of an equal amount of corn oil in the deficient diet.

Prothrombin. After the chicks had been fed the experimental diet for 4 days, they were anesthetized lightly with ether and samples of blood were taken by cardiac puncture. Plasma was analyzed for coagulation activity using Russell's viper venom according to Hjort et al. (2). In mammalian plasma, this procedure detects changes in prothrombin and Factor X (3); however in chicks the assay may be specific for prothrombin since the activity of Factor X is not present. The reference standard for this assay was a pool of plasma obtained from chicks fed for 2 weeks a commercial ration [3] containing an added 0.5 μg of phylloquinone/g.[4] The addition of vitamin K was necessary to ensure uniform maximum prothrombin activity. Aliquots of the reference pool diluted to various concentrations were analyzed with each group of experimental samples. Finally, clotting times were used to calculate the activity of the experimental samples as a percentage of the activity of the reference standard. To evaluate the statistical aspects of this procedure, individual samples of reference plasma were compared with the reference pool. For 78 chicks the average prothrombin was 102 ± 2% [5] and individual values closely approximated a normal distribution.

Received for publication April 21, 1966.

[1] These studies were supported in part under grant no. DA-MO-49-193-62-G41 from the Office of the Surgeon General, Department of the Army and Public Health Service Research grant no. AM09909-01 from the National Institute of Arthritis and Metabolic Diseases. The opinions expressed are those of the authors and not necessarily those of the Department of the Army.

[2] James Manufacturing Company, Fort Atkinson, Wisconsin.

[3] Purina Startena, Ralston Purina Company, St. Louis.

[4] Reference to specific forms of vitamin K is made in accordance with the recommendation of the Nomenclature Commission of the IUPAC (4). The K_2 vitamins are menaquinones (abbreviated MK-n) and vitamin K_1 becomes phylloquinone (abbreviated K). The length of the side chain is designated on the basis of the number of isoprene units (n).

[5] Throughout this report means are accompanied by their standard error.

TABLE 1

Vitamin K-deficient diet for chicks

Soy protein [1]	35.00
DL-Methionine	0.75
Glycine	0.30
Glucose monohydrate [2]	48.95
Vitaminized glucose monohydrate [3]	5.00
Salts 446 [4]	5.00
Cellulose [5]	3.00
Fat-soluble vitamin mix [6]	0.10
Corn oil [7]	1.90

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[2] Cerelose, Corn Products Company, New York.
[3] Vitaminized glucose monohydrate supplied the following vitamins in mg/100 g of diet: thiamine, 1; riboflavin, 1; Ca pantothenate, 5; pyridoxine, 0.5; niacin, 2; folic acid, 0.1; vitamin B_{12}, 0.005; biotin, 0.01; *p*-aminobenzoic acid, 1; inositol, 25; and choline chloride, 250.
[4] Mameesh and Johnson (1).
[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[6] The fat-soluble vitamin mixture supplied the following vitamins in μg/g of diet: vitamin A acetate, 5; vitamin D_3, 0.035; and vitamin E acetate, 60.
[7] Mazola, Corn Products Company, New York.

RESULTS AND DISCUSSIONS

The response of chicks to vitamin K is shown in figure 1. Each point is the mean prothrombin activity of at least 20 chicks. In the upper region of the curve, average normal prothrombin activity was observed in chicks that received the diet containing approximately 0.4 μg of phylloquinone/g. The lowest detectable concentration of vitamin K was less than 0.05 μg/g.

Chicks used in these studies generally weighed about 130 g at 10 days and gained approximately 50 g during the 4 days of the assay; however, during the winter chicks were often stunted by exposure to inclement temperatures during shipment. This prompted an examination of the effect of weight gain on the assay. In table 2 prothrombin levels are shown for normal and underweight chicks fed the deficient diet or a diet containing 0.2 μg of phylloquinone/g. Body weights as low as 80 g at 10 days and a weight gain as low as 30 g during the assay did not alter predictable prothrombin activities.

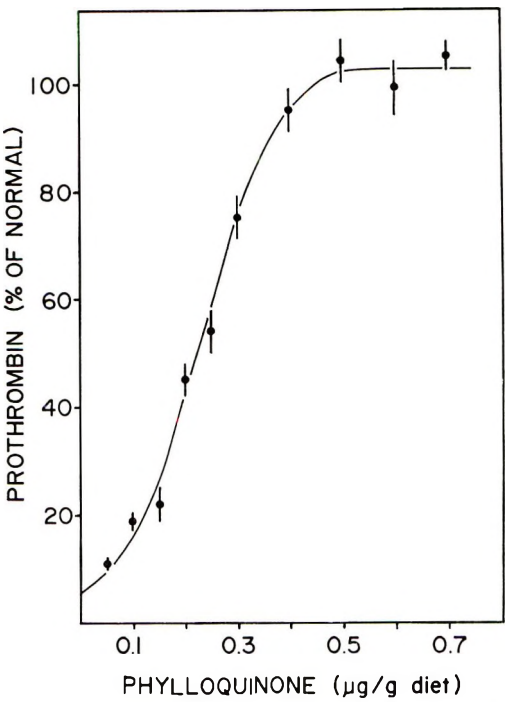


Fig. 1 Response of vitamin K-deficient chicks to diets containing controlled amounts of phylloquinone. Each point is accompanied by the standard error of the mean.

As shown in table 1, the deficient diet contains corn oil; however, several other oils may also be fed without providing significant amounts of vitamin K. The bioassay of several oils at 2% of the diet is shown in table 3. Only peanut oil and one brand of soybean oil contained detectable amounts of vitamin K.

TABLE 2

Comparison of prothrombin in normal and underweight chicks

Diet	Body weight		Prothrombin
	10 days	14 days	
	g	*g*	%
Deficient	106±3(19) [1]	149±4	6±0.3
	132±3(31)	182±5	5±0.4
Supplemented [2]	80±1(10)	114±2	46±7
	129±2(20)	184±5	45±3

[1] Mean ± SE. Number of observations in parentheses.
[2] 0.2 μg of phylloquinone/g of diet after 10 days.

TABLE 3

Bioassay of several dietary oils

Dietary oil	Prothrombin
	%
Corn [1]	5 ± 0.4 [2]
Soy [3]	5 ± 1
Soy [4]	19 ± 1
Cottonseed [5]	5 ± 0.2
Safflower	4 ± 1
Peanut	9 ± 1
Olive	5 ± 0.4

[1] Mazola, Corn Products Company, New York.
[2] Mean ± SE.
[3] Crisco Oil, Procter and Gamble, Cincinnati.
[4] Durkee Famous Foods, Cleveland.
[5] Wesson Oil Sales Company, Fullerton, California.

TABLE 4

Relative molar activities of several forms of vitamin K

Vitamin [1]	Relative molar activity [2]
MK-1	1
K-2	30
MK-2	35
K-4	100
MK-4	156
MK-5	116
MK-7	122
MK-9	78
MK-9(H)	67
MK-10	49
Menadiol diphosphate [3]	164
Menadione	49

[1] Most of the vitamins were donated by Hoffmann-LaRoche, Inc., Nutley, New Jersey, through the generosity of Dr. O. Isler. Menaquinone-7 (MK-7) was donated by Dr. E. A. Doisy. Samples of vitamin K_9(H) (MK-9(H)) were donated by Dr. M. Weber and Dr. C. Coscia.
[2] Phylloquinone (K-4) was arbitrarily assigned an activity of 100.
[3] Synkavite, Hoffmann-LaRoche, Inc.

In an earlier extensive study, Wiss et al. (5) determined the biological activity of several forms of vitamin K using the assay of Dam et al. (6). In the present study we examined the activity of some of these vitamins and extended the assays to more lipophilic forms of vitamin K which have recently assumed biological interest. Each vitamin was compared with phylloquinone which was arbitrarily assigned an activity of 100. All vitamins were assayed at 2 concentrations within the useful region of the curve shown in figure 1. The tabulated values shown in table 4 are the average of these assays. By comparison with earlier studies, the menaquinones appear to have exhibited greater activity, whereas menadione was less active than expected. MK-9 (H) which differs from MK-9 by the presence of a single saturated isoprene unit in the side chain (7) was approximately as active as MK-9. The remarkable inactivity of dietary 2-methyl-3-dimethylallyl-1,4-naphthoquinone (MK-1) first observed by Wiss et al. (5) was also obvious in the present assay.

In addition to studies with pure samples of vitamin K we have also analyzed the vitamin K content of several animal tissues. After some preliminary studies, it was possible to obtain direct assays by mixing 30% of homogenized raw tissue with the standard vitamin K-deficient diet. Tissues obtained from the packing house were of the best grade available; tissues from laboratory animals came from healthy specimens fed commercial chows. The data recorded in table 5 were obtained by the assay of tissue from at least 10 animals. There was a low level of vitamin K in bird liver compared with mammalian species, but among mammals there was no apparent basis or predictability for the concentration of hepatic vitamin K. The richest source we have observed is beef liver which may contain as much as 1.2 μg/g of tissue. Those tissues in which vitamin K was not detected contained less than approximately 0.07 μg/g; i.e., vitamin K was barely detectable in chick and rat liver. In confirmation of the results of Dam (8), Bou-

TABLE 5

Chick bioassay of animal tissue

Tissue	Vitamin K [1]
	μg/g
Liver, beef	0.92
Liver, veal	0.27
Liver, lamb	nd [2]
Liver, dog	0.53
Liver, pig	0.25
Liver, rabbit	0.35
Liver, monkey	0.33
Liver, rat (M)	0.07
Liver, rat (F)	0.10
Liver, guinea pig	0.15
Liver, turkey	nd
Liver, pigeon	nd
Liver, chicken	0.08
Liver, duck	nd
Heart, beef	nd
Kidney, beef	nd
Muscle, beef	nd

[1] Activity expressed as phylloquinone.
[2] None detected.

man and Slater (9) and others, extrahepatic tissues contained little if any vitamin K. In a separate study with rats, comparative assays of fresh and irradiated beef indicated the presence of about 0.04 μg of vitamin K/g of fresh tissue; however, this is below the level of detection in the present assay.

The data presented here emphasize that remarkably small amounts of vitamin K are required for normal function. The concentration in liver, from less than 0.1 to 1 μg/g, defines a potency and effectiveness for vitamin K approximating that of the notably trace vitamins such as biotin and vitamin B_{12}.

ACKNOWLEDGMENTS

The authors are indebted to Mrs. Josephine Amelotti and Carol Kuhn for capable technical assistance.

LITERATURE CITED

1. Mameesh, M. S., and B. C. Johnson 1958 The effect of penicillin on the intestinal synthesis of thiamine in the rat. J. Nutr., 65: 161.
2. Hjort, P., S. I. Rapaport and P. A. Owren 1955 A simple specific one-stage prothrombin assay using Russell's viper venom in cephalin suspension. J. Lab. Clin. Med., 46: 89.
3. Hougie, C. 1956 Effect of Russell's viper venom (Stypven) on Stuart clotting defect. Proc. Soc. Exp. Biol. Med., *93*: 570.
4. IUPAC Biochemical Nomenclature Commission 1965 Nomenclature of quinones with isoprenoid side-chains. Biochim. Biophys. Acta, *107*: 5.
5. Wiss, O., F. Weber, R. Rüegg and O. Isler 1959 Über die biologische Activität der Vitamin K_1 und K_2 und ihrer Isoprenologen. Z. Physiol. Chem., *314*: 245.
6. Dam, H., I. Kruse and E. Søndergaard 1951 Determination of vitamin K by the curative technique in chicks. Acta Physiol. Scand., 22: 238.
7. Gale, P. H. B. H. Arison, N. R. Trenner, A. C. Page, Jr., K. Folkers and A. F. Brodie 1963 Characterization of vitamin $K_9(H)$ from *Mycobacterium phlei*. Biochemistry, 2: 200.
8. Dam, H. 1935 The antihemorrhagic vitamin of the chick. Nature, *135*: 652.
9. Bouman, J., E. C. Slater 1956 Tocopherol content of heart-muscle preparations. Nature, *177*: 1181.

Proceedings of the Thirtieth Annual Meeting of the American Institute of Nutrition

SHELBURNE HOTEL, ATLANTIC CITY, NEW JERSEY
APRIL 12–16, 1966

COUNCIL MEETINGS

The Council of the American Institute of Nutrition met Sunday evening, April 10, and Monday morning and evening, April 11. The actions of the Council were presented at the Institute business meetings and are included in the report of those meetings.

SCIENTIFIC SESSIONS

A total of 348 abstracts of papers was accepted by the Institute; 70 of them were transferred to other societies; 43 were accepted from other societies, making a total of 321 papers programmed by the Institute. These were arranged into 26 regular AIN and 3 intersociety (atherosclerosis) sessions. In addition, two informal conferences were held, Poultry Nutrition and Ruminant Nutrition, and the following half-day symposia were presented:

1. The Relationship of Nutrition to Central Nervous System Development and Function
2. Trace Elements in Nutrition
3. Nutrition Education
4. Improving the Nutritional Value of Cereal Grains by the Use of Gene Mutations and Selective Breeding
5. Interactions of Nutrition and Infection

BUSINESS MEETINGS

Business meetings were held on Tuesday, April 12 and Friday, April 15. Dr. O. L. Kline presided at both meetings.

I. *Proceedings of 1965 meeting*

The Proceedings as published in the Journal of Nutrition, *86:* 439–450, 1965, were approved.

II. *Elections*

The 682 ballots were counted by Drs. Dorothy Arata and Duane Benton. The following were elected:

President-elect:
George M. Briggs

Councilor:
Alfred E. Harper

Nominating Committee:
M. L. Scott, Chairman
C. O. Chichester
D. B. Coursin
B. C. Johnson
R. M. Leverton

III. *Membership Status*

As of April 1, 1966, there were 1,034 members of the Institute: 914 active, 102 retired and 18 honorary members, this being a net increase of 35 members since last year. Fifteen members retired during the year. The Clinical Division reports a total membership of 171.

Notice of the deaths of these members was received this year:

Bertha A. Bisbey (Charter Member), April 3, 1965
C. W. Carrick, February 17, 1966
Amy L. Daniels (Charter Member), January 31, 1965
Richard H. Follis, Jr., December 5, 1965.
Allen R. Hennes, November 12, 1965
Josiah S. Hughes, August 12, 1965
H. H. Mitchell (Charter Member), March 28, 1966
Elsie Z. Moyer, July 1, 1965
Lydia J. Roberts (Charter Member), May 28, 1965
W. D. Salmon (Charter Member), February 5, 1966
Harry H. Sobotka (Charter Member), December 24, 1965
Hertha H. Taussky, November 7, 1965
Robert R. Williams, October 2, 1965

The following resolution in honor of Dr. Bisbey was read:

RESOLVED: That the American Institute of Nutrition assembled at Atlantic City, New Jersey, at its annual meeting, April 12, 1966, place in its minutes for permanent record this statement of deep regret and sorrow at the loss by death of Charter Member, Dr. Bertha A. Bisbey, on April 3, 1965, and further, that special recognition be given to her unique qualities that: enabled her to translate her knowledge of chemistry and her appreciation of the limiting nutrients of of flour into practical recommendations to obtain the "nutritious loaf," which proved useful for the socially underprivileged; permitted her to organize and administer a nutritional research and training program during the early years of our Society; afforded her students the human understanding and encouragement needed to set and obtain high scholastic standards and sound research principles; and gave her the courage and strength to achieve a successful career and to inspire others to make nutrition their life's work.

The following resolution in honor of Dr. Daniels was read:

RESOLVED: That the American Institute recognize the loss by death of Dr. Amy Louise Daniels on January 31, 1965, in her 90th year. Dr. Daniels was truly a pioneer among women scientists. She was a Charter Member of the American Home Economics Association (1909), was the first woman to receive a Ph.D. in Physiological Chemistry from Yale (1912), and was a Charter Member of the AIN.

After teaching in the universities of Missouri and Wisconsin and taking graduate study at Harvard Medical School, Dr. Daniels came to the University of Iowa in 1918 as Professor of Nutrition in the new Child Welfare Station. Until 1925 she was also affiliated with the Department of Pediatrics, and managed a well-baby feeding clinic. Her research was directed toward establishing nutritional requirements for infants and children. She was the first to show that infants given cow's milk feedings needed additional vitamins. The effect of vitamin D on calcium and phosphorus retention of infants, and the magnesium requirements of young children are among her many contributions to nutritional needs during growth.

In 1930, Dr. Daniels was appointed to the first White House Conference on Child Development and in 1937 she received the first Borden Award given by the American Home Economics Association for her work in child nutrition. Failing sight forced her retirement in 1941 and precluded further scientific study. Dr. Daniels' wide range of interests and her keen wit made her a delightful companion. Her high standards and unfailing honesty set an example that will long be remembered by her students and co-workers.

The following resolution in honor of Dr. Mitchell was read:

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annular meeting, April 12, 1966, wishing to express its deep regret and sorrow at the passing of one of its most distinguished Charter Members, Harold Hanson Mitchell, place this statement in its minutes for permanent record.

Educated at the University of Illinois, Dr. Mitchell spent his entire scientific career at that institution, joining the staff in 1911 and continuing until his retirement in 1955. Few investigators have had an influence on nutritional research and concepts equal to that of Dr. Mitchell. His research program was characterized by logical planning and minute attention to experimental details. He was an early advocate of the statistical treatment of experimental data. Among his many research contributions the following are outstanding: the development of the Thomas-Mitchell method of protein evaluation and demonstration of the correlation between protein value and amino acid content; the espousal of controlled feed intake in nutrition studies; the importance of nutrient balance in rations; energy and mineral metabolism studies of farm animals and of man; the factorial approach to determination of nutrient requirements. Together with these research accomplishments Dr. Mitchell is known for his meticulous and astute approach to the scientific literature and for his great ability to summarize and to critically evaluate the issues of his day. He was firm in his opinions, but fair and always a gentleman in their defense.

Attendance in his classes was a rewarding experience. His course outlines were prepared and presented with great clarity and detail, and he encouraged open discussion and evaluation of the data and conclusions. As a culmination of his long teaching and research experience he devoted his retirement years to writing a 2-volume treatise on Comparative Nutrition of Man and Domestic Animals which will long serve as an invaluable reference.

Dr. Mitchell served on the editorial board of the *Journal of Nutrition* for 19 of the first 25 years of its existence. He received the AIN Borden Award in

1945 and was elected a Fellow in 1958. At the time of his death he was the nominee for the 1966 Osborne and Mendel Award which was presented posthumously at the annual banquet. He received the Morrison Award of the American Society of Animal Production in 1950 and was elected a Fellow of that society in 1960.

The circle of his scientific admirers was wide and he had a close circle of intimate friends. His was a full life and a rich one. We who are his beneficiaries realize a great loss but recognize how greatly we have been privileged.

The following resolution in honor of Dr. Roberts was read:

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 12, 1966, place in its minutes for permanent record this statement of deep regret at the loss by death of one of its distinguished Charter Members, Lydia Jane Roberts, May 28, 1965 in Puerto Rico.

And further that high tribute be paid to Dr. Roberts for her versatility in excellence including research, teaching and action programs in nutrition; for her two distinct careers, one at the University of Chicago until retirement in research and teaching, and the other at the University of Puerto Rico in teaching and in community demonstrations of the value of nutrition in improving the living standards of low-income families; for direction of graduate students in research on nutrient and food needs, especially of children and the effects of supplementing diets of children with different foodstuffs; for her contributions to professional and nutrition policy-making groups such as the Food and Nutrition Board of the National Research Council, the Council on Foods and Nutrition of the American Medical Association, the White House Conferences on Children and Youth and the American Dietetic Association; for her contribution and aid to nutrition programs in developing countries and training for the Peace Corps; for her writings, not only research papers but interpretive books, such as Nutrition Work with Children, Patterns of Living for Puerto Rican Families, the Doña Elena Project, and the text book on nutrition planned primarily for use in the Caribbean area; for her dynamic and friendly leadership, her inspiration to students and her ability to win support for nutrition programs not only from other professionals but from legislators and governors.

The following resolution in honor of Dr. Salmon was read:

RESOLVED: That The American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 12, 1966, place in its minutes for permanent record this statement of sincere regret and sorrow at the loss by death of one of its distinguished Charter Members, William D. Salmon, on February 5, 1966.

Dr. Salmon pioneered in nutritional research in the South. Among his many contributions were his work in demonstrating the nature of vitamin B as a complex of vitamins; his observations on the influence of dietary fat on the body fat composition; his discovery that zinc deficiency was the primary cause of parakeratosis in swine; his showing that dietary deficiency, especially of choline, can produce cancer in experimental animals; and providing the evidence that influenced legislation in Alabama requiring the enrichment of white flour, white corn meal, and grits.

He served his institution, Auburn University, with distinction as professor, department head, and professor emeritus, and he had a marked influence on the quality of both its teaching and research. His alma mater, the University of Kentucky, awarded him an honorary Doctor of Science degree in 1958 and named him a Centennial Distinguished Alumnus in 1965. He was one of the most loyal AIN members, serving for twelve years on the editorial board of the *Journal of Nutrition*, and as member or chairman of numerous committees. He was elected a Fellow of the Institute in 1962 and was a nominee for president-elect at the time of his death.

Dr. Salmon had an unfailing sense of humor and a sharp wit to match his keen intellect. His death is a great loss to our Society and to his countless friends.

The following resolution in honor of Dr. Sobotka was read:

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, April 12, 1966, at its annual meeting wishing to express regret and sorrow at the loss by death of one of its distinguished Charter Members, Harry H. Sobotka, place this statement in its minutes for permanent record.

Dr. Harry Sobotka was awarded a Ph.D. in chemistry under Professor Richard Willstätter, a Nobel laureate in enzyme chemistry. His early appointments were those of Research Assistant at the Rockefeller Institute, and Research Associate at the New York Uni-

versity-Bellevue Medical Center. In 1928 he was appointed Director of the Department of Chemistry of the Mount Sinai Hospital in New York City, a post he held until 1965, when he retired.

Dr. Sobotka was well known and honored throughout the scientific world. More than 300 scientific communications and a number of books and monographs attest to the prolific output of the group which he headed; his monograph on the Physiology of the Bile still remains a classic. At the time of his death he was editing a work on alkaloids and awaiting the eighth volume of Advances in Clinical Chemistry which he edited in collaboration with C. P. Stewart of the university of Edinburgh. By his interest in clinical chemistry, Harry Sobotka was instrumental in the founding of the American Association of Clinical Chemists and served as its first president. He received the first Van Slyke Award in 1958 and the Ames Award from the Association in 1962. In 1964 he was awarded an honorary doctorate by the University of Perugia, Italy, and also received the medal of the Societé de Chemie Biologique.

Harry Sobotka was a busy man, ever in search of knowledge. His high standards led him through a long and sucessful career as a scientist. His death leaves a void in our scientific community which will be difficult to fill.

The following resolution in honor of Dr. Williams was read:

RESOLVED: That the American Institute of Nutrition, assembled at its annual meeting in Atlantic City, New Jersey, on April 12, 1966, wishes to express its deep sorrow and profound sense of loss at the passing of one of its most distinguished members and beloved colleagues, Robert R. Williams, on October 2, 1965.

As a scientist, philanthropist, humanitarian and man, Dr. Williams won respect, admiration and affection. His life was, and will continue to be, an inspirational example — a life marked by intense dedication to the high purpose he set for himself, the eradication of dietary diseases which have so long plagued mankind.

His monumental scientific achievement was the identification and synthesis of thiamine, the culmination of an arduous quest spanning a quarter century. While recognition came in the form of many honors from a host of nations, he spurned personal financial reward in order to create the Williams-Waterman Fund for the Combat of Dietary Diseases as a mechanism through which the fruits of his labors and those of his co-workers in this enterprise could be applied to the betterment of humanity.

The bestowal of honors was accompanied by calls to service from many quarters, and Dr. Williams gave of himself unstintingly in positions which demanded judgment, responsibility, broad knowledge and a high order of competence in furthering the cause to which he was devoted. Of particular note were his valuable contributions as a member of the Food and Nutrition Board of the National Research Council and as Chairman of its Committee on Cereals from its founding in 1940 until his retirement in 1959.

In 1957 his colleagues in the American Institute of Nutrition elected him as their President. In this capacity, as in all others he filled, he served with the greatest distinction, regarding it, characteristically, not as a well-deserved honor but as an opportunity for additional service.

Robert R. Williams, one of the foremost pioneers of nutrition, architect of cereal enrichment programs throughout the world, outstanding scientist, warm humanitarian, has an abiding place in history and in the hearts of those who were so fortunate as to know him. Because of what he was, the purpose is clearer, the road straighter, the goal nearer, and those who follow will ever receive inspiration from his life's work.

IV. *New Members*

The membership committee considered the qualifications of 92 nominees. The following 77 nominees, recommended by the Council, were elected to membership at the buisiness meeting:

NEW MEMBERS — 1966*

Abelson, Denis (C)
Abernathy, Richard Paul
Allred, John B.
Baldwin, R. L.
Brown, Elmer B. (C)
Brown, William Duane
Campagnoli, Mario (C)
Carpenter, Lawrence E.
Carpenter, Mary P.
Carroll, Catherine
Chalupa, William
Christakis, George J. (C)
Christensen, Halvor Niels
Cornwell, David G.
Coulson, Walter F.
Dempsey, Hugh (C)
Donald, Elizabeth A.
Doyle, Margaret D.
Ellenbogen, Leon
Evans, Joseph L.
Fougere, William
Gevirtz, Norman (C)
Gortner, Willis A.
Hackler, L. Ross
Hall, Charles A. (C)
Hanna, Fikri M. (C)
Hanson, Lester R.
Harrill, Inez
Hawkins, George E.
Herman, Robert H. (C)
Hopkins, Leon L., Jr.
Jacobs, Francis A.
Jansen, G. Richard
Jones, Don Paul (C)
Kalbfleisch, J. McDowell
Kaufman, Nathan (C)
Kies, Constance V.
Klain, George J.
Klavins, Janis V. (C)
Knittle, Jerome L. (C)
Kornegay, Ervin T.
Kronfeld, David S.
MacDonald, Ian (C)
Majaj, Amin S.
Marion, James E.
McGandy, Robert B. (C)
McKigney, John I.
McRoberts, Milton R.
Meade, Robert J.
Mistry, Sorab Pirozshah
Mookerjea, Sailendu Sekhar
Motzok, I.
Munro, Hamish N. (C)
Navia, Juan M.
Peifer, James J.
Raun, Ned Smith
Sandstead, Harold H. (C)
Savage, J. E.

Schroeder, Henry Alfred
Schulert, Arthur R.
Seward, Coleman R.
Sipple, Horace L.
Smith, John T.
Snook, Jean Twombly
Srebnik, Herbert H.
Tinoco, Joan W. H.
Turk, Donald E.
Vanderveen, John E.
Vaughan, David A.
Wang, Hwa. Lih
Ward, Gerald M.
Welch, James Graham
Wheby Munsey Stephen (C)
Yang, Modesto G.
Yeh, Samuel D. J.
Zalusky, Ralph (C)
Ziffer, Herman (C)

* For institutional affiliations and addresses of new members, see the Federation *Directory of Members* to be published in the fall of 1966.
(C) Also elected to membership in the Clinical Division on April 30, 1966.

Drs. Anne M. Briscoe and Fred Kern, Jr., already members of the American Institute of Nutrition, were elected to membership in the American Society for Clinical Nutrition.

HONORARY MEMBERS

The following scientists were elected to Honorary Membership:

H. A. P. C. Oomen, *Director, Institute for Tropical Hygiene, Amsterdam, Holland*

B. S. Platt, *National Institute for Medical Research, M.R.C. Human Nutrition Research Unit, Mill Hill, London, N.W. England*

Jean Tremolieres, *Institut National d'Hygiene, Paris, France*

V. *President's Report*

President Kline advised the membership that AIN will continue to pursue its efforts to obtain support for developing a word list or guide to nutrition terminology that would serve as a basis for a documentation system for nutrition science literature.

Since the initiation of the Sustaining Associates program last year, 19 organizations have become Sustaining Associates of the AIN. Contributions ranged from $100 to $1000, making a total of $5,500. Sustaining Associates are listed in the *Journal of Nutrition* each month.

The Council has approved a budget of up to $5,000 for the expert advice, consultation and conferences required for revising the leaflet, "Careers in Nutrition."

President Kline advised that, due to the pressures of other duties, the AIN Nutrition Notes editors, M. R. S. Fox and William Boehne, have asked to be relieved of this assignment. A vote of appreciation and thanks was made to the editors for their efforts in making the newsletter such a success.

AIN and the Nutrition Society of Canada will plan for a joint meeting in 1970, hopefully to be expanded to include the recently established Latin American Nutrition Society.

The Council has initiated the procedure that the President will poll the members of the newly elected Nominating Committee as a means for their selecting their own chairman.

It has been a long-standing custom to hold in confidence the identity of the recipients of the annual AIN Awards and also those to be named Fellows until the actual presentation of these honors at the Society banquet. Dr. Kline announced that the AIN Council had taken the important decision to alter this arrangement to permit pre-meeting announcement of Award winners and Fellows. This change was made to allow for more effective publicity concerning the individuals to be honored, their specific scientific accomplishments which won them recognition, and the nature of the Awards and their sponsors. Under the present arrangement our news releases were always competing with all the others resulting from the Federation meetings.

A joint committee representing both AIN and ASCN is being established to undertake a thorough revision of the constitution and by-laws of both societies. The purpose is to improve the precision of both documents and to resolve certain inconsistencies between them.

The American Board of Nutrition has requested the Councils of both AIN and ASCN to accept the responsibility of making a nomination to fill any vacancy that occurs on the Board. The latter will elect one of these nominees to maintain a balance between those working in human nutrition and those in clinical nutrition. The AIN Council has accepted this assignment and the ASCN Council will act upon the request at its next meeting.

VI. *Executive Secretary's Report*

Dr. Waddell reported that the total funds available to the AIN Committee for the support of travel grants to the VIIth International Congress of Nutrition in

EXHIBIT A

Balance Sheet — December 31, 1965

ASSETS		
Cash		$46,097.75
Accounts Receivable		5,938.75
Investments		21,832.68
Furniture and Equipment	$1,645.57	
Less Accumulated Depreciation	(82.28)	1,563.29
Prepaid Expenses		84.00
Total Assets		$75,516.47
LIABILITIES AND FUND CAPITAL		
Accounts Payable		$15,696.50
Fund Capital (see Exhibit B)		59,819.97
Total Liabilities and Fund Capital		$75,516.47

EXHIBIT B

Statement of Income and Expense and Fund Capital
For the Period April 1, 1965 through December 31, 1965

Fund Capital Forwarded, 4/1/65	$40,515.96
Income:	
Page Charges	11,900.00
Interest Income	1,368.20
Membership Dues (897 @ $7.00)	6,279.00
Supporting Members Contribution	50.00
Annual Meeting Registrations	5,552.70
Sale of Special Publications	112.00
Additional Net Subscription Income — Wistar Institute	3,723.75
Editorial Allowance — Wistar Institute	7,275.00
Award Winners Reception Receipts	460.65
Proceeds from Sale of Series K Bond	493.50
Other Income	7.00
Total Income	$77,737.76
Expenses:	
Salaries and Wages	$ 5,341.42
Payroll Taxes	144.70
Pension and Group Insurance Contributions	292.27
Editorial Office Expenditures	3,785.00
Other Personal Service	12.36
Hotel and Travel	4,319.76
Addressing, Mailing and Shipping	308.66
Telephone and Telegraph	215.31
Printing and Engraving	681.29
Supplies and Duplicating	754.94
Rental of Equipment and Space	116.00
Equipment Repairs and Maintenance	25.78
Depreciation Expense	82.28
Dues to AAAS	40.00
Award Winners Reception	460.65
Coffee Lounge — Annual Meeting	134.81
Other Expense	261.60
	$16,976.83
FASEB Business Service Charge	940.96
Total Expense	$17,917.97
Fund Capital at 12/31/65	$59,819.97

Hamburg was $45,000 (National Institute of Arthritis and Metabolic Diseases, $15,000; National Science Foundation, $10,000; Nutrition Foundation, Inc., $5,000; and AIN $15,000). This was appreciably less than had been anticipated, while the number of applications was greater than expected. The Committee awarded 121 travel grants out of a net total of 180 applications.

Two additional group flights to Hamburg and return were announced, supplementing those previously described in the travel folder sent to all AIN members and to applicants for travel grants. These were for trans-Atlantic travel only to suit those who wished to spend more than 21 days abroad but wished to arrange for their own European travel.

VII. *Treasurer's Report*

Dr. W. A. Krehl distributed copies of the financial statement, reproduced here, and introduced to the membership the AIN business manager, Mr. John Rice. The Auditing Committee reported that, on their behalf, Dr. John Bieri of the Bethesda area had examined the records of the AIN finances and found them in order. The Auditing Committee recommended that in the future the auditing committee consist of one or more AIN members residing in the Bethesda area, and that they be instructed to review the financial records of the Institute in the Federation office. AIN's present business arrangement with the Federation includes a professional audit of the AIN accounts within the Federation audit.

VIII. *Dues*

Motion was made, seconded and passed to accept the recommendation of the AIN Council that membership dues remain the same as for last year ($7.00).

IX. *Editor's Report — Journal of Nutrition*

The editor of the *Journal of Nutrition*, Dr. Richard H. Barnes, submitted his report for the calendar year 1965 with a comparison to 1963 and 1964. He called particular attention to the current guide for authors and asked that it be followed for manuscripts submitted to the Journal.

It was reported that the AIN Committee on Publication Management has been asked to investigate the costs and procedures for moving the redactory services of the journal to Beaumont and submit a recommendation to the Council.

The report and proposed budget were approved and are summarized below:

Editing and Publication Operations (Calendar year)

	1963	1964	1965
Volumes published	79, 80, 81	82, 83, 84	85, 86, 87
Pages published (including papers, biographies, announcements, and proceedings)	1436	1339	1384
(Scientific papers only)	(1383)	(1267)	(1313)
Papers published (including 3 biographies)	204	191	189
Papers submitted	312	293	303
Papers rejected	113	112	98
Rejection rate (based on no. papers submitted)	36%	38%	33%
Supplements published	—	—	—
Letters to the Editor		1	2
Operating Schedule			
Avg time for date of receipt of manuscript to mailing to Wistar Press:			
Avg no. days with reviewer	21.2	20.7	21.5
Avg no. days out for revision	24.8	22.6	22.2
Avg no. days in office, in mail or in unavoidable delay	34.9	29.2	30.6
Avg total *days*	80.9	72.5	74.3
Avg time from date of receipt of manuscript to mailing by Wistar Press:			
Avg no. months with Editorial Office	2.6	2.4	2.5
Avg no. months with Wistar Press	3.2	3.2	3.0
Avg *total months* from date of receipt to mailing of Journal	5.8	5.6	5.5

Summary of Finances in the Operation of the Editor's Office, *Journal of Nutrition*

July 1, 1965 — December 31, 1965

Balance brought forward	$ 959.27
Receipts, AIN	6,000.00
Total receipts and balance available	6,959.27
Expenditures	4,751.29
Balance	$2,207.98

(This statement covers a period of only 6 months because the beginning of the fiscal year was changed by the AIN business office from July 1 to January 1.)

The Council approved the budget proposed by Dr. Barnes for the Editor's Office for the year starting January 1, 1966.

X. *Report of the Clinical Division*: J. F. Mueller

Dr. Mueller advised the membership of the coming ASCN annual meeting in Atlantic City and invited all members to attend and participate in the Second McCollum Award banquet.

Dr. Mueller reminded the members of the Norman Jolliffe Fellowship Award. The purpose of the award is to stimulate interest in clinical nutrition among medical students in the U. S. and to give financial support to those active in the teaching of clinical nutrition in American medical schools.

XI. *Reports of Committees and Representatives*

A. *Standing Committee on Experimental Animal Nutrition:* G. F. Combs

The purpose and function of the Committee on Experimental Animal Nutrition as stated in the operating procedure approved by the Council are as follows: *Purpose:* (a) To emphasize the role that research workers in basic and applied experimental animal nutrition have played and will continue to play in the development of the science of nutrition. (b) To arouse greater interest on the part of these scientists in the functions of the AIN. *Functions:* (a) To provide representation of the experimental animal nutritionists in the affairs of the AIN Council. (b) To be responsible to the Council on matters pertaining to experimental animal nutritionists. (c) To work with the Ruminant Nutrition and Poultry Nutrition Conferences in behalf of the Council. (d) To initiate steps necessary for the conduct of specific activities and meetings, including symposia, considered desirable in the accomplishments of the purposes set forth above. (e) To develop and initiate other approaches designed to stimulate the general interest and participation of research workers in basic and applied experimental animal sciences in the functions and activities of the American Institute of Nutrition.

A symposium on "Nutrition Education" was organized for the annual meeting by a subcommittee on Nutrition and Education, chaired by J. K. Loosli. Participants in the symposia included John B. Youmans and George V. Mann, Co-chairmen, and the following speakers: W. H. Griffith, A. E. Harper, F. W. Hill, John F. Mueller, and Dena C. Cederquist.

In addition, two conferences were organized for the annual meeting as follows: (a) 31st Annual Poultry Nutrition Conference, chaired by F. H. Kratzer. A major part of this program dealt with "Effect of Nutrition on Connective Tissue and Cartilage Formation in Chickens." (b) 7th Annual Ruminant Nutrition Conference, chaired by R. L. Reid. The topic of this conference was "Food Intake by Ruminant Animals."

Efforts to collect a series of slides depicting nutritional deficiency disease in experimental animals have been initiated (see Proceedings of the Twenty-ninth Annual Meeting, Journal of Nutrition, 86: 445, 1965). The response by AIN members to requests for appropriate slides has been limited as yet, but this project will be given additional emphasis by the committee during the coming year. Even now the number of slides available to the committee is very encouraging. Members are asked to send slides to the committee chairman or to the Excutive Secretary.

B. *Joint Committee on Biochemical Nomenclature:* S. R. Ames

The Committee confined its activities principally to problems of vitamin nomenclature and to the establishment of working liaisons with other nomenclature groups.

The nomenclature of compounds of interest to biochemists is continually under review by committees of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Results of their deliberations are published and become accepted terminology for scientific journals. The International Union of Nutritional Sciences (IUNS) occasionally has had representatives at CBN deliberations. In this country, the Office of Biochemical Nomenclature, NAS-NRC (OBN) was formally launched on July 1, 1965, with the appointment of Waldo E. Cohn as director. The objectives of OBN are defined "to coordinate information concerning biochemical nomenclature efforts of national and international organizations and to take appropriate steps for the stimulation of efforts and the dissemination of this information in appropriate journals and by other means."

Nutritionists both here and abroad appear to have taken a relatively passive role in the establishment of biochemical nomenclature and more active participation is desirable. There is necessity for immediate action in some areas, especially the vitamins and related compounds.

The Committee considers that generic descriptors for the vitamins are useful and necessary. Because clarification of the IUPAC-IUB-CBN position in this area was essential, CBN was asked

if the designation of suitable generic descriptors of the vitamins was within its province. CBN advised that it is not now nor does it propose to become in the forseeable future concerned with the nomenclature of vitamins as vitamins. It considered the problem of generic descriptors of vitamins as lying outside its province and within the province of IUNS. Where CBN has concerned itself with substances with vitamin activities, it has done so on a chemical basis not on an activity basis. Our Committee is thus free to work with IUNS to establish suitable generic descriptors for the vitamins without conflict of interest with CBN.

The following system of nomenclature for the stereoisomers of tocopherols and their esters was proposed by the Committee and accepted by the Council.

α-Tocopherol:

Following its isolation from natural sources, naturally occurring α-tocopherol was termed α-tocopherol. Later, to distinguish it from synthetic α-tocopherol, naturally occurring α-tocopherol was referred to as *d*-α-tocopherol. Recent evidence has shown it to be 2D,4′D,8′D-α-tocopherol (2R,4′R,8′R-α-tocopherol). In view of its natural occurrence and in agreement with IUPAC practices for other vitamins having asymetric centers, the name α-tocopherol is proposed for the pure subtance hitherto known as *d*-α-tocopherol.

Racemic-α-tocopherol:

Racemic-α-tocopherol, synthesized from racemic isophytol, is a mixture of the eight possible stereoisomers of α-tocopherol. It was incorrectly termed *dl*-α-tocopherol (DL-α-tocopherol) implying identity with the previously synthesized 2DL,4′D,8′D-α-tocopherol derived from natural phytol (see below). To resolve the present confusion and in recognition of its being racemic at three asymetric centers, the trivial name racemic-α-tocopherol is proposed for the mixture of eight stereoisomers hitherto known as *dl*-α-tocopherol.

2DL-α-tocopherol:

The synthetic α-tocopherol derived from natural phytol was the first substance to be named *dl*-α-tocopherol and has been uniformly confused with racemic-α-tocopherol (see above) following synthesis of the later substance from racemic isophytol. The synthetic α-tocopherol derived from natural phytol has been shown to be 2DL,4′D,8′D-α-tocopherol (2RS,4′R,8′R-α-tocopherol), a mixture of only 2 of the 8 possible stereoisomers of α-tocophcrol. As the acetate ester, it was termed *dl*-α-tocopheryl acetate which as a 1% solution in oil was the former International Standard. Synthetic α-tocopheryl acetate derived from natural phytol (2DL,4′D,8′D-α-tocopheryl acetate) is presently being specified for the Animal Nutrition Research Council Vitamin E Reference Standard. To resolve the present confusion and in recognition of its being racemic at only the 2 position, the name 2DL-α-tocopherol is proposed for the mixture of 2 stereoisomers hitherto known as synthetic α-tocopherol from natural phytol.

2L-α-Tocopherol:

2L,4′D,8′D-α-Tocopherol (2S,4′R,8′R-α-tocopherol) is the *l*-epimer of α-tocopherol. It has been prepared in pure form by resolution of 2DL-α-tocopherol (see above) and its biochemical and nutritional properties are being extensively investigated. In view of the importance of this stereoisomer in research on the biochemistry of α-tocopherol, the name 2L-α-tocopherol is proposed for the substance hitherto known as the *l*-epimer of α-tocopherol.

Tocopheryl esters:

In view of historical precedent and since no change seems justified, the retention of the -yl suffix to refer to tocopheryl esters is proposed (e.g., α-tocopheryl acetate).

β-, γ- and δ-Tocopherols:

The above proposals can be directly applied to the corresponding stereoisomers of β,- γ- and δ-tocopherols.

With the formation of OBN, the Joint Committee on Biochemical Nomenclature becomes superfluous and will be discontinued. The Committee recommends the formation of a permanent Nomenclature Committee of AIN. This committee would serve to evaluate and make recommendations in areas not being reveiwed by CBN and OBN and in cooperation with IUNS would resolve nomenclature problems that are peculiar to nutritionists.

C. *Ad hoc Committee on International Nutrition*: W. N. Pearson

This Committee recently carried out a survey of the AIN membership to determine the extent to which individual members were involved in international nutrition programs. The results of this survey were the basis of a report, prepared by the Committee last year, showing the number of members who had actually worked in a foreign country, those members who expected to participate in foreign nutrition programs in the immediate future, and the names and addresses of 625 foreign students trained in the U. S. by members of the AIN. Copies of this report are available from the office of the Executive Secretary.

Dr. Pearson reported to the Council that the Committee, having completed the above report, had not embarked on any further programs during the past year, but he recommended the continuance of the Committee and the work which had been started.

President-elect Schaefer, in his informal comments to the members, reported that the Council had taken positive action to continue this work and that he would appoint a strong committee to carry it on. Specifically, the latter was charged with developing a contract between the Agency for International Development and AIN in support of the AID "Food for Peace" program. This would include, among other things, the development of a roster of nutrition scientists working, or available for work, on foreign assignments; foreign students being trained in the

U. S. and the location and availability of those who have completed their training; and a review of ongoing research associated with foreign institutes.

D. *Representative to the Food and Agriculture Organization:* B. S. Schweigert

FAO has been active in a number of fields of interest to nutritionists during the past year and of particular significance is the summary in the November-December issue in 1965 of FREEDOM FROM HUNGER for the 20 years of program activities in this field. It is appropriate to point out that while extensive progress has been achieved in a number of countries in improving food production and health, expanding world population in relation to food supplies has resulted in an even greater concern to achieve "freedom from hunger" in 1966.

Three other reports of interest published by FAO or jointly with WHO are appropriate for mention: (1) Protein Requirement, Report no. 37; (2) Specifications for the Identity and Purity of Food Additives and Their Toxicological Evaluation: Food Color and some Antimicrobials and Antioxidants, Report no. 38; and (3) Symposium on Industrial Feeding and Canteen Management in Europe, Report no. 36.

E. *Representative to National Research Council Division and Boards:* G. F. Combs

The Division of Biology, NRC-NAS, under the able leadership of Dr. A. G. Norman, through its Food and Nutrition Board, Argicultural Board, Agricultural Research Institute, Institute of Laboratory Animal Resources, U. S. National Committee of International Union of Nutritional Sciences, U. S. National Committee for the International Biological Program, U. S. National Committee for the International Union of Biological Sciences and the U. S. National Union for Pure and Applied Biophysics, has had another most productive year.

The Food and Nutrition Board, which completed its 25th anniversary with its 68th meeting, has published an historical brochure, *Twenty-five Years in Retrospect.*

Other publications of the Food and Nutrition Board during the year include: Pub. 1282, *Pre-School Child Malnutrition — Primary Deterrent to Human Progress,* and Pub. 1270, *Some Considerations in the Use of Human Subjects in Safety Evaluation of Pesticides and Food Chemicals.* Also, the first edition of the *Foods Chemicals Codex* will be available in the summer of 1966 with specifications of identity and purity for about 500 food chemicals. Other new reports progressing include: *The Role of Cereals in World Nutrition, Nutritional Diseases Around the World — Geographic Nutrition,* and *Dietary Fat and Human Health.*

Other activities include a program in food microbiology which deals with: 1) improving the reporting of food-borne diseases; 2) standard methods of microbiological examination of foods, and; 3) microbiological control measures under conditions of good manufacturing practices.

The Committee on Marine Protein Resources Development, with the help of Mr. George K. Parman, Executive Secretary, has continued to be active in its advisory service on the research and production of marine protein concentrates for human consumption and the effective utilization of such products. This committee has stated that the fish protein concentrate, from whole hake, as prepared by the Bureau of Commercial Fisheries process, is safe, nutritious, wholesome and fit for human consumption.

The Agricultural Board is now printing revised publications on nutrient requirements of dairy cattle, poultry and rabbits. A new publication on nutrient composition of feedstuffs was released. A special steering committee of the Agricultural Board arranged and conducted a public symposium on the scientific aspects of pest control, (January 31 — February 3, 1966) a *Proceedings* which will appear around May 1, 1966.

The affiliated Agricultural Research Institute held its annual meeting on October 18–19, 1965, on the theme of *World Food Needs and Production — Present and Future, Proceedings* of which are published. The 1966 program will deal with *The Role of Animal Agriculture in Meeting World Food Needs.*

A *Symposium on Research in Agriculture* also was sponsored jointly by USDA and the National Academy of Sciences on February 23–25, 1966. The Agricultural Research Institute is cooperating with USDA and state agricultural experiment stations in preparing a national inventory of agriculture. Publication is expected by midsummer, 1966, after it has been analyzed.

The Institute of Laboratory Resources is currently updating and revising *Laboratory Animals II, Animals for Research; Standards* for the care, breeding and management of mice, rats, hamsters, guinea pigs, cats, dogs, rabbits, primates (Macaca mulatta); the shipping of dogs, the utilization of test animals; and the shipment of laboratory primates. In preparation are poultry standards, genetic standards, laboratory animal quality standards, laboratory animal procurement standards, health standards for international shipment of laboratory animals, and primate standards (other than rhesus).

The U.S. National Committee for the International Biological Program has progressed nicely in planning the basis for participation of U.S. scientists, including nutrition scientists, in IBP. The preliminary framework of the U.S. program of IBP is presented in USNC, IBP Pub. no. 1, NRC-NAS, August 1, 1965.

Members of the AIN should be fully aware of the nature of this program and unique opportunities which it can provide for nutrition research scientists interested in international nutrition.

F. *Representative to the Federation:* A. E. Schaefer

The Federation Board acted to approve the addition of another wing to the new building at Beaumont (Milton O. Lee Building).

The Federation has established a patent policy concerning inventions by employees which will be voted on by the Board after review by legal counsel.

The Federation bylaws have been revised to define functions and duties of Executive Director, Comptroller, Treasurer. Affairs of the Federation are in good order; the new Executive Director, Dr. J. F. A. McManus is continuing the leadership of the Federation in a commendable manner.

G. *U.S. National Committee, IUNS:* G. F. Combs

The U.S. National Committee of the International Union of Nutritional Sciences held two meetings during the year and continues to support the admission of IUNS into the International Council of Scientific Unions. To this end, the Committee has encouraged strengthening the present program of the IUNS to include participation in the International Biological Program, to develop a documentation of nutrition science at the international level, to encourage a common nomenclature for international use, and to maintain a list of nutrition scientists by countries throughout the world.

A revised Constitution and By-laws for IUNS has been prepared for presentation to the delegates at the forthcoming Congress. The Committee has encouraged the appointment of a nutrition scientist to act as coordinator of nutrition science pertinent to the International Biological Program.

The Committee continues to encourage the development of stronger nutrition societies in other countries as a means of strengthening the IUNS.

H. *Ad hoc Committee on Meeting Evaluation:* Carl D. Douglass

Dr. Douglass advised the membership that AIN had formed a committee to assess the effectiveness of the annual meeting in serving the needs of the membership. To provide the basis for sound recommendations, the committee asks that members give diligent care to the AIN questionnaire prepared by this committee which will be sent to all members.

I. *Affiliation with AIBS:* R. W. Engel

The AIN committee considering the feasibility of AIN affiliation with AIBS advised that they had no recommendation to make at this time.

J. *International Biological Program.* See U.S. National Committee, IUNS, XI, G, of these Proceedings.

XII. *New Business*

A. Resolution on Fluoridation. The following resolution was adopted by the membership at its meeting on April 15, 1966:

> WHEREAS the addition of appropriate amounts of fluoride, to those municipal water supplies which are deficient in fluoride, has been shown to improve the durability of dental enamel and to decrease the rate of dental decay, and
> Whereas this well-tested public health measure has the support of the U.S. Public Health Service, the American Medical Association, the American Dental Association, and many other science-oriented groups, therefore
> BE IT RESOLVED that the American Institute of Nutrition at its annual meeting April 15, 1966 recognizes fluoridation as a safe, effective and low-cost means of improving dental health by improving nutrition.

B. Future Federation meetings;

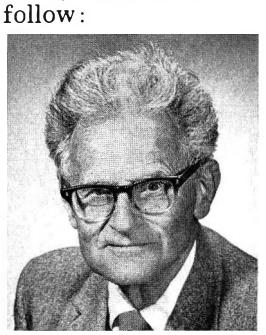
1967: April 16–21, Chicago
1968: April 15–20, Atlantic City
1969: April 13–18, Atlantic City

C. The next Council meeting will be held on October 29, 1966, in Washington, D.C.

ANNUAL DINNER AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held Thursday, April 14, 1966 at the Shelburne Hotel, with 377 individuals attending. Dr. Kline presided.

Dr. Charlotte Young, as chairman of the Fellows Committee, introduced the newly appointed Fellows, whose citations follow:



MAX KLEIBER

MAX KLEIBER—in recognition of his distinguished career as an inspiring teacher, author and research scientist; for his attempt to make nutrition a quantitative science by expressing biological concepts in mathematical and universal terms; for his unraveling of the conditions which influence the metabolic rate of the body; for his discovery of the relationship between body size and metabolic rate; for his contributions concerning the evaluation of diets for animals, particularly his "replacement equivalent" method of investigating the utilization of dietary energy; for his pioneering use of isotopes in the clarification of the biogenesis of the constituents of milk. His success as a teacher and lecturer is due in part to his independence of thought but in large measure to his great sense of humor. He received the AIN Borden Award in 1952 and the Morrison Award of the American Society of Animal Science in 1953.

SAMUEL LEPKOVSKY

SAMUEL LEPKOVSKY — for his distinguished career of research in both fundamental and applied nutrition; for his contributions to the early discoveries of the B vitamins including: the relationship of vitamin B_6 to xanthurenic acid excretion and to the level of dietary protein, the identity and structure of riboflavin, the relationship of thiamine need to the fat and carbohydrate of the diet, and the metabolism of pantothenic acid; for his contributions to our understanding of the mechanisms of digestion, hunger and satiety. His modest unself-seeking and generous attitude toward his work and his fellow scientists is well-known throughout the world of nutrition. His quiet voice has cleared up many a controversy and his chuckle has smoothed out many arguments.

JOHN B. YOUMANS

JOHN B. YOUMANS — for a distinguished career as physician and medical educator whose leadership in clinical nutrition has profoundly affected nutritional science; for his design and application of the technic of nutritional survey in the United States and abroad prior to World War II and his major contributions to ICNND; for his effective guidance in clinical nutrition and research for the U. S. Army through the Office of the Surgeon General and for the American Medical Association through long membership on the Council on Foods and Nutrition and as its Scientific Director. As a teacher he has exerted a telling effect upon the careers of many members of AIN.

Dr. Kline, as toastmaster, presided at the presentation of the following awards:

BORDEN AWARD IN NUTRITION

RALPH T. HOLMAN

The 1966 Borden Award of $1000 and a gold medal was presented to Dr. Ralph Theodore Holman, Professor of Biochemistry, Hormel Institute, University of Minnesota. The award was made in recognition of Dr. Holman's outstanding contributions to knowledge of the metabolism and the requirements of those important constituents of milk, the fatty acids. He has determined the dietary requirements for polyunsaturated fatty acids in several species, including man, and the factors which influence these requirements. He has developed several methods for the analysis of fatty acids and has carried out extensive investigations of the interrelationships of fatty acids with other lipids. He has made estimates of the dietary linoleate intake of men and of infants and assessed the requirements of the latter from serum fatty acid analyses.

THE CONRAD A. ELVEHJEM AWARD FOR PUBLIC SERVICE IN NUTRITION

CHARLES GLEN KING

The first Conrad A. Elvehjem Award of $1000 and a scroll was presented to Dr. Charles Glen King, Associate Director, Institute of Nutrition Sciences, Columbia University. The award was made in recognition of Dr. King's long and distinguished service to the public as exemplified by the following: his isolation of vitamin C permanently benefits man; he has been unsparing in his efforts to further the cause of good nutrition through professional societies and national and international agencies; as teacher and counselor he has infected students and associates with his spirit of public service. Also as the first Director, and later President, of the Nutrition Foundation he established a pattern for the support by industry of nutritional research and education which has been widely imitated. This Foundation has had a profound impact upon the development of nutritional science and the application of research findings to the benefit of mankind everywhere. Dr. King has indelibly imprinted the importance of nutrition on the institutions of government, of industry, and of education.

MEAD JOHNSON AWARD FOR RESEARCH IN NUTRITION

M. DANIEL LANE

The 1966 Mead Johnson and Company Award of $1000 and a scroll was awarded to Dr. M. Daniel Lane, Associate Professor of Biochemistry, New York University School of Medicine. Dr. Lane was selected in consideration of his fundamental contributions to our understanding of the mode of action of biotin in enzymatic carboxylation reactions, and of his pioneering demonstration that in biotin enzymes the vitamin is covalently bound in an amide linkage involving its carboxyl group and the epsilon amino group of lysyl residues of the protein.

OSBORNE AND MENDEL AWARD

HAROLD H. MITCHELL

The 1966 Osborne and Mendel Award of $1000 and a scroll was awarded posthumously to Dr. Harold H. Mitchell, Professor Emeritus of Animal Nutrition, University of Illinois. It was presented in recognition of his pre-eminent studies in mammalian protein, mineral and energy metabolism, culminating in the authorship of a two- volume compendium, "Comparative Nutrition of Man and Domestic Animals." His many publications on the biological evaluation of proteins had a profound influence on methodology and understanding of this important field and illustrate the thoroughness of his approach to scientific matters. His interpretive reviews on calcium requirements, fluorine toxicity, and the effect of environmental stress on nutrient metabolism revealed his unusual capability as a meticulous critic. His influence was felt also by the excellence of his teaching and the accomplishments of many students. A charter member and Fellow of the Institute, he served for 24 years on the editorial board of the *Journal of Nutrition.* He received the AIN Borden Award in 1945, and the Morrison Award from the American Society of Animal Production in 1950.

AMERICAN INSTITUTE OF NUTRITION

Founded April 11, 1933; Incorporated November 16, 1934; Member of Federation 1940

OFFICERS, 1966–1967

President: A. E. Schaefer, Nutrition Section, Office of International Research, National Institutes of Health, Bethesda, Maryland.

President-Elect: G. M. Briggs, Department of Nutritional Sciences, University of California, Berkeley, California.

Past President: O. L. Kline, 3509 Rodman Street, N.W., Washington, D. C.

Secretary: W. N. Pearson, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee (1969).

Treasurer: W. A. Krehl, Clinical Research Center, University of Iowa, Iowa City, Iowa (1968).

Councilors: N. S. Scrimshaw (1967), E. L. R. Stokstad (1968), A. E. Harper (1969), A. B. Eisenstein (1969).

Executive Secretary: James Waddell, 9650 Rockville Pike, Bethesda, Maryland 20014.

COMMITTEES

Nominating Committee: M. L. Scott, chairman; D. B. Coursin, B. C. Johnson, Ruth Leverton, C. O. Chichester.

Membership Committee: K. W. King (1968), chairman; C. H. Hill (1967), Dena C. Cederquist (1969), G. V. Mann (1970), B. L. O'Dell (1971).

Nominating Committee for Mead Johnson Award: P. L. Day (1967), chairman; A. R. Kemmerer (1968), L. M. Henderson (1969).

Nominating Committee for Borden Award: P. H. Weswig (1968), chairman; E. L. Hove (1967), K. E. Harshbarger (1968).

Nominating Committee for Osborne-Mendel Award: M. O. Schultze (1967), chairman; Alex Black (1968), L. E. Holt, Jr. (1969).

Nominating Committee for Conrad A. Elvehjem Award: E. E. Howe (1967), chairman; F. W. Quackenbush (1968), R. E. Shank (1969).

Fellows Committee: S. L. Hansard (1967), chairman; P. E. Johnson (1967), W. H. Griffith (1968), Agnes F. Morgan (1969), R. M. Forbes (1969).

Committee on Honorary Memberships: Paul György, chairman; Grace A. Goldsmith, R. W. Engel.

Auditing Committee: W. A. Gortner, chairman; G. V. Vahouny.

Finance Committee: W. A. Krehl, chairman; H. W. Howard, D. V. Frost, Grace A. Goldsmith, James Waddell (ex officio).

Symposia Committee: E. L. R. Stokstad, chairman; F. J. Ingelfinger, S. M. Garn, Lucille S. Hurley, Doris H. Calloway, W. S. Hartroft, C. G. King.

Committee on Publication Management: W. J. Darby (1968), chairman; F. L. Iber (1967), D. M. Hegsted (1967), R. W. Engel (1968), J. F. Mueller (1968), James Waddell (continuing) secretary. Ex officio: W. N. Pearson, A. B. Eisenstein.

Committee on Nomenclature: S. R. Ames, chairman; P. L. Harris, H. H. Williams, H. E. Sauberlich, M. E. Shils.

Public Information Committee: M. S. Read (1968), chairman; A. L. Forbes (1967), C. J. Ackerman (1967), Ruth L. Pike (1967), Mary A. Williams (1967), P. L. White (1968), J. T. Sime (1968), R. S. Goodhart (1968).

Committee on Experimental Animal Nutrition: G. F. Combs (1969), chairman; W. H. Pfander (1967), vice-chairman; R. W. Luecke (1969), E. W. Crampton (1968), F. W. Hill (1968), J. K. Loosli (1967). Ex officio: Hans Fisher (1967), D. S. Kronfeld (1967), A. E. Schaefer (1967).

Ad hoc Committee on Science Fairs: C. W. Carlson, chairman; E. L. Wisman, Helen M. Dyer.

Ad hoc Committee on Sustaining Associates: C. H. Krieger, chairman; W. R. Graham, H. L. Sipple, James Waddell (ex officio).

Ad hoc Committee on International Nutrition: R. W. Engel, chairman; Guillermo Arroyave, D. B. Hand, W. G. Unglaub, L. J. Teply, J. B. Youmans, W. J. McGanity, G. F. Combs, W. N. Pearson (ex officio).

Ad hoc Committee on Nutrition Education: A. E. Harper, chairman; A. L. Black, Doris H. Calloway, G. F. Combs, K. W. King, H. A. Schneider, T. B. Van Itallie, C. W. Woodruff.

Ad hoc Committee on Recommended Constitutional Changes: J. C. Fritz (chairman), W. H. Griffith, L. M. Henderson, R. S. Goodhart, J. F. Mueller, I. C. Plough, G. M. Briggs, James Waddell (ex officio).

Ad hoc Committee on Evaluation of Meetings: C. D. Douglass, chairman; S. R. Ames, Myron Brin, Mary A. Morrison, C. H. Hill, K. W. King, J. M. Iacono, James Waddell (ex officio).

U. S. National Committee, IUNS: O. L. Kline (1967), chairman; C. G. King (1967), W. J. Darby (1967), W. A. Krehl (1967), W. M. Beeson (1968), A. L. Forbes (1968), R. W. Engel (1968), G. F. Combs (1969), Charlotte Young (1969). Ex officio (voting) A. E. Schaefer. Ex officio (non-voting): A. G. Norman, R. K. Cannan, Harrison Brown, E. C. Rowan.

REPRESENTATIVES

National Research Council Boards and Divisions: G. F. Combs (1969).

American Association for the Advancement of Science: R. L. Lyman (delegate), Ruth M. Leverton (alternate).

National Society for Medical Research: H. C. Spruth, H. A. Schneider.

Food and Agriculture Organization: H. E. Sauberlich (1968).

Federation Public Information Committee: M. S. Read (1968).

Federation Proceedings Editorial Board: P. L. Harris (1967).

Federation Proceedings Translation Supplement: J. A. Stekol (1968).

Editorial Board
The Journal of Nutrition

R. H. Barnes, Editor (1969); H. H. Williams, Associate Editor; E. Neige Todhunter, Biographical Editor; Roslyn B. Alfin-Slater (1970), George H. Beaton (1970), George M. Briggs (1967), Samuel J. Fomon (1969), R. M. Forbes (1967), L. M. Henderson (1968), F. W. Hill (1968), Jules Hirsch (1967), E. E. Howe (1967), B. Connor Johnson (1970), Gennard Matrone (1968), Paul M. Newberne (1969), Boyd L. O'Dell (1969), William N. Pearson (1970), H. E. Sauberlich (1969), Clara A. Storvick (1968).

Officers, American Society for Clinical Nutrition
(A division of the American Institute of Nutrition)

President, R. E. Hodges; President-Elect, R. E. Shank; Past President, C. S. Davidson; Secretary Treasurer, A. B. Eisenstein, 818 So. Meramec Avenue, Clayton, Missouri; Councilors: R. M. Kark (1967), W. S. Hartroft (1968), J. J. Will (1969). W. A. Krehl (ex officio).

Editorial Board
American Journal of Clinical Nutrition

W. A. Krehl, Editor-in-Chief; R. E. Hodges, Associate Editor; Margaret J. Albrink, Ernest Beutler, G. J. Gabuzda, Francisco Grande, S. A. Hashim, Victor Herbert, Robert Jackson, C. M. Leevy, M. E. Shils, D. M. Watkin, George M. Owen, H. E. Sauberlich.

JAMES WADDELL, *Executive Secretary*
American Institute of Nutrition