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GEORGE R. COWGILL, *Editor*
Yale Nutrition Laboratory, 333 Cedar Street
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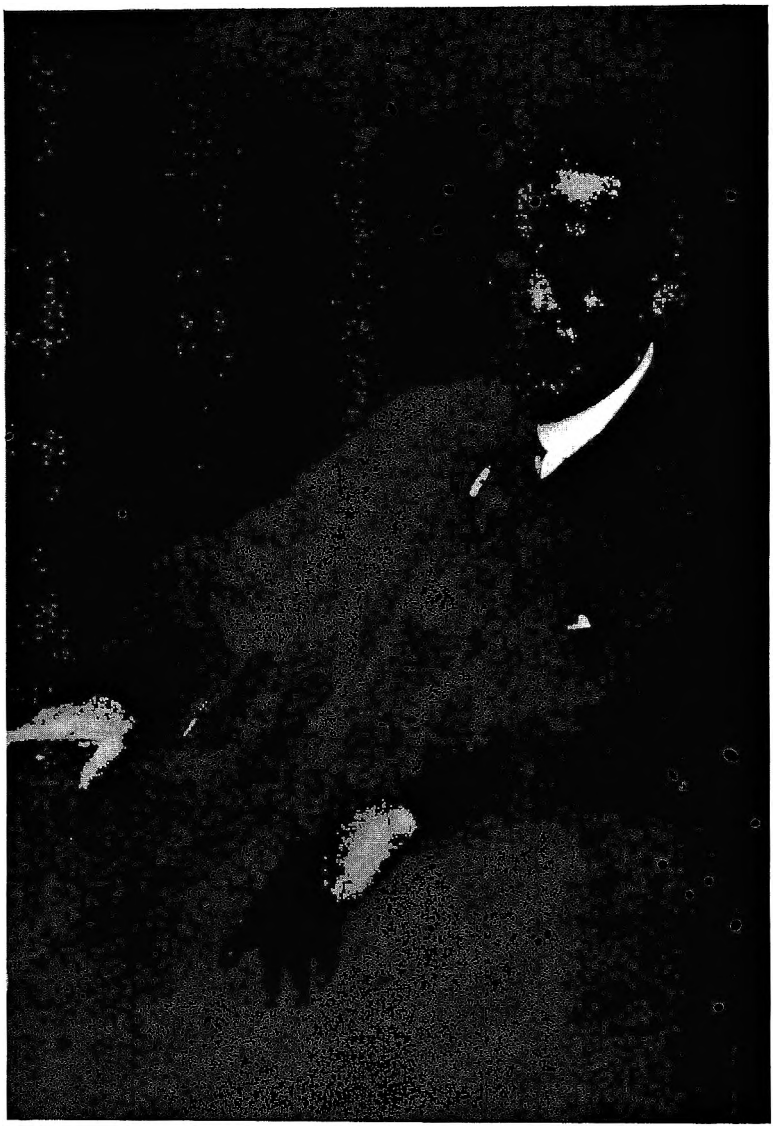

THOMAS BURR OSBORNE

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(August 5, 1859 – January 29, 1929)

To students of nutrition, the name of Thomas Burr Osborne is almost invariably associated with that of Lafayette B. Mendel. These two distinguished investigators collaborated from 1909 until Osborne retired in 1928, and published jointly somewhat more than 100 papers on various aspects of nutrition. This was the period in which the existence of vitamins was first clearly demonstrated, and the explanation of the essential role of proteins in nutrition was obtained. Osborne and Mendel were acknowledged leaders in the field, and their accomplishments during that important 20-year period still exert a powerful influence upon the progress of the science. It is the purpose of the present article to consider the background of the share that Osborne brought to this fruitful partnership, and to show the significance of his particular fund of knowledge and experience in relation to the development of modern concepts of animal nutrition.

Osborne was born in New Haven, Connecticut, on August 5, 1859, the son of Arthur Dimon Osborne and Frances Louisa Blake. His father, trained as a lawyer, was for many years the president of a local bank, and the Osborne family can be traced back in the records of New Haven to the start of the colony in 1639. On his mother's side, Osborne was also descended from old New Haven families; the Blakes and Whitneys have long been known for their eminence in mechanical invention and in New England industry.

Osborne was educated at the Hopkins Grammar School in New Haven and at Yale University where he was graduated in 1881. His boyhood interest in scientific matters had been greatly encouraged by an uncle, Eli Whitney Blake, Jr., professor of physics at Brown University, and their relationship

may be illustrated by a single incident. When Alexander Graham Bell gave the first public demonstration of the telephone in Providence on October 9, 1876, Osborne was present at his uncle's invitation, and subsequently prepared a school essay on the telephone which was illustrated with a home-made working model. At this time Osborne also developed a taste for ornithology and contributed many early observations on birds which were recorded in C. Hart Merriam's "Review of the Birds of Connecticut." His interest in birds was lifelong.

Osborne's undergraduate career at Yale was not outstandingly distinguished in the conventional courses of study, but his extra-curricular activities were most significant. He was a leading member of a small group of students known as the Yale Society of Natural History, and surviving manuscripts of papers read at their meetings indicate that this was a very serious-minded group indeed; 6 of them in later years became members of the National Academy of Sciences. Entirely apart from his university connections, Osborne concerned himself with the development of a machine of importance in the wheat milling industry. His "Electric Middlings Purifier" was patented in 1880 in this country and abroad, and was used for several years until superseded by more efficient devices.

After graduation, Osborne remained at Yale where, for a year, he studied medicine, but then shifted over to the graduate school and began the serious study of chemistry under W. G. Mixter. He soon became a laboratory assistant in analytical chemistry, and his first scientific paper was published in 1884; it dealt with the separation of zinc and nickel. His dissertation for the Ph.D. degree, which was awarded in 1885, was on the determination of niobium in columbite, the mineral from Connecticut in which niobium had been discovered by Hatchett[1] in 1801. One more year was spent at Yale as an

[1] Hatchett investigated a specimen which had been sent to the Royal Society of London, England, more than a century earlier by John Winthrop, the first governor of Connecticut. Osborne's specimen was obtained in Branchville, Connecticut. The original specimen probably came from near Mystic, Connecticut.

instructor, but in May, 1886, Osborne took the step that defined his future career. He accepted the invitation of S. W. Johnson, professor of agricultural chemistry in the Sheffield Scientific School of Yale University and Director of The Connecticut Agricultural Experiment Station, to join the staff of the Station as an analytical chemist. In the same year, he married Elizabeth Annah Johnson, Professor Johnson's daughter.

Osborne's career, in particular his complete devotion to chemistry, can perhaps best be understood in terms of his close personal relationship with Johnson. Chemistry was a close bond of union, but their other interests and activities were widely different although complementary. Johnson was for 40 years one of the most distinguished members of the faculty of the Sheffield Scientific School. He was pre-eminently a teacher and administrator, a propagandist of the doctrine that agriculture is essentially a scientific pursuit and, above all, a public-spirited man who took every possible opportunity to give scientific advice and aid to the farmer. He was a bibliophile who collected an amazing personal library of scientific books and journals, still preserved in the Osborne Library at the Station, and he read and digested them all. Osborne was perhaps his most eminent pupil although, since the list contains such names as Russell H. Chittenden and Henry P. Armsby, this might be debated. At all events, Osborne had the advantage of almost daily contact with Johnson throughout the formative years of his scientific life. The relationship was one of complete mutual respect and deep affection. They engaged in frequent discussions of the experimental work as the investigations proceeded and, when the time came to prepare a formal paper, the elderly scholar provided the historical background and the literary critique, pointed out the weak spots in the arguments and saw to it that no important detail was omitted. As a result, it is possible to this day to repeat the preparation of a protein from Osborne's description, and to obtain substantially identical results. Osborne's training as a scientific investigator was thus a long and

thorough one, and was obtained under the happiest possible conditions.

Osborne's first assignment as a member of the station staff was to carry out combustion analyses for carbon and hydrogen in a series of preparations of carbohydrates that Johnson had obtained from various plant gums. Their nature as pentose sugars was soon established, but the publication of Kiliani's discovery that arabinose is a pentose sugar, and the extensive investigations of these substances then going on in Germany, led Johnson to decide not to publish the results. Accordingly, for the next year or so, Osborne shared in the general research work of the laboratory. However, in 1888, his investigations took an entirely new direction. At that time an addition to the income of the station in the form of Federal funds became available under the Hatch act of 1887. Johnson thereupon took a step of unusual boldness; he suggested that Osborne should undertake the investigation of the proteins of plant seeds as a full-time and independent project.

It must be remembered that the agricultural experiment stations, which were then being established throughout the country, were almost exclusively devoted to service activities with the object of providing practical aid to the farmer Fundamental research occupied only a minor place, and in many stations was carefully avoided for fear of criticism from unimaginative members of the legislature. However, Johnson, over the years, had convinced the Connecticut community that fundamental research was far more likely to render aid to the farmer in the long run than a program of mere testing and demonstration, and he therefore felt free to make use of the new income to support such an activity. His selection of a project reveals strikingly the breadth of his grasp of contemporary problems in agriculture. Although it had been fully appreciated since the time of Magendie that the food of man and animals must contain a certain proportion of nitrogenous material of the kind then frequently referred to as albuminous substances, there had been only one long-con-

tinued and serious attempt to prepare the nitrogenous components of food materials in a state of purity, and to study their complex relationships. This was the work on the proteins of seeds carried out in Germany by Heinrich Ritthausen. The theoretical approach to the subject in these early years was dominated by Liebig's statement that there are only 4 proteins in nature, albumin, casein, fibrin and gelatin, that these substances are formed by plants, and that animals acquire them either directly or indirectly by the ingestion of plant material. Liebig had obtained this last idea from the Dutch investigator Mulder to whom in turn it had been suggested, more or less as a working hypothesis, in a personal letter from Berzelius in 1838.[2]

This primitive view of the situation had been greatly broadened by Ritthausen, and the publication of his book "*Die Eiweisskörper der Getreidearten, Hülsenfrüchte und Ölsamen*" in 1872 confirmed Johnson's high opinion of the accomplishments of the man whom he had met briefly while a student in Leipzig in 1853. The subsequent publications from Ritthausen's laboratory had been followed with close attention, and the 1890 revision of Johnson's celebrated text-book "How Crops Grow" includes a chapter on proteins which is by far the most comprehensive and illuminating treatment of the subject of its period. In it Ritthausen's results take their rightful place as the most significant observations available, in spite of the fact that his work had been largely ignored and discredited in Germany.

Osborne's investigations of the proteins of seeds began with a study of oats. This particular seed was chosen because it had been investigated some 40 years earlier by Johnson's former teacher, J. P. Norton, at Yale, but had been little studied subsequently in spite of its wide use in animal feed-

[2] It was in this letter of July 10, 1838 that Berzelius coined the term "protein" which he derived from a Greek adjective meaning to be in the first rank or position. The specific reference was to the importance of the position of proteins in the nutrition of animals.

ing. Osborne's first account of the proteins of this seed was published in the annual report of the Connecticut Station for 1890, and in the American Chemical Journal in 1891. During the next 10 years, about 40 papers appeared in which the proteins of no less than 32 different species of seeds important as food materials were described. Each seed was found to yield two or more preparations which had different properties, and each of these was isolated where possible by several different techniques. Every precaution was taken to insure that the final preparations should represent pure and homogeneous material free from all contamination with non-protein substances. The fundamental criterion of purity was the reproducibility of the analysis for carbon, hydrogen, nitrogen and sulfur in preparations obtained by various modifications of the procedure, and use was also made of the constancy of such properties as coagulation temperature, general solubility relationships, and, with many globulins, the capacity to separate from solution in crystalline form.

Throughout this early period, Osborne was still more or less influenced by the contemporary notion that the same protein may occur in the seeds of different plant species. By 1894, for example, he had obtained crystalline or amorphous globulins from hempseed, castor beans, squash seed, flaxseed, wheat, maize and cottonseed which were as close to each other in ultimate composition as were individual preparations of the globulin from any one of these seeds. He accordingly, wrote, "as the properties of the preparations obtained from all of these sources are substantially alike, there can be little doubt that one and the same proteid exists in them all." For this globulin the name edestin, derived from the Greek for the word "edible," was proposed.

As his experience broadened, however, Osborne's suspicions of the validity of this conclusion were aroused. By 1896, he had found that the globulins of the peach seed and almond were indistinguishable from each other as were those of the walnut and filbert, but these pairs of globulins were different

from each other and from the so-called edestin of the various seeds mentioned. Furthermore, the excelsin of the Brazil nut, the avenalin of the oat and the conglutin of lupine seed were also obviously different from all of these. Thus, although these globulins had previously all been grouped together under the term "vitellin," this name had obviously been applied to at least 6 different substances. Osborne accordingly suggested that the term vitellin should be abandoned since its further use could only lead to confusion.

By 1899, Osborne had encountered several other instances in which proteins indistinguishable from each other by any current technique could be prepared from seeds of different although allied species, legumin from the pea, lentil, horse bean and vetch being examples. Nevertheless, differences among preparations from different species were frequently noted and, with the wealth of material he had at hand, Osborne was now in a position to undertake a program of far more detailed examination of the preparations than had before been possible.

Osborne, with his associates, subjected the great collection of preparations of proteins to the most minute examination. He showed that sulfur is normally present in two different forms of combination, one of which could be liberated as hydrogen sulfide under proper conditions, and he sought with considerable success for stoichiometric relationships between these two forms of sulfur. The solubility limits in solutions of ammonium sulfate and other salts were studied as well as the capacity to combine with acid and with base and the intensity of the tryptophan reaction; the content of carbohydrate groups and such physical properties as specific rotation and heats of combustion were also examined. It is clear, however, from the papers published between 1899 and 1906, that Osborne was becoming more and more impressed with the possibilities offered by chemical analysis of his preparations for the amino acids that they yielded after hydrolysis.

For the origin of this idea one must turn to Ritthausen, who, as early as 1872 (*Die Eiweisskörper*, p. 231 f.), had made use of determinations of glutamic and aspartic acids in his efforts to discriminate among the so-called plant caseins. He had emphasized the significance of the differences in composition between these proteins and the alcohol-soluble proteins of cereal grains. To be sure, his data were far from accurate, and he obviously recognized their limitations. Nevertheless, the fundamental principle was clearly expressed, for these were the first analyses of their kind, and Osborne carried on from the point where Ritthausen had left the problem. In 1899, for the first time, a moderately accurate chemical method for the examination of the composition of proteins had become available. Hausmann, a student of Hofmeister, published a simple technique whereby one could determine the proportions of the nitrogen of a protein liberated by acid hydrolysis as ammonia, and as amino acids which could be precipitated by phosphotungstic acid. The method required only one gram of the protein, at that time regarded as an extremely small sample, and thus could be widely applied and the data checked by repetition. In the following year, Kossel and Kutscher published their method to determine the three basic amino acids, histidine, arginine and lysine, the first method in the history of protein chemistry which had any reasonable claim to quantitative accuracy. Osborne fully appreciated the possibilities offered by these methods for discrimination among similar protein preparations, and

[3] In this discussion of Osborne's background and contributions to protein chemistry and hence to nutrition, much emphasis has been placed upon the relationship of his work to that of Ritthausen. This is essential if one is to appreciate Osborne's proper position in the history of biochemistry. The proteins of plant seeds were the main consideration in programs of research which were continuous from about 1860 until 1928 in two laboratories under two outstanding scientists. These programs overlapped in point of time for about a decade in the nineties, but the general theme and the high standards with which the work was accomplished were unchanged throughout. Osborne had the advantage of more modern methods and sounder theory and, accordingly, accomplished more of lasting significance.

promptly initiated a program of amino acid analysis of proteins that was to continue for many years.

With the aid of the Hausmann method, he was able to show, in 1903, that the preparations of the so-called edestin from 8 different seeds were for the most part undoubtedly different from each other. Only the preparations from hempseed, castor bean and cottonseed were alike with respect to the proportions of amide, basic and non-basic nitrogen and, of these, the globulin from cottonseed, unlike the others, was found to give a strong Molisch reaction for carbohydrate. Osborne accordingly concluded that the term edestin should be applied only to the globulins of hemp and castor bean seeds, and expressed serious doubt that even these were identical since experience with the proteins of various other seeds showed that close resemblances were to be found only in the proteins from closely related botanical species. The name edestin has, from that time, been restricted to the chief crystalline globulin of hempseed.

However, the application of the Kossel method in any broadly planned investigation of seed proteins required much time. No less than 50 gm of highly purified material were needed for a single analysis, and the complex and difficult procedure required at least a month for a single determination of the three bases in one protein. Closely agreeing check analyses could be obtained only after considerable experience had been obtained. Thus, it was not until 1908 that Osborne was in a position to publish the results of determinations of the basic amino acids in proteins. The paper contained the data for 26 different proteins; on many of these, duplicate and even triplicate analyses had been carried out. Agreement among the replications was only moderately good in many instances, and Osborne accordingly adopted the practice of quoting the highest figure rather than an average as the most reliable result. The argument was that, since the method depended essentially upon isolation of the base in a state of purity, the analysis which gave the highest result was the most successful.

Meanwhile, the chemical examination of the proteins was being carried out along two other entirely different lines. The proportion of glutamic acid which could be isolated as the hydrochloride from samples of from 30 to as much as 75 gm of protein was determined, and data were obtained for 25 different proteins. A little later, when data for aspartic acid also became available, Osborne drew attention to the close correlation between the results for the proportion of ammonia yielded by proteins and the proportions that could be calculated on the assumption that the dicarboxylic acids are combined in the protein molecule as amides. This was the first direct evidence to be obtained in favor of an hypothesis suggested many years before by several investigators, including Ritthausen.

The other line of investigation became the major activity in Osborne's laboratory from 1906 until 1911. This was the analysis of proteins by the Fischer ester distillation method, and some 25 papers were published in which the results of the examination of a wide assortment of both seed and animal proteins were described. It is difficult today to appreciate what one of these analyses meant in terms of plain physical labor. The first of these papers describes the analysis of the gliadin, glutenin and leucosin of wheat. No less than 1100 gm of highly purified gliadin were hydrolyzed for the determination of glutamic acid and the mono-amino acids, 300 gm for the separate determination of cystine by direct isolation from a neutralized hydrolysate, and 219 gm for the direct isolation of tyrosine; in addition, the determination of the bases required 50 gm. To be sure, not all of the analyses were carried out on quite this lavish scale, but large quantities were essential since the data depended upon the isolation of each of the amino acids in sufficiently pure form, as demonstrated by macro determinations of carbon, hydrogen and nitrogen, to be weighed. That Osborne and his few assistants accomplished so much in what was to them, at the start, a new and untried field may still be a matter for wonder.

It would be pleasant to record that the result of all this industry was to establish in final form the amino acid composition of a wide variety of proteins. Unfortunately, this is far from being the case. The weights of the hydrolytic products of a protein should add to about 115% of the weight of the protein because of the water taken up during hydrolysis. Many of Osborne's earlier analyses gave results that added to little more than one-half of this figure, and, even after experience had shown the necessity for many precautions that had been neglected in the early attempts, the results still fell far short of a complete analysis. The unsatisfactory nature of the data finally led Osborne, in 1910, to a study of the sources of loss. A mixture of 326 gm of pure amino acids was made up to imitate the composition of zein, so far as this was known, and was subjected to esterification and the conventional methods of separation of the individual substances. The recovery of the amino acids was only 66%, and the individual losses ranged from 100% of the serine to 20% of the leucine taken. Osborne pointed out that if the loss of each amino acid in this experiment was applied as a correction upon the results for a parallel analysis of zein itself, a moderately satisfactory accounting of the composition of the protein could be obtained. The unknown deficit on this assumption amounted to only 7% of the molecule, and neither serine nor cystine had been isolated although both were probably present. However, it was obvious that further progress was contingent on improvement in the methods.

Osborne, by 1910, had thus practically exhausted the potentialities of the contemporary chemical methods for the examination of the proteins. He had become convinced that the detection of differences between proteins from different sources was of far greater significance than any emphasis upon their similarity, for the data for amino acid composition, despite the inevitable errors, had led to many clear differentiations between proteins hitherto regarded as identical. Nevertheless, the evidence frequently was still far from satisfactory, and Osborne therefore turned to the sensitive

biological methods then coming into prominence. In collaboration with H. Gideon Wells of Chicago, a program of study of the anaphylaxis reactions of the seed proteins was initiated; Osborne outlined the problems and supplied the preparations, and Wells carried out the tests. During the next 6 years, most of the remaining puzzles regarding differentiation were solved and reported in a series of 7 papers in which the biological reactions of many preparations were carefully examined. The outcome was that, with only two or three minor exceptions in which the preparations had been obtained from seeds of closely allied species, it became possible to assert that the proteins of seeds are specific substances; each is different from the others in some respect.

This conclusion is one of Osborne's greatest contributions to fundamental protein chemistry. It was henceforth necessary to consider the proteins of plants from the same point of view as those of animal origin for which evidence of specificity was rapidly accumulating. Today, it is a matter of assumption that proteins are specific substances, and differences in amino acid composition have been demonstrated even in such a relatively simple instance as the insulin derived from different animal species. The theoretical approach has turned completely away from the views that Liebig had enunciated somewhat more than a century ago, and Osborne must be credited for a large share of the early evidence that brought about this revolution.

It is clear that, by 1909, Osborne was reaching a point in his investigations where it was necessary to consider the direction in which further progress could be made. A somewhat similar point had been reached about 10 years previously, and the manner in which he had met the challenge has already been set forth. It is of interest to note that, whether consciously or not, he again chose to follow a suggestion that had been made by Ritthausen in 1872. The wide differences Osborne had observed in the amino acid composition raised the question of the relative effectiveness of proteins in nutrition. This problem had also arisen in Ritthausen's mind.

On page 234 of his book there is a short section under the title, "*Ungleicher Werth der Proteinstoffe der Samen bei der Ernährung,*" which begins with the statement:

> "Now that investigation has shown that the proteins of those seeds which are especially suited for the nutrition of man and animals differ extensively from each other in composition, the questions at once arise whether their value or over-all effect as foods may differ from each other, whether they are equivalent to each other, or whether according to their higher or lower carbon and nitrogen content they have a greater or smaller nutritive value."

After some speculative discussion of these points, he concluded:

> "Various, although not very well-established, facts indicate that animal and plant proteins, even though of closely similar composition, are not exactly alike. Thus the conversion of the latter into the former in the components of the animal organism cannot be so simple a process as is commonly held, but must be quite complex. Among these facts are the observations that animal proteins on hydrolysis with sulfuric acid yield no glutamic acid[4] whereas all seed proteins do. Also, that the other decomposition products, such as tyrosine, leucine, aspartic acid, etc., are formed in different amounts, as the researches of the author, of Dr. Kreusler and of Habermann and Hlasiwetz have shown. Is it not possible that the circumstance that certain plant proteins have been named plant casein and plant albumin has masked the idea that they differ from the animal proteins of the same name; that, in spite of similar composition and behavior, differences exist which make it essential to consider the substances as distinct, and to deal with them separately rather than merely to regard them as identical?"

Here, Ritthausen clearly recognized the inadequacy of Liebig's early views, and the vast store of information that Osborne had accumulated by 1909 made it feasible to undertake an experimental study of the problem. Accordingly, he enlisted the help of Lafayette B. Mendel, professor of physiological chemistry at Yale and a close friend of many years, in a joint program of investigation of the relative nutritive properties of proteins.

[4] Ritthausen received private word, evidently while his book was in press, of Hlasiwetz and Habermann's success in isolating glutamic acid hydrochloride from casein and egg albumin after hydrolysis with hydrochloric acid. In a "*Nachtrag*" on the last page, he mentions this, but points out that Hlasiwetz had also failed to obtain glutamic acid after hydrolysis with sulfuric acid.

The first problem was to develop a technique for the feeding of small animals whereby the food intake could be measured with accuracy. Rats were chosen as the experimental animal since they would readily eat the artificial food mixtures provided, and the tests could be conducted at no great cost. The first experiments were begun on July 5, 1909. They consisted of feeding a pasty mixture of ground dog-biscuit and lard to mature albino rats procured from a local pet shop. The object was to determine whether rats could be maintained for long periods on a monotonous diet, and whether a sufficiently accurate record of the food intake could be obtained. Having established this, and, incidentally, having been forced to make a fresh start when a fire destroyed the laboratory the following January, attempts were made to devise a simple artificial diet of protein, carbohydrate, fat and salts upon which a grown rat could also be maintained. A mixture which contained 18% of casein, 15% of sugar, 29.5% of starch, 30% of lard, 5% of agar and 2.5% of a salt mixture was found to be moderately successful for periods of several months, and was established as a basal diet to which diets containing various seed proteins were to be compared. This was a notable advance. Meanwhile, steps were taken to establish a breeding colony[5] so that the effect of the diets on the growth of young animals could be studied.

The first full year of nutrition experiments was one of many failures and few successes. It was found possible to maintain grown rats for two or three months on the basal diet, and on a few experimental diets in which one or another of the seed proteins was included. However, failure evidenced by severe loss of weight sooner or later ensued, and the animals could be saved from death only by putting them on a mixed dog-biscuit food which was supplemented with raw carrots and ground sunflower seed. After the animal had

[5] This colony has been maintained at the Experiment Station to the present day without the introduction of new blood. Descendants of the colony are often mentioned in the literature as rats of the Osborne and Mendel strain or sometimes, and improperly, as the Yale strain.

regained its weight, a return to the experimental diet soon brought about another decline.

Osborne and Mendel devoted much thought to these observations. They had had a moderate degree of success with diets in which whole milk powder was used as the source of protein and salts, and yet failure ultimately resulted when casein and an artificial salt mixture were fed. They accordingly directed their attention to the nutritive effect of what they called the non-protein constituents of milk. Milk was acidified to precipitate the casein, the serum was boiled to coagulate the soluble proteins, and the filtered aqueous solution of the lactose and salts was evaporated to dryness. This preparation was called "protein-free milk." When used at a level such that the salt intake was similar to that in the milk powder diet (whole milk powder 60%, starch 16.7% and lard 23.3%) that had given nutritive success, it provided a food which gave excellent rates of growth with a number of seed proteins. Furthermore, when this product was added to the diets upon which rats were undergoing nutritive decline, prompt recovery ensued. With diets that contained zein or gliadin as the chief protein, and upon which rats did not grow, the addition of protein-free milk brought about no advantage. Accordingly, the deficiency in these particular diets was clearly to be attributed to the protein. Osborne and Mendel wrote, in 1911, "Thus at length we have found a method of controlling or furnishing some of the most essential non-protein factors in the diet, so that the value of the individual proteins can be investigated under much more favorable conditions than formerly."

With the use of protein-free milk fed at the rate of 28.2% of the diet, they were able to show that rats which received thoroughly purified casein, ovalbumin, lactalbumin, edestin, glutenin or glycinin as the sole source of protein grew at a normal rate; rats that received gliadin or hordein were maintained for long periods of time but did not grow at all, while rats supplied with zein rapidly declined in weight and soon died unless transferred to a more complete diet. They pointed

out that gliadin and hordein are recognized to be deficient in glycine and lysine while zein lacks tryptophan as well as these amino acids.

Osborne and Mendel next tried to account for the success of diets that contained protein-free milk. This material had been included primarily as a source of inorganic salts in what might be assumed to be a "correct" proportion. A mixture of inorganic salts made up to imitate the composition of the salts of milk as closely as possible was prepared and, with its aid, they obtained growth for two months or a little more, but nutritive failure then ensued. Recovery was obtained when protein-free milk was returned to the diet in place of the salt mixture.

However, they were unwilling at this time to accept the notion that some indefinite growth hormone present in the milk preparation might be responsible for its successful use. Their chief interest appears to have been centered upon the capacity of the animal body to synthesize amino acids, as was demonstrated by experiments in which gliadin furnished the sole protein for periods in excess of 200 days. Young animals could be maintained for many months without growth, and a mature female on the gliadin diet was successfully bred and its young were grown for several months on casein, edestin or milk food diets, although one of these young placed on the gliadin diet did not grow. Nevertheless, there were certain strict limitations upon the amino acid-synthesizing capacity of the rat. This was demonstrated by experiments in which rats fed zein or gelatin were shown to lose weight rapidly even when protein-free milk was included in the food. They grew, however, if a casein or edestin food were substituted for one-half of the zein diet. This behavior they believed to be associated with the lack of tryptophan in zein and gelatin.

Early in 1913, however, Osborne and Mendel were becoming more and more impressed with the failure of the diets which contained the artificial salt mixture to provide for more than temporary growth or maintenance. They noted that nutritive

collapse came suddenly, and that recovery could be brought about only by a prompt change to a diet that contained protein-free milk. They discussed the evidence in the literature that certain food products contain something that is essential for the normal activity of the cells, Hopkins' then recent experiments on accessory factors in milk being recalled in particular. Nevertheless, instances were accumulating in which rats on presumably adequate diets which contained protein-free milk also suddenly declined after from 60 to 100 days, and such animals could be saved only by transfer to a dried milk diet. Even the protein-free milk foods were therefore deficient in something that is essential for good growth.

The possibility was at first considered that the deficiency might be inorganic. Their preparations of artificial salt mixtures had been made with ordinary laboratory reagents, but they now repeated the preparation with the purest reagents obtainable. The food containing this product failed in every case but one to promote growth for more than a few weeks. A new salt mixture (the well-known "Artificial IV salts") was therefore prepared which contained trace amounts of iodine, manganese, fluoride and aluminum. This gave a much improved result, but was still inferior to preparations of protein-free milk. As this result seemed to dispose of the possibility that the deficiency in the so-called "purified" diets was inorganic, Osborne and Mendel then closely examined the composition of these diets in comparison with their milk food. This had been prepared with whole milk powder, and the obvious difference lay in the inclusion of milk fat in it. The proteins, carbohydrates and salts were essentially the same, and the process of heating employed in preparing protein-free milk was considered to be no more serious in its potential effects than that used in drying whole milk commercially. Accordingly, they carried out feeding experiments in which a part of the lard in the paste foods was replaced by butter. Such a diet furnished to an animal, which was declining in weight upon a diet that contained protein-free milk as the

source of salts, and lard as the exclusive source of fat, immediately gave rates of growth superior to those shown by the so-called "curve of normal growth" of the colony. They wrote, "It would seem, therefore, as if a substance exerting a marked influence upon growth were present in butter, and that this substance is largely, if not wholly, removed in the preparation of our natural 'protein-free milk'." This was the discovery of the existence of the nutritive factor which was later designated vitamin A. The report of this work was submitted to the Journal of Biological Chemistry on June 21, 1913.

In so active a field as the investigation of the nutrition of animals had become at this time, priority of publication of a discovery is largely a matter of chance. It so happens that McCollum and Davis on June 1, 1913 had submitted a paper to the same journal in which they described experiments which revealed the existence of an essential nutritive factor in the ether extract of egg yolk as well as in butter. Accordingly, in the history of nutrition, priority of publication of the discovery of this hitherto unknown factor is properly awarded to them. However, when two laboratories independently arrive at the same conclusion at approximately the same time, the position of both is greatly strengthened, and wide and immediate acceptance of the results becomes inevitable. This was true in the present instance. What had hitherto been a somewhat vague working hypothesis that was discussed in terms of "accessory food factors," or "antiscorbutic" or "antineuritic" substances for which the term "vitamine" had been proposed by Funk, now became a matter that could be investigated in terms of definite chemical substances, the presence or absence of which could be demonstrated in various food materials. Although years were to elapse before the full complexities of the situation were revealed and the exact nature of any of these factors was established, the fundamental principle which converted animal feeding from an art into something which approaches an exact science had now been enunciated, and progress was from that time rapid.

Osborne and Mendel were now able to return to the problem outlined in their earlier experiments on the feeding of the chemically deficient proteins gliadin and zein. With the use of diets that contained butterfat, these experiments could be repeated under greatly improved conditions, for the occasional inexplicable failures no longer impaired the interpretation. They retained "protein-free milk" in the food mixtures, and showed that, when zein was the only protein, every rat whether young or old promptly and rapidly lost weight. When 0.54% of tryptophan was added to this diet (i.e., 3% of the protein), a young rat was maintained at an unchanged weight of 50 gm for 183 days. When lysine was then added, growth at once occurred. After its weight had doubled, this rat was fed various mixed food diets and grew and survived for another year. By similar experiments in which lysine was alternately added and withheld, the capacity to grow was shown to be completely dependent upon the supply of lysine. With gliadin as the sole protein, rats grew extremely slowly, if at all. The addition of lysine stimulated the rate of growth, and growth ceased if lysine was withheld. Accordingly, the requirement for lysine is a quantitative one, the amount present in this unique protein being inadequate to permit growth but sufficient to allow of long-continued maintenance. Tryptophan is present in gliadin in adequate amount. Osborne and Mendel were able to conclude that "the relative values of the different proteins in nutrition are based upon the content of those special amino acids which cannot be synthesized in the animal body and which are indispensable for certain distinct, as yet not clearly defined, processes which we express as maintenance or repair." They concluded this paper, published in 1914, with the sentence, "Newer trials may indicate the desirability of increasing the proportion of arginine present in zein foods; and still other adjustments may be required to promote ideal growth in this or different species. The way to successful investigation has been opened."

This demonstration of the reason for the differences in the nutritive effect of proteins is perhaps Osborne and Mendel's greatest achievement. Although they left the problem of the identification of the indispensable amino acids in a notably incomplete condition, they had gone as far as was possible at a time when it was necessary to rely on the use of native proteins characterized by the absence of one or other of these substances. Osborne had shrewdly taken advantage of his unique knowledge of the amino acid content of the seed proteins, and his broad experience had led him to the selection for investigation of those few substances which were best calculated to shed light upon the problem. Furthermore, methionine was unknown at this time, and Rose's later success in clarifying the whole matter was dependent upon his discovery of threonine and its recognition as one of the essential amino acids.

It is indeed revealing to read these early papers of Osborne and Mendel today in the light of modern knowledge of vitamins. We are taught that the most important qualification of the investigator is the preservation of an open mind; that one must free himself from preconceived notions and rely strictly upon logic in the interpretation of experimental results. Yet there was a period of two years or more when Osborne and Mendel showed themselves to be strangely resistant to the idea that there may be organic substances without which life is impossible but which are required by the animal organism in, literally, only trace amounts. Not until they had thoroughly exhausted the possibility that their failures may have resulted from the absence of some inorganic component could they bring themselves to consider seriously the concept that the deficiency in their diets was organic in its general nature. Furthermore, extremely little was known at this time about the precise and detailed composition of such materials as natural fats or carbohydrates to say nothing of the chemical composition of milk or of a plant or animal tissue. However this may be, it is certain that, as soon as the experiments admitted of but one interpretation, Osborne

and Mendel reversed their field and threw themselves enthusiastically into the investigation of the nature and distribution of the essential unknown substance in natural fats. The high esteem in which cod liver oil had been held for many years by the medical profession soon received its logical interpretation, and several other natural fats were studied. Furthermore, they devoted much time to a study of the phenomena of growth, its retardation and acceleration in response to manipulations of the diet. It was shown that growth could be suppressed for many months in various ways but that the animal never lost the capacity to grow if an adequate diet were subsequently supplied.

The relative nutritive value of a number of proteins was subjected to far more thorough examination than had heretofore been possible, and it was found that many observations could be accounted for in terms of the supply of the two essential amino acids then recognized. The benefits to be obtained by employing a protein high in these amino acids as a supplement to one in which they were more or less deficient were shown, and a rational explanation was obtained for the use in animal feeding of many mixtures of protein-containing foods that had long been known to be advantageous. By 1917, Osborne and Mendel had recognized that protein-free milk was a source of the so-called "water-soluble vitamin," and were discussing the role of vitamins in nutrition and examining various plant tissues as possible sources. This last was a topic that occupied them for a number of years. The period from 1914 to 1922 was an extremely productive one, and papers appeared at the rate of from 9 to 14 a year in each of which some phase of the general problem was discussed and illustrated by carefully planned and executed feeding experiments. As an example of the importance of some of this work, attention may be directed to two brief papers published in 1918 which described diets on which chickens could be raised to maturity under laboratory conditions. The study was made in an effort to find an experimental animal that could be more conveniently used than the rat, and which

would give the required evidence more quickly. Although not successful in this, the fact, established for the first time, that chickens could be raised to maturity in cages by proper attention to the diet later led others to the investigations upon which the modern poultry industry is founded.

Another example is furnished by the work with yeast. Since yeast was found to be a potent source of the water-soluble vitamin, an effort was made to obtain some information concerning the chemical nature of this substance. The outcome was the description of a method for the preparation of a concentrate rich in this material which was the first of its kind. It was later produced commercially and had wide use. The observation that alfalfa leaves are a rich source of vitamin A led to studies of the chemical composition of this plant that were continued for many years in Osborne's laboratory.

During the last few years of Osborne's active scientific life, his attention became more and more directed towards the chemical aspects of the problems that arose from the nutrition studies. The nutrition work in collaboration with Mendel continued, the main interest being their concern with various problems of growth. But Osborne himself became deeply interested in the nature of the proteins of green leaves and in the chemical composition of the soluble components of leaf tissue. Preparations were obtained of the proteins of spinach and alfalfa leaves, but Osborne frankly confessed to his associates that he was completely baffled by the unusual properties of these proteins. They could be isolated only in an altered or denatured condition, and when Chibnall came to the laboratory in 1922 for a two-year period, Osborne was happy to turn this difficult problem over to him just as he turned the problem of the nitrogenous components of the alfalfa leaf over to Vickery. In 1923 and 1924, he spent many happy months revising and bringing up to date his monograph on the vegetable proteins which had been first published in 1909, and much of his time in these last years was spent in conferences with his associates, in talking with the scores of visitors who came to see him, and in supervision of the work of the laboratory rather than in active participation at the

bench. The writer has many recollections, however, of Osborne's sudden incursions into the laboratory with demands for this or that preparation of an amino acid which he would gravely subject to color tests and then purify by recrystallization if impurity were found or suspected. Only a few weeks before his death in January 1929 and many months after his retirement, he appeared at the laboratory one morning with some freshly shelled pecans, a nut which he had never chanced to investigate, and expressed the desire to prepare the globulin from them. This package is still a treasured memento in the laboratory vault along with the innumerable bottles of the amino acids and proteins to which he had devoted his life.

Osborne was a typical member of what has come to be remembered by the younger generation of scientists as the "old guard." He had great personal dignity and unusual social charm, and was unfailingly kindly and generous with his advice to all who consulted him. However, in the laboratory, he was definitely the leader who went about his work with absolute singleness of purpose. He disciplined himself and required discipline of others; work was what counted, and all else was subordinated to the solution of the problems upon which he was engaged. Nevertheless, he was personally a shy and retiring man to whom the delivery of a public lecture even to a small group was a severe trial. He had a most unusual critical faculty which enabled him to analyze problems acutely, to uncover the fallacies in the arguments and to plan the experiments which would lead to the correct result. No amount of labor was ever spared to arrive at the truth, and perhaps his outstanding characteristic was his capacity cheerfully to scrap months of work and start afresh "to do it right," as he sometimes put it. He lived for his work and allowed nothing to interfere with it.

Osborne's social relationships were somewhat circumscribed as he had little or no interest in music or the arts or in athletics. He was at his best with a small group of friends at home or at his club where he showed himself to be an able and charming conversationalist, and an acute debator

with a broad knowledge both of science and of business and political affairs. His relaxation was taken in the summer when he spent several months at his place in Holderness in New Hampshire driving about the countryside, in fishing, and hunting in the fall.

Osborne was fortunate in seeing the field of science to which he devoted his life progress from an obscure and little known specialty, which had attracted only three or 4 first-class minds in all the world, to a branch of biochemistry that at the time of his death had become one of fundamental importance. He was also fortunate in that recognition of his eminence came early, at first in the unusual form of having all of his papers translated and republished in German periodicals as they appeared. This practice was started by Griessmayer who, in 1896, published the papers that had appeared up to that date in book form and who continued to translate them until 1907. In 1910, Yale conferred an honorary degree upon him and in the same year he was elected to the National Academy. Many other honors followed these, but perhaps the one that he appreciated most was the first award of the Thomas Burr Osborne gold medal given him by the American Association of Cereal Chemists in 1928 in recognition of his contributions to cereal chemistry.

Only a few of the seniors among us now have personal recollections of Osborne. He did not have a large group of former students who keep his memory alive, for his life was spent entirely in the laboratory, and his career as a teacher ended in 1886 when he left Yale and took up his work at the station. Only a very few of his assistants and associates now survive, and most of his New Haven friends have long since gone. But it is thoroughly appropriate that this volume of the Journal of Nutrition should be devoted to recalling his memory. He was a great man and a great biochemist, and one has only to leaf through any volume of this journal to find the traces of his footsteps on many pages.

H. B. VICKERY

The Connecticut Agricultural Experiment Station
New Haven, Connecticut

THE EFFECT OF METHIONINE SUPPLEMENTATION UPON THE TUMOR-HOST RELATIONSHIP IN THE RAT [1]

J. B. ALLISON, R. W. WANNEMACHER, JR., R. HILF AND C. A. HETZEL
Bureau of Biological Research and Department of Physiology and Biochemistry, Rutgers University, New Brunswick, New Jersey

THREE FIGURES

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The nutritional demands of tissues of an animal sometimes may be revealed more adequately by studying the effects of various stresses upon food requirements. For that reason the effects of toxic agents, hormonal or amino acid imbalances, protein or calorie starvation, and cancer are being studied in our laboratories (Roth et al., 50; Wase et al., '52; Allison et al., '54a, '54b, '55; Allison, '55). One of the most interesting stresses which we have found to reveal nutritional demands of normal tissues has been a transplanted sarcoma which grows independently of many constituents in the diet.

This sarcoma developed most rapidly in rats fed a semisynthetic diet containing 12% casein. Increasing the casein content of the diet to 25 or 35% or supplementing the 12% casein with methionine did not alter the rate of growth of the tumor but did favor the development and maintenance of normal tissues. N, N′, N″-triethylenephosphoramide (TEPA) reduced the development of both body and tumor but supplementation with methionine favored the maintenance of nor-

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mal tissues, resulting in longer survival times (Allison et al., '54a). The addition of more nitrogen in the form of glycine or guanidoacetic acid with the methionine increased still further the gain in weight of the normal tissues (Allison et al., '55; Hilf, '55). More work has been done to differentiate the effect of adding this additional nitrogen on the growth of the body versus the growth of the tumor. Since the nitrogen-sparing effect of methionine has been associated with the reduction in the formation of urea, the excretion of urea nitrogen from tumor-bearing animals and from normal rats fed a protein-free diet was measured. A preliminary partial comparison was sought in this way between the depleting effect of the tumor and of simple nitrogen starvation. Data were also obtained on the effect of the various diets on the electrophoretic patterns of the plasma.

METHODS

The semi-synthetic diet and the methods of transplanting and measuring the growth of the tumor were described previously (Allison et al., '54a). Ten male Wistar rats were placed on each diet at the time of transplantation, one group receiving the 12% casein diet, while others were fed the same diet supplemented respectively with 0.67% of DL-methionine, 0.67% of guanidoacetic acid, 1.28% of glycine, 1.53% of alanine and 1.95% of ammonium citrate; 0.67% of methionine plus 0.67% of guanidoacetic acid, 0.67% of methionine plus 1.28% glycine, or the methionine plus 1.53% of alanine. Previous studies (Baron and Allison, '54; Brown, '49; Hilf, '55) demonstrated that these percentages of methionine and guanidoacetic acid were near optimum for maintenance in adults, for growth in young animals and for the development of body weight in tumor-bearing rats fed the 12% casein diet. Except for methionine, the glycine and other supplements were added in equivalent amounts of nitrogen to the guanidoacetic acid. All groups were pair-fed. A similar experiment was performed by pair-feeding supplemented and unsupplemented protein-free diets to normal rats.

Where urea was measured, animals were placed in metabolism cages with two rats to each cage. Urine was collected over three-day periods at intervals throughout the experiment. Urea and ammonia were determined by the Conway method as modified by Steinetz ('39). The tumors were allowed to develop to lethal proportions in the animals fed the 12% casein diet, such a tumor usually weighing 20 or more grams three weeks after transplantation. A few animals were discarded when the transplants did not show appreciable growth and were considered as abnormal takes. At the termination of the experiment, blood serum was studied electrophoretically by the method of Tiselius (Longsworth, '42).

RESULTS AND DISCUSSION

The ratio of gain in body weight to an increase in tumor weight has been used as one overall measure of the effect of the tumor upon the body, the larger the ratio the greater the resistance of the body to the depleting effects of the tumor (Allison et al., '54a). The data in figure 1 A illustrate the effect on this ratio of supplementing the casein diet with methionine (M), glycine (Gl), alanine (Al) and ammonium citrate (NH_4), respectively. Supplementation with methionine increased the ratio, an increase which is illustrated also by the bars under B. The lines through the tops of the bars record the standard errors. Supplementing with a mixture of methionine and guanidoacetic acid increased the ratio still further. A similar although not always as marked an increase was obtained by supplementing with a mixture of methionine and glycine. The glycine was added in equivalent amounts of nitrogen to guanidoacetic acid. Adding alanine with the methionine did not increase the ratio significantly above the value for methionine alone. These data suggest that methionine plays a specific role in increasing the ratio between body weight gain and tumor weight but that glycine and guanidoacetic acid enhance that role.

The variations in the ratios between body weight gain and tumor weight in animals fed the 12% casein diet are illus-

trated in figure 2 A. The circles represent one experiment which was terminated at the end of 21 days at which time the tumor weight averaged approximately 16 gm in 7 rats. Since the data can be described by a straight line drawn through the origin, the growth of the tumor can be considered to be independent of the gain in body weight, the average

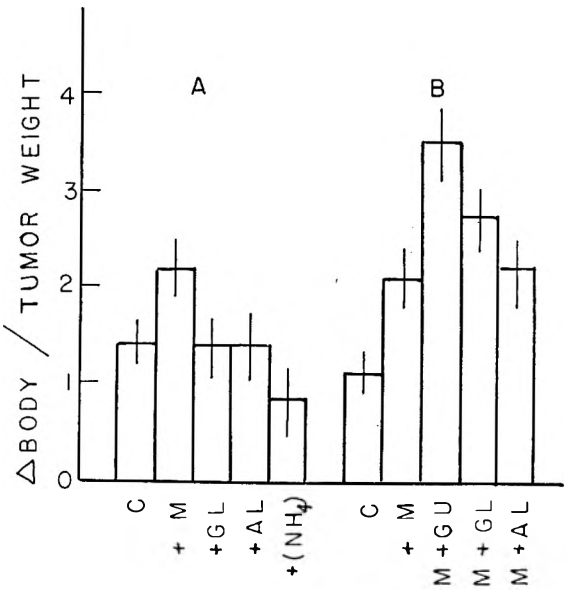

Fig. 1 (A) The bars illustrate the average body weight gain/tumor ratios in 10 rats fed a 12% casein diet (C), and in other groups of 10 rats each fed the casein diet supplemented with methionine (M), with glycin (Gl), with alanine (Al), and with ammonium citrate (NH_4), respectively. (B) Similar data obtained in animals fed the casein diet supplemented also with methionine (M) and various mixtures of methionine and guanidoacetic acid, and glycine and alanine. Lines are standard errors (20 rats to a group).

weight of the tumor being the reciprocal of the slope. This independence of the growth of the tumor was also suggested by the previous data (Allison et al., '54a). The circles with the bar illustrate data obtained during a second experiment which terminated at the end of 17 days at which time the tumors averaged approximately 28 gm. The slope of the line drawn through the points was calculated to be equivalent to

a tumor of this size. The gain in weight of a majority of the animals in both experiments was less than 50 gm and the average body weight gain/tumor ratio for the 15 rats was 1.95 with a standard error of $\pm$ 0.27.

The data plotted in figure 2 B were obtained using tumor-bearing rats which were pair-fed to the corresponding groups in figure 2 A using the 12% casein diet supplemented with 0.67% of DL-methionine. Supplementation with methionine did not alter the independence of the growth of the tumor to

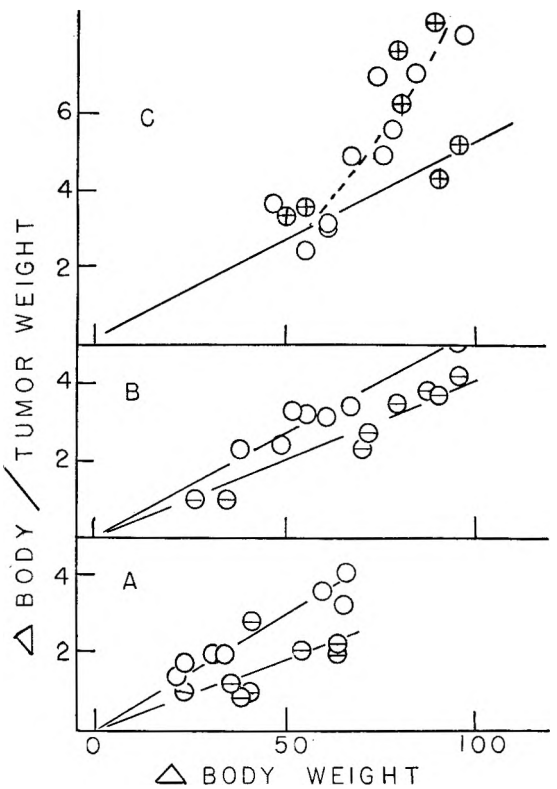

Fig. 2 (A) The open circles illustrate body weight gain/tumor ratios in rats fed a 12% casein diet. The circles with lines illustrate similar data obtained on another group of tumor-bearing rats. (B) Data obtained on rats fed the casein diet supplemented with methionine. (C) Data obtained on rats fed diet supplemented with methionine plus glycine (open circles) and methionine plus guanidoacetic acid (circles with cross lines).

body weight gain. A majority of the animals, however, gained 50 gm or more during the experimental period and the average body weight gain/tumor ratio for the 15 rats was 3.38 ± 0.3 which is significantly higher than the ratio obtained when the rats were fed the unsupplemented diet. Even though the growth of the tumor was not altered by supplementation, the greater body weight of the animals given additional methionine was shown previously to increase their length of life (Allison et al., '54a).

The open circles in figure 2 C illustrate the effect of adding 1.28% glycine together with the methionine, on the body weight gain/tumor ratio. The solid line drawn in figure 2 C reproduces the line drawn through the open circles in figure 2 B. Thus the addition of glycine with the methionine caused the ratio to increase markedly in animals that gained more than 50 gm in body weight. These data can be interpreted to mean that the relationship between tumor and host has been altered by this type of supplementation so that the growth of the tumor is suppressed in these larger animals. Similar data were obtained when 0.67% of guanidoacetic acid was added together with the 0.67% of methionine (illustrated by the circles with the cross bars in figure 2 C). The average increase in body weight with respect to the tumor in 18 rats fed methionine plus glycine was 4.27 ± 0.44. Similarly, the average ratio for 19 rats fed the 12% casein supplemented with methionine and guanidoacetic acid was 4.6 ± 0.4. Both these ratios are significantly larger than the one obtained for supplementation with methionine alone.

The nitrogen-sparing effect of methionine in the rat may be correlated, in part at least, with a reduction in catabolism of amino acids and in the excretion of urea nitrogen (Allison, '53). Some of the effects of the growing tumor on the body of the animal may be associated with depletion of protein stores in the normal tissues (Mider, '51; Fenninger and Mider, '54), a situation which often leads to a reduction in the excretion of urea nitrogen in a depleted animal. A preliminary comparison was sought, therefore, between the effects

of methionine on the excretion of urea nitrogen in protein-depleted and in tumor-bearing rats.

The data in figure 3 B illustrate the effect of these various forms of supplementation on the excretion of urea nitrogen when added to a protein-free diet fed to normal rats. The

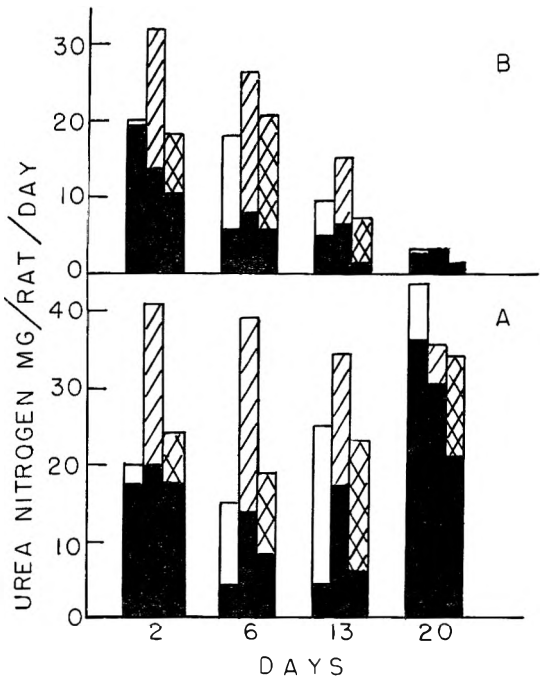

Fig. 3 (A) The excretion of urea nitrogen 20 days after transplantation in tumor-bearing animals fed 12% casein diet (white bars), supplemented with 1.28% of glycine (bars with slanted lines), supplemented with 0.67% of guanidoacetic acid (bars with cross lines). The effect of adding 0.67% of methionine to each one of these diets is illustrated by the black bars. (B) Similar data obtained using rats fed the protein-free diet to which was added the various supplements.

white bars illustrate the average excretion of urea nitrogen in a group of rats fed the unsupplemented protein-free diet. The bars with slanted lines illustrate the effect of adding 1.28% of glycine, and the bars with crossed lines record the excretion of urea in animals fed the protein-free diet plus 0.67% of guanidoacetic acid. The effect of adding 0.67% of

methionine to each one of these diets is illustrated by the black bars. Initially, the addition of methionine did not reduce the excretion of urea nitrogen below the control group. Adding 1.28% of glycine to the protein-free diet increased the excretion of urea nitrogen but supplementation with methionine reduced this excretion markedly. Supplementation with both methionine and guanidoacetic acid also reduced the excretion of urea in animals fed a nitrogen-deficient diet. The effect of continued feeding of these nitrogen-deficient diets on the excretion of urea nitrogen is illustrated by the bars over days 6, 13 and 20. In general, there was a gradual decrease in the excretion of urea nitrogen in all groups of animals until a minimum was reached at 20 days. This type of conservation of nitrogen by animals fed a protein-free diet has been described previously (Allison, '53). The effect of the protein depletion upon the utilization of glycine is particularly dramatic, the glycine not being catabolized to form urea nitrogen in the depleted rat. It is possible that the effect of adding glycine with methionine may have different effects on the metabolism of the animal than adding a combination of methionine and guanidoacetic acid.

This pattern of excretion in the protein-depleted animals may be compared to a pattern of excretion in tumor-bearing animals as illustrated in figure 3 A. The excretion of urea nitrogen in tumor-bearing animals fed 12% casein is illustrated by the white bars. The bars with slanted lines illustrate the excretion of urea nitrogen in tumor-bearing animals fed this diet supplemented with 1.28% of glycine. The bars with crossed lines illustrate the effect of adding 0.67% of guanidoacetic acid. The effect of adding methionine to each one of these diets upon the excretion of urea nitrogen in the tumor-bearing animals is illustrated by the black bars. The bars over 6 and 13 days suggest that the animal is being depleted somewhat in protein stores with a reduction in excretion of urea nitrogen but as the tumor grows large and develops to lethal proportions there is a marked increase in the loss of urea. Although the normal tissues of the body may

be quite depleted in protein stores at 20 days, the high excretion of urea nitrogen demonstrates a high catabolic activity associated with the tumor (see Fenninger and Mider, '54). This excretion is least, however, in tumor-bearing animals fed the combination supplements of methionine plus glycine or methionine plus guanidoacetic acid. The nitrogen-conserving effect of these combinations may account, at least in part, for the improvement in the development in the gain in weight of the body of the animal and the actual reduction in the development of the tumor.

Associated with the excretion of methionine was a slight but significant increase in excretion of ammonia nitrogen. The average excretion of ammonia nitrogen in the tumor-bearing animal fed 12% of casein varied from around 11 to 15 mg of nitrogen/day/rat. Adding methionine to the diet increased excretion of ammonia to values as high as 17 mg/day/rat. The highest excretion of ammonia was obtained in the presence of methionine plus glycine where values as large as 22 mg/day/rat were obtained. A similar increase in excretion in ammonia nitrogen was associated with methionine supplementation in the animals fed a protein-free diet. Adding methionine alone or with glycine or guanidoacetic acid also increased the excretion of inorganic sulfur in the tumor-bearing animals from appoximately 5 mg to 10 mg/day/rat, an excretion that gradually decreased by several milligrams as the tumor developed maximally. These data can be interpreted, at least in part, to mean that the methionine is partly catabolized, possibly contributing to energy pathways involved in synthetic processes.

That the growing tumor does deplete the plasma protein stores of the animal is illustrated by the electrophoretic patterns of serum proteins recorded in table 1. The pattern obtained with sera from rats fed a protein-free diet over a period of 20 days is very similar to that obtained using sera from tumor-bearing animals, 20 days after transplantation (see review by Mider, '51). Supplementation of the 12% casein diet with 0.67% of methionine in the tumor-bearing

animals increased the total protein and albumin/globulin ratio. Attempts to get good electrophoretic patterns in the other tumor-bearing animals were unsuccessful because of a lipemia which has been described for other transplanted tumors (Mider, '51). The value of supplementation, however,

TABLE 1

Electrophoretic patterns of pooled sera from groups of rats fed different diets

DIET	RAT	TOTAL PROTEIN	ALBUMIN	GLOBULIN				A/G
				α	α_2	β	γ	
				grams per cent				
Protein-free [1]	C [2]	5.24	1.73	1.22	0.53	1.25	0.47	0.51
Casein 12%	T [3]	4.81	1.60	1.49	0.56	0.90	0.21	0.51
Casein 12% + methionine (M)	T	5.49	2.00	1.60	0.50	1.12	0.33	0.57
Casein 12% [1]	C	5.83	2.19	1.38	0.56	1.29	0.44	0.60
Casein 12% + methionine (M)	C	6.40	2.51	1.58	0.60	1.33	0.45	0.64
Casein 12% + guanidoacetic acid (G)	C	5.49	1.93	1.38	0.60	1.18	0.37	0.56
Casein 12% + M + G	C	6.51	2.72	1.57	0.69	1.20	0.40	0.72
Casein 18% [1]	C	6.42	2.57	1.55	0.70	1.21	0.49	0.65
Casein 18% + methionine (M)	C	6.76	2.63	1.58	0.60	1.35	0.41	0.67

[1] = Data from Allison ('55).
[2] C = Normal.
[3] T = Tumor-bearing.

in normal animals is illustrated by the electrophoretic patterns for sera from rats fed diets containing 12% of casein supplemented with methionine, guanidoacetic acid, and then a mixture of the two. The methionine-guanidoacetic acid-supplemented diet was equivalent in its effect on albumin/globulin ratio to the diet containing 18% of casein.

SUMMARY

Data are presented to demonstrate that the gain in weight of a transplanted sarcoma was independent of the gain in body weight of rats fed a 12% casein diet. Supplementation with methionine did not alter this independence but did increase the rate of gain of the body with respect to the tumor. Adding more nitrogen in the form of glycine or guanidoacetic acid together with the methionine increased this rate of gain of the body still further and evidence is presented to show that the rate of growth of the tumor was depressed in the larger animals fed the mixed supplements.

The reduction in excretion of urea nitrogen in the presence of these various methionine supplements was demonstrated in normal rats fed a protein-free diet and in tumor-bearing rats fed the 12% casein diet. The catabolic activity, as measured by excretion of urea nitrogen, differed between protein-starved and tumor-bearing rats, the excretion decreasing to exceedingly low values in the protein-starved while it increased to high values as the tumor became large in the tumor-bearing rats. The mixed supplements of methionine and either guanidoacetic acid or glycine reduced the excretion of urea in the tumor-bearing animals when the loss of urea nitrogen was exceptionally high.

The depleting effects of the tumor and of a protein-free diet were similar when measured in terms of electrophoretic patterns of plasma. The increase in plasma protein stores through methionine supplementation was also detected by these patterns.

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DIET AND SERUM CHOLESTEROL IN MAN:

LACK OF EFFECT OF DIETARY CHOLESTEROL[1,2]

ANCEL KEYS, J. T. ANDERSON, OLAF MICKELSEN,[3]
SADYE F. ADELSON AND FLAMINIO FIDANZA[4]

Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, the Agricultural Research Service, U. S. Department of Agriculture, and the Metabolism Unit, Hastings State Hospital, Hastings, Minnesota

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There is no longer much argument against the theory that the diet of man has a powerful effect on the concentration of cholesterol and related substances in his blood (Keys and Anderson, '54; Mann and Stare, '54; Keys et al., '55b) but this merely emphasizes the importance of the question as to the role of the several dietary elements in this effect. The first item for consideration, obviously, is the cholesterol in the diet. We have already reported briefly both survey and experimental evidence indicating that the cholesterol in ordinary human diets has substantially no effect on the concentration in the blood (Keys, '49, '52a, b; Keys and Anderson, '54).

The present paper is a report of dietary experiments on men under completely controlled conditions, as well as of both cross-sectional and longitudinal studies on men in Minnesota and on two samples of men in Sardinia. In the latter studies the men lived at home and ate as usual.

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[2] This investigation was supported (in part) by research grant H-10 from the United States Public Health Service.

[3] Present address: Institute for Arthritis and Metabolic Diseases, Bethesda, Maryland.

[4] Present address: Institute of Physiology, University of Naples.

SUBJECTS AND METHODS

All of the subjects were men between the ages of 20 and 60 and were pronounced to be physically healthy on the basis of medical examinations, including electrocardiography. The subjects in the prolonged controlled experiments were schizophrenic patients in a special metabolic unit in Hastings State Hospital. They, too, were similarly judged to be physically "normal." The men in the longitudinal surveys, as well as in the detailed measurements of diets made by members of the nutrition research staff of the U. S. Department of Agriculture, were professional and business men, aged 45 to 55 in 1947, who have cooperated with this laboratory for the past 9 years. The surveys on the Island of Sardinia covered coal miners in the small mining town of Bacu Abis and moderately active men employed by the city of Cagliari (policemen and firemen) and by the University of Cagliari Medical School (attendants, mechanics, etc.).

In the Minnesota surveys, except for those done in cooperation with the U. S. Department of Agriculture, and in the experiments with the rice-fruit diet, serum total cholesterol was measured by the Liebermann-Burchard reaction applied to the Bloor extract. In all of the other surveys and experiments the method of Abell et al. ('52), as modified by us (Anderson and Keys, '55), was used. The latter method consistently yields results somewhat lower than the older (Bloor) method but there is a very close correlation between the two. Cholesterol in the beta lipoprotein fraction of the serum was measured on the materials separated by paper electrophoresis as described by Anderson and Keys ('55). All blood samples were drawn from antecubital veins in the morning, with the subjects in the basal state. All analyses were in duplicate.

Dietary cholesterol was computed from the values for foods given in table 1. It is probable that the values for meats are too high in this table. In surveys, except for those where the diets were measured by the U. S. Department of Agriculture group, the dietary intake of cholesterol-containing foods was

estimated by individual interviews, with particular attention to the use of eggs, cream, milk, butter, cheese, ice cream, meats, fish and chicken. The individual cholesterol values thus obtained are only crude estimates, of course, but it should be noted that the consumption of cholesterol-containing foods is more accurately recollected than the rest of the diet.

On the Island of Sardinia the estimation of dietary cholesterol was particularly simple. Because of the low and relatively invariable consumption of all cholesterol-containing

TABLE 1

Approximate cholesterol contents of foods used in the dietary calculations [1]

CONSTITUENT	CHOLESTEROL	CONSTITUENT	CHOLESTEROL
	mg/100 gm edible portion		*mg/100 gm edible portion*
Butter	280	Liver, beef	260
Cheese	120	Liver, calf	350
Cream, 20% fat	70	Margarine [4]	70
Egg [2]	625	Meat [5]	100
Fish [3]	60	Milk, cow	11
Ice cream, 12.5% fat	60	Milk, cow, skim	3
		Poultry	80

[1] Based on reports of Okey ('45), Nataf ('48), Pihl ('52) and on analyses made by Okey and Strisower, cited in Dobbin et al. ('51).

[2] One medium egg contains 300 mg of cholesterol.

[3] High-fat fish such as Atlantic herring or mackerel contain 80 mg of cholesterol.

[4] Contains 65% of animal fat. Vegetable oils contain no cholesterol.

[5] Beef, veal, pork, and lamb. Recent analyses by Del Vecchio et al. ('55), using digitionin precipitation, give lower results than these values from the older literature.

foods except eggs, the individual habit of eating eggs almost wholly accounted for the variation in total cholesterol intake. Among these Sardinians scarcely 10% eat butter, cream or ice cream except on rare occasions, only small portions of meat are eaten a few times a week, milk is consumed only in the morning coffee, the bread and "pasta" (spaghetti, etc.) is made without shortening and olive oil is the sole cooking fat. But the mean for the total of 187 men was 4.92 eggs per week, eaten as such, and the range of consumption was from zero to 30 eggs per week.

In the surveys conducted in cooperation with the U. S. Department of Agriculture, elaborate efforts were made to obtain a complete and accurate measurement of every item of food and drink consumed by the individual men during the period of one or two weeks of study. Enthusiastic cooperation of the wives as well as of the men themselves was a condition for acceptance of the subjects in this program. Portions of food eaten at home were weighed on a gram scale or measured in 8 oz. cups or standard-sized spoons, recipes were checked and care was taken to allow for meat trimmings and other table waste. In these surveys most of the men ate their noon meals during the week in restaurants or clubs. The portions of food at these and other meals were carefully estimated or measured with a pocket rule by the men. In addition, the cooperation of these establishments was frequently enlisted so that the accuracy of the records of lunch consumption could approach that of the records made in the home by the wives of these subjects. Between-meal and evening snacks were also recorded. Every effort was made to assure that normal dietary habits of the individuals were not changed during the period of the study.

RESULTS

Six surveys in Minnesota. Simple cross-sectional surveys always raise the question as to whether the dietary variable is properly isolated. This is particularly true of dietary cholesterol which conceivably may be related, directly or inversely, to other characteristics of the diet or mode of life which, in turn, have an effect on the serum level. This possibility is much reduced when the population is homogeneous in regard to age, economic status, ethnic pattern, clinical status and occupation. The validity of the results is further increased when, as in the case of the 6 surveys to be summarized below, it is found that both absolute body size and relative obesity are not significantly related to either the diet or the serum cholesterol.

The results of 6 surveys in Minnesota are summarized in table 2. Clearly, any relationship between serum cholesterol and that consumed in the diet must be very small indeed in such men. It appears, however, that there may be a tendency for men who consume the least cholesterol to have slightly

TABLE 2

Serum total cholesterol in successive quintiles of cholesterol intake from lowest to highest (1 to 5) in 6 surveys of Minnesota men. For each survey the mean serum value of the middle quintile is taken as 100 and all other values are expressed as percentage of this.

NO. OF SUBJECTS	AGE RANGE	CHOLESTEROL INTAKE QUINTILES [1]				
		1	2	3	4	5
160	18–25	105.9 ± 3.8 [2]	109.7 ± 3.5 [2]	100 ± 3.7 [2]	106.7 ± 2.9 [2]	111.1 ± 3.5 [2]
86	20–27	92.9 ± 5.6	89.3 ± 3.7	100 ± 5.2	98.4 ± 5.9	109.4 ± 5.0
236	42–56	95.9 ± 2.2	103.0 ± 2.4	100 ± 2.7	99.1 ± 2.4	99.0 ± 2.3
214	45–55	98.5 ± 2.4	101.2 ± 2.8	100 ± 2.6	102.1 ± 3.5	101.6 ± 3.0
208	45–55	95.3 ± 2.8	96.6 ± 3.0	100 ± 2.5	94.0 ± 2.7	103.3 ± 2.5
168	48–58	93.5 ± 3.5	95.1 ± 2.8	100 ± 2.6	92.9 ± 2.8	101.7 ± 2.5
1072	18–58	97.18	100.06	100.0	98.82	103.41

[1] The distribution of cholesterol intakes varied somewhat in the 6 series but, in general, they approximated less than 500 mg of cholesterol in the daily diet for the lowest quintile, 500 to 680 mg for the second quintile, 680 to 850 mg for the third, 850 to 980 mg for the 4th and over 980 for the highest quintile.

[2] Mean and standard error.

lower concentrations of cholesterol than the average while those who consume the most cholesterol have the opposite tendency, the deviations in each case being about 3% of the grand mean. Complete independence prevails over the middle range of intakes (second through 4th quintiles) including 60% of the subjects.

However, even these slight trends are not necessarily indicative of any effect of dietary cholesterol itself. In the first place, the dietary cholesterol is expressed in absolute units and differences in these absolute amounts do not necessarily imply a corresponding difference in the character of the diets consumed by the different men. Two men differing in body size and physical activity and therefore consuming different total amounts of food when they are in calorie balance will, of course, consume different total amounts of cholesterol even though the composition of the diet is exactly the same. We do not have information to show that the men in different quintiles were, in fact, all at the same average level of total metabolism. However, we cannot explain more than a small part of the differences in intake as possibly being a reflection of differences in the total mass of the diet; the average cholesterol intake in the top quintile was more than three times that in the bottom quintile.

A more serious question is whether the observed variations in cholesterol intake are independent of important differences in other characteristics of the dietary composition. As a matter of fact, we find that cholesterol intake in the American diets is almost always related, to some extent, to the intake of animal protein and animal fat in the diet. For instance, in table 5, below, it will be noticed that the cholesterol intake of the men on high-fat diets average 20% more than for the men on low-fat diets.

Longitudinal studies on businessmen. Among 286 clinically healthy Minnesota business and professional men studied annually since 1947, there were 33 men whose jobs, diets and body weights were very constant over a period of 4 years and whose dietary intakes of cholesterol were consistently in the lower third of the distribution of the intakes of the entire group of 286. With these men it is possible to contrast 35 of their fellows, similarly constant in work, diet and body weight, whose average cholesterol intake was consistently in the upper third of the distribution. The summarized comparison of these men is given in table 3. It is clear that the

long-standing habit of consuming more or less dietary cholesterol produced no significant difference in the average serum cholesterol level between the two groups.

In this same population of businessmen there were 64 men who made major changes in their dietary cholesterol from one year to another but who maintained their dietary habits relatively constant otherwise and who did not change body weight by more than 5 lbs. The altered cholesterol intakes resulted from exchanging eggs, fat meats and dairy products for oleomargarine, vegetable fats, cereals and lean meats or

TABLE 3

Serum total cholesterol concentration of Minnesota businessmen whose diets were consistently either low or high in cholesterol for 4 years

CHOLESTEROL IN DIET [1]	NO. OF MEN	AV. AGE	REL. BODY WT.	SERUM CHOLESTEROL
			% [2]	*mg %*
Low	35	49.8	102.9 ± 13.9 [3]	248.9 ± 41.4 [3]
High	35	51.2	99.7 ± 11.4	256.2 ± 42.9

[1] The "Low" cholesterol men always received less than 600 mg daily, their average intake being 401 mg, while the "High" intake men always received more than 850 mg, their average diet providing 1010 mg daily.

[2] Relative body weight is the body weight expressed as a percentage of the value, for the same age, sex and height, given in the standard U.S. table of average weights, first published in Medico-actuarial Soc. America (N.Y.), Vol. 1, 1912.

[3] Mean and standard deviation.

fish in the diet. The men who increased their dietary cholesterol did so either without deliberate intent (incidental to a change in domestic status — marriage, divorce or death of the wife), or because they were abandoning a previous effort to curtail cholesterol intake; the result was at least a doubling of the previous cholesterol intake. The men who decreased their cholesterol intake did so in some cases incidental to changed domestic status but most of them had been persuaded, often by the family doctor, that cholesterol in the diet should be avoided; their cholesterol intakes decreased by 50% or more. The serum cholesterol findings on these men are summarized in table 4. When these men changed their cholesterol

intakes, either up or down, there was no significant change in the serum level. The standard error of the mean of the differences, before and after, was ± 5.86 mg % for the men who increased their cholesterol intake and ± 5.05 for those who decreased the cholesterol in the diet.

U. S. Department of Agriculture collaborative survey. In the Spring of 1953, and again in 1954, the nutrition research staff from the Agricultural Research Service of the U. S. Department of Agriculture carried out a very careful study on the diets consumed by 119 men in the same general group of middle-aged businessmen mentioned above. Because of the possibility that different relationships might obtain at dif-

TABLE 4

Serum total cholesterol in Minnesota businessmen before and 4 to 12 months after changing their dietary intake of cholesterol

DIET CHANGE [1]	NO. OF MEN	SERUM CHOLESTEROL	
		Before	After
		mg %	
Increase	23	245.0 ± 39.8 [2]	249.4 ± 48.8
Decrease	41	241.5 ± 45.2	241.9 ± 42.8

[1] The mean change in daily cholesterol intake was + 350 mg for the mean in the "Increase" group and — 480 mg for those in the "Decrease" group.

[2] Mean and standard deviation.

ferent levels of total fat in the diet, the analysis was made separately for the 61 men whose diets provided more than 40% of the calories from fats and for the 58 men whose diets were less rich in fats. The findings in regard to dietary cholesterol, together with the serum cholesterol values, are summarized in table 5.

It will be observed that there was considerable variation in the cholesterol intakes of these men, both in absolute units and in concentration per 1000 calories, but in neither unit is there a significant correlation with the serum cholesterol concentration.

Experiments with modified rice-fruit diets. A simple way to demonstrate an effect of the diet on the serum cholesterol

concentration is to use a rice-fruit diet but it is erroneous to credit the effects to the absence of cholesterol from that diet. Proof of this is given in table 6, which summarizes the results of completely controlled experiments on men in Hastings State Hospital. A modified rice-fruit diet was devised which

TABLE 5

Dietary cholesterol intake and correlation coefficients between intake and serum total cholesterol in middle-aged business and professional men on high-fat (more than 40% of calories from fats) and lower-fat (less than 40%) diets.

	ITEM	High fat 61 MEN	58 MEN Low fat	TOTAL 119 men
1	Intake, mg/day	825.6 ± 206.7 [1]	707.4 ± 240.3 [1]	768.0 ± 230.5 [1]
2	Intake, mg/1000 Cal.	330.5 ± 83.5	287.9 ± 89.7	309.8 ± 88.8
3	r, serum vs. 1 [2]	— 0.176	— 0.037	— 0.057
4	r, serum vs. 2 [2]	— 0.134	0.001	— 0.024

[1] Mean and standard deviation.

[2] To reach the 5% level of probability, with N = 61, 58, and 119, r should be 0.250, 0.257 or 0.180, respectively.

TABLE 6

Mean serum total cholesterol values from 4 experiments in which men changed from the house diet (H)[1] to a modified rice-fruit diet (R_1 or R_3)[2] or the reverse

EXP. AND DIET	NO. OF MEN	SERUM TOTAL CHOLESTEROL Mean	Δ	SE, Δ	EXP. AND DIET	NO. OF MEN	SERUM TOTAL CHOLESTEROL Mean	Δ	SE, Δ
		mg %	*mg %*	*mg %*			*mg %*	*mg %*	*mg %*
50 A	6				50 B	7			
H		217.2			H		211.7		
R_1		183.5	— 33.7	± 11.0	R_3		170.7	— 41.0	± 15.2
50 C	6				50 D	7			
R_1		181.8			R_3		198.8		
H		228.6	46.8	± 7.2	H		256.1	57.3	± 10.9

[1] House diet.

[2] Rice-fruit diet R_1 contained zero cholesterol.
Rice-fruit diet R_3 provided 500 to 600 mg of cholesterol daily.

included salt to improve taste and a daily allowance of 11 gm of fat, either in egg yolks (diet R_3) or in the form of all-vegetable oleomargarine (diet R_1), so that the daily cholesterol intake would be 500 to 600 mg or zero, respectively. The serum cholesterol was measured at the end of 4 weeks on

diet R_1 or R_3 and the control values were obtained from blood samples drawn either before or a month after the dietary restriction had been removed. Calorie balance was maintained throughout.

Table 6 shows that the change from the "house" diet to the modified rice-fruit diet for 4 weeks produced a marked drop, averaging 15 to 20%, in the blood serum cholesterol, and this was not significantly affected by the presence or absence of cholesterol. Further, change from the rice-fruit diet back to an ordinary diet produced even greater increases, averaging 20 to 30%, and this rise was at least as great when

TABLE 7

Serum total cholesterol values in milligrams per cent, of 5 men on diet R_3 and after changing to diet R_1

SUBJECT	DIET R_3 Weeks 3 and 4	DIET R_1			
		1 Week	2 Weeks	3 Weeks	4 Weeks
Br	150	146	150	139	145
Er	181	200	188	184	188
Hi	209	208	204	158	228
Pr	247	251	255	233	239
Tw	162	168	159	139	150
Mean	190	195	191	171	191

the previous diet provided a good deal of cholesterol, i.e. diet R_3, as when it provided none (R_1).

A more direct comparison was provided by an experiment on 5 men who, after 4 weeks of subsistence on diet R_3, were changed to diet R_1 and continued for another 4 weeks. The findings are given in table 7. Obviously, the removal of the 500 to 600 mg of cholesterol from the daily diet was without effect.

Experiments with a mixed diet. Critical experiments with mixed diets of ordinary food require that all conditions, including all nutrients in the diet and all factors, such as exercise, that influence the metabolism, are constant except for the single experimental variable of the dietary cholesterol.

In order to assure this, schizophrenic men were selected for their ability to cooperate and they were housed and fed in a separate unit at the Hastings State Hospital. A series of rotating menus was developed so that the daily nutrient intake varied little though the diet was not particularly monotonous. An average level of 400 to 450 mg of cholesterol daily was aimed at and special cookies were provided which could add to this about 1000 mg daily of pure cholesterol when desired. The mode of life of the subjects was carefully standardized in regard to exercise, recreation, and so on, and this standard was maintained 24 hours a day by a special staff of aides.

After a month of standardization in the metabolic unit, subsisting on measured portions of the regular hospital diet, the men subsisted on the experimental diet for 8 weeks during which time all items of food were measured as served and each man's rejections or extra servings were also recorded. Careful attention was given to maintain the food consumption at the calorie balance point.

In experiment 54 A, 13 men consumed 374 mg of cholesterol daily for the first 4 weeks and then received an average of 1369 mg daily for the final 4 weeks. In experiment 54 B, 14 men went through the reverse order of change in cholesterol intake. The average values for dietary items of interest and for the serum total cholesterol at the end of each 4-week period are given for both experiments in table 8. There are suggestions of a trivial response of the serum cholesterol to the diet but in neither experiment is this statistically significant.

Surveys in Sardinia. On the Island of Sardinia the dietary pattern of the general population is very uniform in most respects and the consumption of cholesterol in the diet is relatively trivial except for that provided by eggs which are eaten in widely varying amounts. Hence, in dietary surveys it is readily possible to separate population samples into relatively low and high cholesterol intakes with the rest of their diets being substantially the same.

Table 9 summarizes the results of two surveys in Sardinia in 1955, one on moderately active municipal employees in Cagliari and one on poor coal miners in the small mining town of Bacu Abis. It will be observed that a two-fold dif-

TABLE 8

Mean diets, body weights and serum total cholesterol values for experiments on two groups of men [1] *together with means and standard errors of the individual changes in serum cholesterol, period 1 minus period 2.*[2]

	EXP. 54 A		EXP. 54 B	
	Period 1	Period 2	Period 1	Period 2
Calories/day	2974	3010	3201	3151
Protein, gm/day	64.7	63.2	67.1	68.4
Butterfat, gm/day	34.5	35.0	32.2	35.8
Total fat, gm/day	65.5	66.4	68.1	66.5
Diet cholesterol, mg/day	374	1369	1388	477
Body weight, kg	65.6	65.5	67.9	67.6
Serum total chol., mg %	211.5	213.6	214.7	206.2
Δ Serum chol., mg %	— 2.08 ± 3.72		8.50 ± 4.22	

[1] Experiment A, 13 men.
Experiment B, 14 men.

[2] Note that in experiment 54 A the cholesterol intake in period 2 was 366% of that in period 1, while in 54 B the cholesterol intake in period 2 was only 34% of that in period 1.

TABLE 9

Serum cholesterol values of men in Sardinia classified according to cholesterol intake. For each man his cholesterol value was expressed as a percentage of the means for all men of his age in the regional sample and the tabular values for serum are the means and standard errors of these percentages.

PLACE	LOW CHOLESTEROL INTAKE			HIGH CHOLESTEROL INTAKE		
	No.	Intake	Serum	No.	Intake	Serum
		mg/day			*mg/day*	
Cagliari	67	220	101.1 ± 2.7	40	430	97.6 ± 3.2
Bacu Abis	38	230	98.7 ± 2.6	39	610	101.3 ± 2.6

ference in cholesterol intake was not associated with a significant difference in the serum of the men of Cagliari. In the men of Bacu Abis the intake difference was almost three-fold yet the serum levels were not statistically different.

DISCUSSION

The foregoing evidence is definitive, we think, in showing that variations in the intake of cholesterol over the whole range of natural diets do not influence the serum level of physically normal adult men so long as other elements in the diet are constant. The results of experiments on 5 normal subjects reported by Mayer et al. ('54) are in agreement for the limited range of cholesterol intakes tried. The findings, too, of Kinsell et al. ('52) and of Ahrens et al. ('55) on hospital patients are confirmatory though the formula diets used in these experiments are radically different from any natural diets and are not, therefore, fully comparable.

According to the results of the carefully controlled experiments of Heymann and Rack ('43), the same rule applies to infants and children. In regard to women, Moses ('52) and Moses et al. ('52) found that the addition of 2 gm of cholesterol to the daily diet of pregnant women did not increase the normal trend to hypercholesterolemia in pregnancy.

The findings in two series of experiments reported in the literature may seem to be in disagreement but on closer scrutiny the results cannot be cited as showing an effect on dietary cholesterol. Okey and Stewart ('33) obtained a small positive response in young women when the yolks of two eggs were added to the daily diet but the fact that this involved a daily addition of about 100 Cal. of fats was not controlled. Similarly, in a prolonged experiment on 60 volunteers (Groen et al. '52), the dietary cholesterol changes were accompanied by substantial changes of the diet in other respects. For example, the "high" cholesterol diet (940 mg daily), provided 100 gm of proteins, 128 gm of fats and 2618 Cal., while for the "standard average" diet the subjects consumed an average of 75 gm of proteins, 99 gm of fats and 2391 Cal. Gillum et al. ('55) reporting on a survey on men and women aged 50 to over 80 years found that the dietary and serum cholesterol values were correlated with a coefficient value of $r = +0.12$. This value may be statistically significant because of the large number (53) of subjects,

but it is obviously biologically trivial even if the dubious proposition is accepted that the cholesterol was the only effective variable in the diets of their subjects. Wilkinson et al. ('50) and Gertler et al. ('50) were unable to find any correlation between cholesterol intake and that in the blood in similar surveys.

It is probable that when cholesterol is artificially added to the diet in increasingly large amounts a point will be reached where there will be a positive effect on the serum level in man. We have shown that the intake of a single meal containing more than 10 gm of cholesterol tends to result in a slight increase in the serum cholesterol for a few hours but the effect is so small that relatively large numbers of experiments are required to prove it statistically (Keys et al., '55b). With intakes of 30 gm of cholesterol per day, for 29 days, Messinger et al. ('50) produced serum elevations in 4 out of 5 subjects but the serum increases were very small; when these huge cholesterol doses were given with large amounts of cream or egg yolk fat the serum rose sharply by averages of 10 to 20%. Kinsell et al. ('52) had negative results with equally large amounts of cholesterol given by mouth to patients with metabolic disorders but these results may not be comparable because of the highly abnormal character of the diet in other respects.

When different populations with divergent dietary patterns are compared it is easy to point out an apparent relationship between cholesterol intake and the concentration in the blood. In general, whenever the population pattern of diet involves a low cholesterol intake, the serum level is also low but this seems to be readily explicable in terms of other peculiarities of the diets that are correlated with their average cholesterol content (Keys et al., '54; Keys et al., '55a, b).

In several of the studies reported here (the surveys in Sardinia, the U.S.D.A. survey of 1954, and the experiments with a mixed diet) measurements were made of the cholesterol in the beta lipoprotein fraction of the serum in addition to the total. This major fraction of the total serum cholesterol

also appeared to be independent of the cholesterol intake in the diet. For example, in the Cagliari survey the mean percentage of the total serum cholesterol represented by that in the beta lipoprotein fraction, and its standard error, proved to be 75.93 ± 0.86 and 75.67 ± 1.04 in the low- and in the high-cholesterol-intak groups, respectively.

Finally, it may be asked why the human serum concentration of cholesterol is so remarkably independent of the amount of cholesterol supplied in the diet. After all, a daily intake of 1 gm of cholesterol, which characterizes some high-cholesterol diets, is something like a daily supply equal to 10% of the total amount of cholesterol in the entire blood volume. A very considerable proportion of this is absorbed from the intestine. But much more than this exogenous supply may be provided in the bile poured into the intestine and it would seem that this endogenous supply, synthesized by the liver, is easily regulated to adjust to the exogenous variations. A very different state of affairs prevails in the rabbit and the chick, of course, but it seems that most carnivores resemble man in this respect.

SUMMARY

1. Two cross sectional surveys in Minnesota on young men and 4 on older men showed no relationship between dietary cholesterol and the total serum cholesterol concentration over most of the ordinary intake range characteristic of American diets.

2. Two surveys on the Island of Sardinia failed to show any difference in the serum cholesterol concentrations of men of the same age, physical activity, relative body weight and general dietary pattern but differing markedly in cholesterol intake.

3. Careful study during 4 years of 33 men whose diets were consistently very low in cholesterol showed that their serum values did not differ from 35 men of the same age and economic status whose diets were very high in cholesterol.

4. Comparisons made of 23 men before and after they had voluntarily doubled their cholesterol intakes and of 41 men who halved theirs failed to show any response in the serum cholesterol level in 4 to 12 months while the rest of the diet was more or less constant.

5. A detailed study of the complete dietary intakes of 119 Minnesota businessmen failed to show any significant increase of serum cholesterol with increasing dietary cholesterol intake.

6. In 4 completely controlled experiments on men the addition to or removal from the diet of 500 to 600 mg of cholesterol daily had no effect on the serum cholesterol fall produced by a rice-fruit diet or on the rise in changing from a rice-fruit diet to an ordinary American diet.

7. In a completely controlled experiment on 5 physically healthy men the change from a rice-fruit diet containing 500 mg of cholesterol daily to the same diet devoid of cholesterol had no effect on the serum level.

8. In a similar experiment with 13 men receiving 66 gm of fat daily there was no significant effect in changing from a cholesterol intake of 374 mg/day to one of 1369 mg/day. In another 12 men the reverse change was likewise without effect on the blood serum.

9. It is concluded that in adult men the serum cholesterol level is essentially independent of the cholesterol intake over the whole range of natural human diets. It is probable that infants, children and women are similar.

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PAIR FEEDING AS A CONTROL PROCEDURE IN METABOLIC STUDIES OF THE X-IRRADIATED RAT [1]

W. O. CASTER AND W. D. ARMSTRONG
Department of Physiological Chemistry, University of Minnesota, Minneapolis

FOUR FIGURES

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Whenever an experimental animal shows a simultaneous decrease in food intake and loss of body weight, one is confronted with the problem of discovering the extent to which inanition determines the final experimental result. This is a major problem in the study of the metabolic effects of total body x-irradiation in the rat.

A common way of circumventing this problem is to employ pair-feeding techniques which equate the food intakes of the control and experimental animals by using either: (a) the pair-starved control, i.e., reducing the food intake of the control group so as to parallel that of the experimental group, or (b) the tube-fed experimental and control, i.e., forcibly maintaining a constant food intake in the experimental as well as the control animals. There is little doubt that pair-feeding procedures will equate the amounts of food passing through the mouth of the control and experimental animals. A more critical problem is the extent to which these procedures equate the digestive and metabolic mechanisms within these different animals. It is to this problem, as it applies to the acutely x-irradiated rat, that this work is addressed.

[1] This work was supported under terms of Contract AT(11-1)-105 between the Atomic Energy Commission and the University of Minnesota.

EXPERIMENTAL

Four groups of 20 male white rats,[2] 300 gm in weight, were maintained in individual cages with free access to water. The first group of rats received an LD_{50} dose (700 r) of total body x-irradiation. The second group was not x-irradiated, but their intake of food [3] was restricted so as to equal that of the first group. The third and 4th groups were fed a milk diet twice daily by stomach tube at a constant level just sufficient to maintain the 4th (control) group at constant body weight. The third group received 700 r of total body x-irradiation, while the 4th group received none. Body weights were recorded daily, and at fixed periods groups of animals were killed for tissue weight determination.

RESULTS

In the first 4 days following total body irradiation with an LD_{50} dose of x-ray there occurs, in the rat: (1) a 75% decrease in food intake, (2) a 15% loss in body weight, and (3) a 50% increase in water intake. During the first two days of the post-irradiation period, the food intake is substantially zero. From two to 4 days following irradiation the food intake averages approximately half of the pre-irradiation value. Thereafter, the food intake approaches normal levels. The greatest increase in water intake comes during the first two days following irradiation, and the figures for weight loss and increased water intake in the post-irradiation period are roughly equivalent to those obtained during an equivalent period of starvation.

From the two lower lines in figure 1 it is seen that the weight loss of the irradiated animal (group 1) is much the same as that of the pair-starved control (group 2). This is in agreement with other observations (Bowers et al., '53; Smith et al., '51; Smith, Ackermann and Smith, '52; Smith and Tyree, '54). The two upper lines in this figure represent

[2] Holtzman Co., Madison, Wisconsin.

[3] Purina Chow.

the body weights observed when both the control and irradiated animals are fed a constant amount of milk diet administered twice daily by stomach tube. When the food intake was maintained constant by this means, it was again found that the weight loss of the irradiated (group 3) and control (group 4) animals are, within experimental limits, equal. Smith, Ackermann and Smith ('52) have reported the same effect when a diet containing a pancreatic digest of

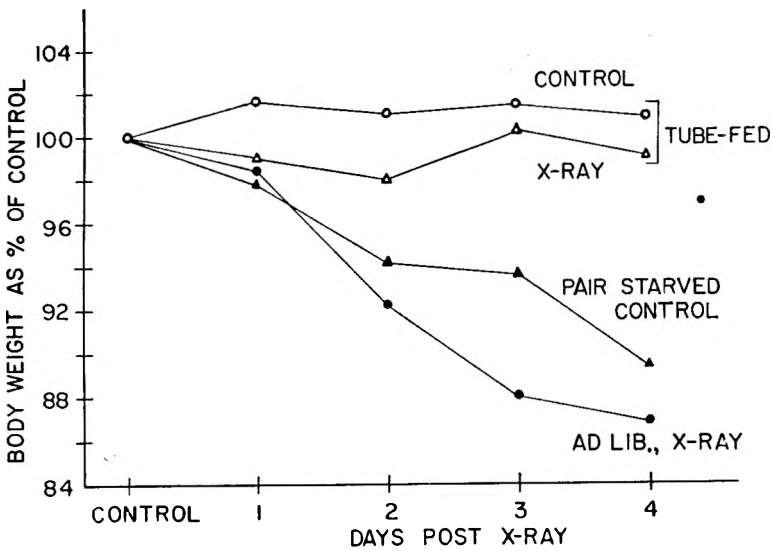

Fig. 1 Total body weight changes observed in x-irradiated and pair-fed rats.

casein [4] was tube-fed. Figure 1, then, summarizes evidence which can be given as proof that the weight loss of radiation sickness is due quantitatively to starvation.

From the evidence in figure 1, it would seem possible to avoid all weight loss problems by tube-feeding the animals. When the distribution of tissue weight is studied by dissection procedures, quite a different picture emerges. This is largely due to the marked difference in the weight of the gastrointestinal contents and the difference in the loss of muscle mass

[4] Amigen, Mead, Johnson and Co., Evansville, Indiana.

observed when the results of the two experimental procedures are compared.

Tube-feeding. The fact that the tube-fed rat fails to lose weight following irradiation is explained by the coincidence that the increase in the weight of gastric contents is nearly equal to the loss in tissue weight resulting from irradiation. As seen in figure 2, the tube-fed rat loses tissue weight following irradiation at substantially the same rate as the rat fed ad

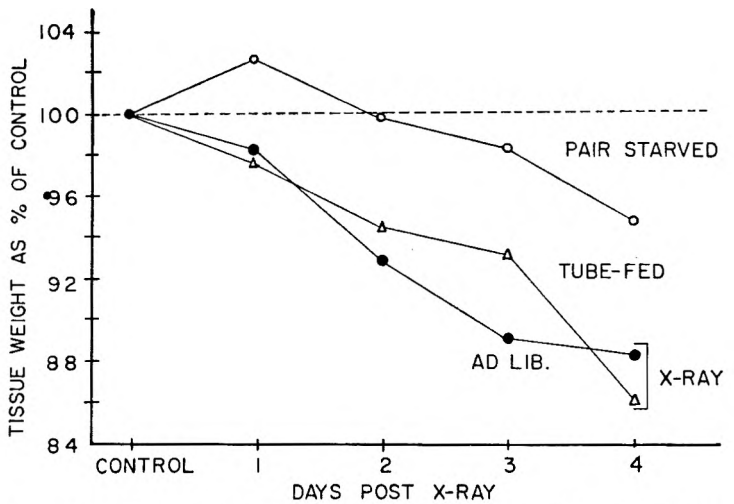

Fig. 2 Total tissue weight changes observed in x-irradiated and pair-fed rats.

libitum. During this time, however, the tube-fed rats accumulate as much as 50 to 60 gm of excess gastric contents.

Figure 3 shows the relative sizes of the stomachs of the control and 4-day post-irradiated animals. All of these stomachs were removed about 18 hours after the last tube-feeding. Similar gastric retention effects have been noted by Smith, Ackermann and Smith ('52).

It therefore appears that tube-feeding the irradiated animal does not prevent the loss in the tissue weight characteristic of irradiation. It does superimpose an additional stress upon an already complex situation, and may well result in

misleading information concerning body weight loss and metabolic balance.

Pair-starved control. The weight loss of the irradiated rat and its pair-starved control were found to be roughly parallel as shown in figure 1. Here again the parallelism disappears when the animals are dissected and the tissue weight changes compared (fig. 2).

Again, one of the major differences comes in the weight of material retained in the stomach. Much of the weight loss of the starving rat is due to that of gastrointestinal contents.

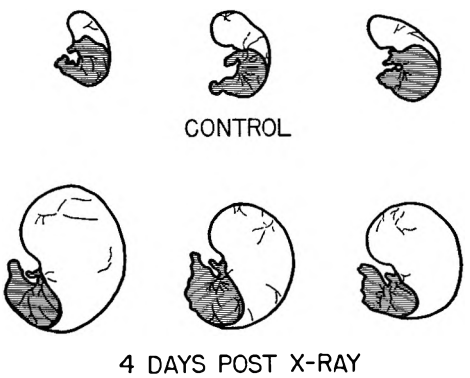

Fig. 3 General size and appearance of the stomachs of tube-fed rats at 18 hours after their last feeding. The cross-hatched area represents the pyloric portion.

In many rats this loss actually exceeded that of the loss in body weight during the first 24 hours; indicating that the total tissue weight actually increased slightly in the initial period. This slight increase may be associated with a change in hydration, since the water intake increased by two- to three-fold during the first day of starvation.

Figure 4 shows the basic differences in the weight changes observed in these two conditions. Several facts stand out. The main weight loss following irradiation is due to a loss of muscle mass. In the starving animal, however, there is no loss of muscle until after the third day, while the main loss of weight results from a decrease in gastrointestinal

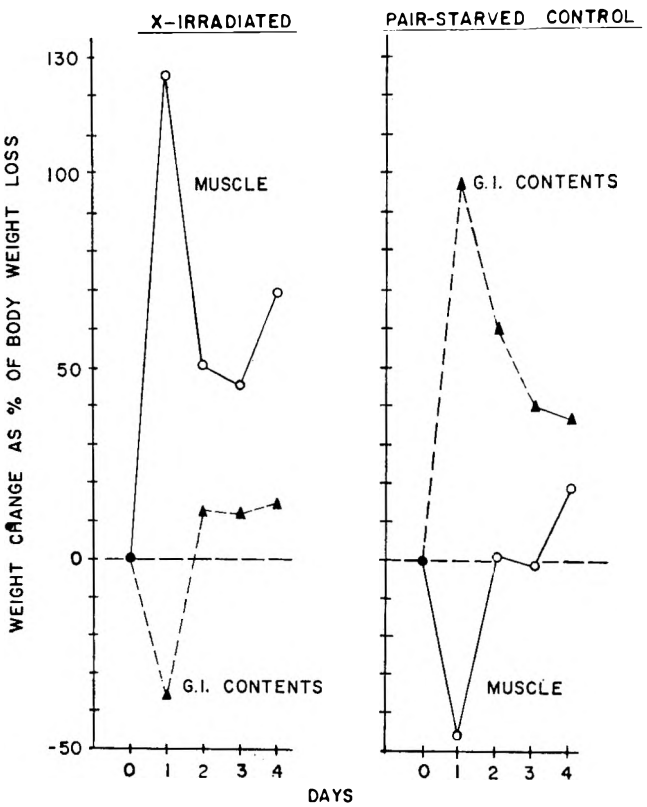

Fig. 4 A comparison of the changes in muscle mass and gastrointestinal content weight in the x-irradiated and in the starved rat.

contents, body fat, and liver weight. In the irradiated rat, fed ab libitum, on the other hand, there is an increase in gastrointestinal content weight but a prompt loss of tissue weight.

Even larger differences between these two conditions can be observed, of course, in the case of the so-called "radiosensitive" tissues, e.g., spleen, thymus and small intestine. These tissues, however, are too small to have much effect upon total body weight.

DISCUSSION

Pair-feeding techniques are frequently used and advocated in the study of radiation effects on the presumption that these

procedures allow one to eliminate the effects of inanition from the other effects of radiation, and thus simplify the interpretation of the data. The difficulties of this approach can best be illustrated by taking a specific example. In electrolyte balance studies (Bowers et al., '53), the irradiated rat has been compared with his pair-starved control. To be exact, the pair-starved control balances were subtracted from the irradiated animal balances, and the data expressed as balance differences. As might be expected from figure 4, during the first stress days the irradiated animal loses substantial amounts of muscle potassium, and the starved animal loses substantial amounts of gastrointestinal potassium. In this comparison, then, one subtracts a muscle potassium loss from a gastric potassium loss, and labels the difference as the potassium loss attributable to "pure" irradiation effects. Quite obviously this use of the pair-fed control can only lead to gross confusion concerning the "pure" metabolic effects of irradiation. Furthermore, the lack of appropriate control data prevents a reinterpretation of the experimental data on any other basis.

All of this raises some very practical problems concerning the conditions under which it may be justifiable to use this pair-feeding technique. Physiological and statistical considerations suggest that the following three conditions should be met: (1) The net effects (total-body potassium losses) of the stress (irradiation) are similar to those observed in starvation, (2) these outward effects are caused by the same metabolic alterations in each case, and (3) there is evidence of the independence of (lack of interaction between) the stress (irradiation) and starvation effects.

Since the starving and irradiated animals are losing equivalent amounts of potassium (Bowers et al., '53), the first condition is satisfied. However, the clear difference in the nature of the internal mechanisms involved points to a failure to meet the second condition. This is not an isolated or unique case. In a number of the situations which have received careful study, the metabolic changes in the irradiated animal were

either uncorrelated or directly opposed to those observed in the pair-starved control. Differences in oxygen uptake (Kirschner et al., '49), nitrogen excretion (Gustafson and Koletsky, '52), liver glycogen level (Ross and Ely, '51; Denson et al., '53), glucose absorption (Goodman, Lewis and Schuck, '52), plasma lipid level (Rosenthal, '49), and tissue weight loss (fig. 4) are examples of this, and point to the basic metabolic differences in these two experimental states.

An alternative approach is to use the normal (fed ad libitum) control as a standard for comparison, and to express the effects of irradiation as deviations from the normal state. This does not commit one to interpreting the data in any predetermined manner, and thus allows one to test alternative hypotheses. If, as in the present case, the inanition hypothesis fails, one is free to search for other possible causes for the observed deviations.

SUMMARY AND CONCLUSIONS

Assumptions implicit in the use of pair-feeding techniques have been discussed, and the logical consequences of using the pair-starved control as a basis for interpreting the potassium loss of radiation sickness have been explored. This type of experimental design assumes that the metabolic effects of starvation are major factors in determining the over-all experimental result. In the example studied this was not true. When this a priori assumption is not fully justified, erroneous conclusions result, and there is a loss of the total data. In many cases, therefore, the non-critical use of pair-feeding techniques may constitute a distinct hazard.

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THE EFFECT OF PROTEIN LEVEL ON THE TRYPTOPHAN REQUIREMENT OF THE GROWING CHICK[1]

PAUL GRIMINGER,[2] H. M. SCOTT AND R. M. FORBES
Illinois Agricultural Experiment Station, Urbana

TWO FIGURES

(Recieved for publication December 13, 1955)

Grau ('48) and Grau and Kamei ('50) demonstrated an increase, at a decreasing rate, of the absolute lysine and sulfur amino acid requirements of the growing chick when the protein level of the diet was increased. Almquist ('49) found that at supernormal protein levels the methionine requirement was a constant percentage of the protein; similar observations were made by Almquist and Merritt ('50) with arginine.

If all essential amino acids, even at supernormal levels, had to be supplied as a constant percentage of the protein, higher levels of proteins deficient in one or more amino acids would only serve to accentuate an imbalance. If there were, however, a decrease in the requirements, expressed as percentage of the protein, with increasing protein levels, it might be possible to overcome moderate amino acid deficiencies by feeding more of the deficient protein. Barnes et al. ('45) have shown with rats that by increasing the level of properly heated soy-flour, deficient in methionine, or wheat gluten, deficient in lysine, above what is generally considered optimum, a level of growth

[1] The experimental data in this paper are taken from a thesis submitted by the senior author to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the Ph.D. degree in Animal Nutrition.

[2] Present address: Department of Poultry Husbandry, University of Nebraska, Lincoln, Nebraska.

could be obtained that compared favorably with the one obtained by feeding egg protein.

The experiments reported in this paper were designed to study the relationship of the tryptophan requirement of the chick to dietary protein levels by feeding graded levels of tryptophan and protein and using weight gain as the criterion.

EXPERIMENTAL

Male chicks, originating from a mating of New Hampshire males to Columbian females were reared in thermostatically controlled, electrically heated batteries equipped with raised wire floors. After 10 days on a pretest diet (diet 0) they were distributed by weight into groups of 5 chicks each, two groups being assigned at random to each treatment. Six levels of tryptophan were fed with each of the 4 experimental diets (diets A, B, C and D) containing 10, 20, 30 and 40% of protein respectively, making a total of 24 treatments. The composition of the diets is shown in table 1. The chicks were kept on the experimental rations for 10 days. Feed and water were kept before them at all times. Individual weights of chicks were recorded every other day, and gain/feed ratios were calculated for each treatment for the total experimental period. No death losses occurred during the experimental period, but two groups of chicks on the 40% protein diet in experiment 2 had to be excluded from further consideration due to a refusal to eat the diet during the first two days. No such problem was encountered with any of the other groups. The experiments were carried out in an air-conditioned room, kept at 78°F. and 40 to 50% relative humidity.

A number of preliminary trials was required in order to devise a diet which would be suitable for these experiments. The main difficulty seemed to lie in the hygroscopic nature of hydrolyzed casein. This difficulty was largely overcome by using dextrin as the carbohydrate. Alfalfa meal, added to provide unidentified factors and improve the texture of the diet, was the only source of tryptophan in the basal diets. The sun-

cured sample used in these experiments was analyzed by the Kjeldahl-Wilfarth-Gunning method for nitrogen and microbiologically for tryptophan, using barium hydroxide hydrolysis and *S. faecalis,* according to a modification of the method of Miller and Ruttinger ('50). The alfalfa sample was found to contain 20% of protein (N × 6.25) and 0.30% of trypto-

TABLE 1

Basal diets used in experiments 1 and 2

INGREDIENTS	DIET 0	DIET A	DIET B	DIET C	DIET D
	%	%	%	%	%
Cerelose	57.92				
Dextrin		76.46	63.84	51.16	38.46
Casein, crude	18.00				
Casein, acid hydrolyzed [1]	2.00	10.90	22.42	34.00	45.60
Gelatin	12.24				
Alfalfa meal, sun-cured	3.00	3.00	3.00	3.00	3.00
Salt mixture [2]	5.34	5.34	5.34	5.34	5.34
Corn oil, refined	1.00	3.00	3.00	3.00	3.00
Choline Cl	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.30	0.10	0.20	0.30	0.40
L-Arginine HCl		0.50	1.00	1.50	2.00
Glycine		0.50	1.00	1.50	2.00
Total [3]	100.00	100.00	100.00	100.00	100.00

[1] HY-CASE, a salt-free product of Sheffield Chemical Company, Inc., Norwich, N. Y.

[2] Fisher et al. ('54a).

[3] Plus the following vitamins (milligrams per kilogram diet): thiamine HCl 100.0; riboflavin 16.0; niacin 100.0; calcium pantothenate 20.0; pyridoxine 6.0; folic acid 4.0; biotin 0.6; vitamin B_{12} 0.02, inositol 100.0; para-aminobenzoic acid 2.0; ascorbic acid 250.0; Menadione 5.0; α-tocopherol acetate 20.0; 10,000 I.U. vitamin A and 600 I.U. vitamin D_3. Procaine penicillin G was added at the level of 15 mg/kg.

phan. The hydrolyzed casein was supplemented with methionine, arginine and glycine so that except for the small amount of protein supplied by the alfalfa meal, the ratio of all amino acids except tryptophan to each other did not differ materially in the 4 experimental diets. Due to the inefficient conversion o orally administered D-tryptophan to the L isomer, reported by Morrison ('55), only the natural isomer was used to supple

ment the basal diets. The high level of nicotinic acid included in the diets (100 mg/kg) should serve to minimize any stress on the tryptophan requirement due to conversion of this amino acid into the vitamin.

In the first experiment not enough points were obtained below what appeared to be the minimum requirement, and the experiment was therefore repeated with differently spaced levels of tryptophan. Although there was a slight difference between the original starting weights of the chicks at 10 days of age in experiments 1 and 2 (114 gm versus 122 gm), analysis of variance of the gains at those levels which were common to both experiments indicated that the weight gain data of the two experiments could be pooled for the purpose of further considerations.

RESULTS AND DISCUSSION

The results of these experiments are shown in table 2. Growth on the 10% protein diet was only about half as good as that on the 20% diet. Thirty percent of protein did not improve growth over the 20% level while the highest level (40%) definitely depressed growth. From inspection of the data it appears that the gain/feed values follow the same trend as the growth data.

In order to obtain an estimate of the minimum tryptophan requirement at the different protein levels, a least squares method was employed. The intersection of an ascending line with a line parallel to the y axis, both fitted to the sum of gains of the chicks in each group, was considered the best estimate of the minimum requirement. The results of these calculations are presented graphically in figures 1 and 2. The minimum requirements found in this way for the 4 protein levels were 0.09, 0.143, 0.182, and 0.20% of tryptophan of the diet, or 0.9, 0.72, 0.61, and 0.5% of the protein in each diet, respectively. These results indicate that in the presence of adequate niacin the absolute dietary requirement of growing chicks for tryptophan increases with higher protein levels, though at a slower rate than the latter. Expressed in terms of

TABLE 2

Effect of protein level on the tryptophan requirement of the chick [1]

DIET	L-TRYPTOPHAN		EXP. 1		EXP. 2	
			Av. gains	gain/feed	Av gains	gain/feed
	% of protein	*% of diet*	*gm*		*gm*	
A (10% protein)	0.6	0.06			28	0.13
	0.7	0.07	52	0.23	40	0.20
	0.8	0.08			50	0.22
	0.9	0.09	64	0.26	58	0.24
	1.0	0.10			60	0.25
	1.1	0.11	66	0.27	62	0.27
	1.3	0.13	52	0.24		
	1.7	0.17	58	0.24		
	2.1	0.21	64	0.27		
B (20% protein)	0.55	0.11			54	0.29
	0.65	0.13	86 (74)[2]	0.38 (0.39)[2]	90	0.42
	0.75	0.15			112	0.49
	0.85	0.17	115 (117)	0.47 (0.48)	110	0.49
	0.95	0.19			107	0.49
	1.05	0.21	108 (116)	0.45 (0.47)	103	0.48
	1.25	0.25	116 (107)	0.48 (0.47)		
	1.45	0.29	117 (112)	0.48 (0.49)		
	1.65	0.33	107 (102)	0.46 (0.42)		
C (30% protein)	0.37	0.11			22	0.18
	0.43	0.13	44	0.29	48	0.32
	0.50	0.15			63	0.38
	0.57	0.17	92	0.45	82	0.45
	0.63	0.19			100	0.51
	0.70	0.21	96	0.48	106	0.52
	0.83	0.25	96	0.46		
	1.03	0.31	98	0.46		
	1.23	0.37	100	0.47		
D (40% protein)	0.32	0.13	32	0.24	28 [3]	0.24
	0.37	0.15			42 [3]	0.33
	0.42	0.17	62	0.39	69 [4]	
	0.47	0.19			70 [3]	0.41
	0.52	0.21	91	0.46	80 [3]	0.46
	0.57	0.23			80 [4]	
	0.62	0.25	82	0.48		
	0.82	0.33	73	0.42		
	1.00	0.40	82	0.45		

[1] Averages of 10 chicks, except if indicated otherwise, during 10-day experimental period.

[2] Controls not receiving penicillin in parentheses.

[3] Averages of 8 chicks.

[4] Averages of 4 chicks.

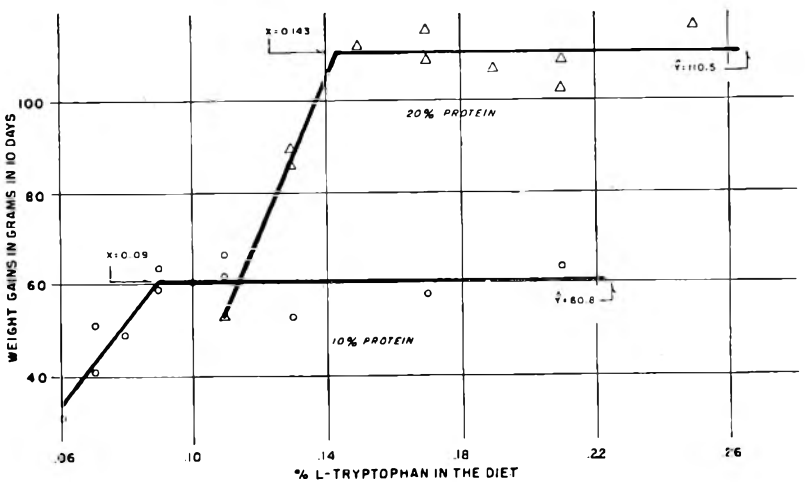

Fig. 1 Effect of graded levels of L-tryptophan on gains during a 10-day experimental period with diets containing 10 and 20% of protein. Each circle (10% protein) or triangle (20% protein) represents average of two pens of 5 chicks each; the 0.29 and 0.33 tryptophan levels on the 20% protein diet were omitted from this graph for technical reasons.

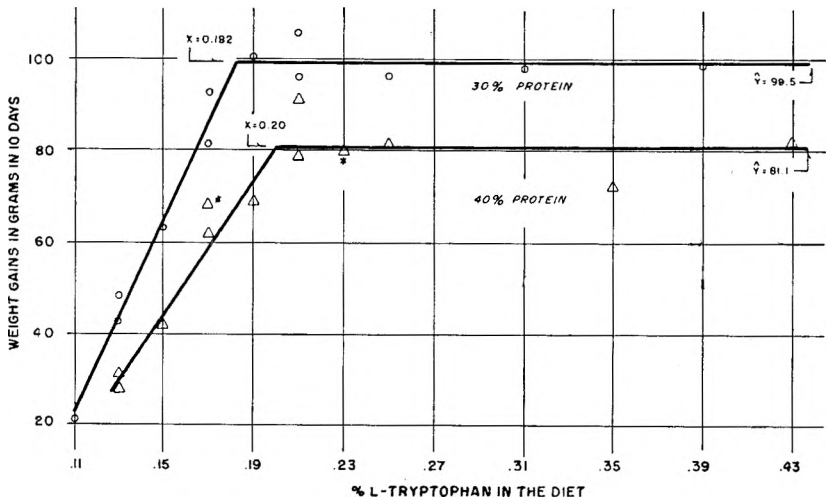

Fig. 2 Effect of graded levels of L-tryptophan on gains during a 10-day experimental period with diets containing 30 and 40% protein. Each circle (30% protein) represents average of two lots of 5 chicks each; each triangle (40% protein) represents two lots of 4 or 5 chicks each; triangles marked with * represent one lot of 4 chicks only.

percentage of dietary protein, the tryptophan requirement appears to decrease at a decreasing rate.

When a maintenance requirement for tryptophan is estimated and subtracted from the figures obtained for the 10 and 20% protein levels, the remaining amounts of tryptophan required for growth parallel the gains, being in both cases approximately twice as large for the 20 as for the 10% level. It is not so simple to explain the increased requirement at the supernormal protein levels, i.e., where an increase of protein did not result in increased growth.

Some consideration has to be given to the occurrence of more efficient feed utilization at protein levels above those required for optimum growth. A greater gain/feed ratio at equal gains is the result of decreased feed intake of all nutrients, including the amino acids. To maintain a certain absolute amount of tryptophan intake, a more efficient diet would therefore have to contain a higher percentage of all nutrients. Most nutrients will be present in sufficient excess to allow for this slight increase, but a nutrient that is limiting and fed at graded levels might conceivably be shown to be required in larger amounts. It seems, however, that this factor could not explain more than a small part of the increased requirement at supernormal levels.

When a protein deficient in an amino acid is fed, the utilization of this protein is limited by the extent of the deficiency. It is possible that the organism in its attempt to remove the excess amino acids also loses a certain amount of the limiting amino acid. A similar situation is created when excess amino acids, due to a supernormal protein level, have to be removed from the body. The increased dietary requirement for the limiting amino acid might be an expression of these losses. It remains to be determined where these losses occur.

Sauberlich and Salmon ('55) have shown with rats that an imbalance of tryptophan created by the addition of protein sources deficient in this amino acid does not result in lowered digestibility and absorption of the protein. They observed an actual increase of amino acids in the urine, including an

increase in tryptophan, the limiting amino acid. This increase alone, however, could not explain quantitatively the increased dietary requirement. It is thus necessary to assume an increased breakdown of tryptophan in the body, probably in the wake of the increased catabolism of amino acids following high protein intake.

Jones and Combs ('51) reported that aureomycin supplementation to a practical type diet suboptimal in tryptophan appeared to spare this amino acid. In order to obtain an indication whether a similar response to antibiotic would occur under the conditions of this study, diet B in experiment 1 was fed with and without penicillin. Analysis of variance of the results of this comparison showed highly significant differences for tryptophan levels, but no significant difference for penicillin, or for the interaction of these two sources of variance. This agrees with the findings of Biely et al. ('52) that antibiotics do not improve growth on a diet low in tryptophan. It must not be overlooked that in the present investigation all chicks received antibiotic during their first 10 days, and that the batteries and quarters were washed and disinfected before the experiment was initiated, a procedure which tends to reduce the antibiotic response (Coates, '53). Should, however, the action of antibiotic be explained only or mainly by the type of environment, there seems to be little reason to believe in the possibility of a tryptophan-sparing action of antibiotics.

The L-tryptophan requirement indicated in these experiments for the 20% protein level, in the presence of adequate niacin, agrees reasonably well with the results of Fisher et al. ('54b) and Morrison ('55) who also used hydrolyzed casein and the natural isomer in their studies. In a study of the relationship of rate of growth of chicks to the tryptophan requirement (Griminger, '55) the latter was calculated at 0.17% of a diet containing approximately 28% protein for both fast- and slow-growing chicks. These results are in good agreement with the present experiment. A number of other investigators have shown that the requirement does not exceed 0.20%, but

little work has been done with graded levels close enough to each other to establish the actual minimum requirement. Also, the use of the racemic mixture with various assumptions concerning the availability to the chick of the D isomer, and the use of higher protein levels might sometimes cause requirements to appear higher than they really are.

In contrast with methionine, considerable excess of dietary tryptophan above the requirement for optimum growth did not seem to exert a growth-depressing effect on the chicks. This is in agreement with observations made by Fisher et al. ('54b) and by other authors.

It is of interest to observe that work done by Salmon ('54) showed that the tryptophan requirement of the rat, with niacin provided at adequate levels, increases with increasing protein levels but at a slower rate. These results closely parallel those obtained in the present investigation with chicks.

SUMMARY

The dietary tryptophan requirement of growing male crossbred chicks has been shown to increase with increasing protein levels, though at a slower rate than the latter. When the diet contained 10, 20, 30 or 40% protein, the minimum requirement for tryptophan was estimated to be 0.09, 0.143, 0.182, and 0.20% of the diet respectively. Thus, a protein causing a slight tryptophan deficiency when incorporated into a diet at the 20% level might conceivably supply sufficient tryptophan for optimum growth when incorporated into the diet at a higher level.

Supplementation with an antibiotic did not appear to have a sparing effect on the requirement for dietary tryptophan.

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VITAMIN B_{12} CONTENT OF MILK AND MILK PRODUCTS AS DETERMINED BY RAT ASSAY

ARTHUR M. HARTMAN, LESLIE P. DRYDEN AND GEORGE H. RIEDEL

Dairy Husbandry Research Branch, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland

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Practically all of the values reported in the literature for the vitamin B_{12} content of cow's milk have been determined by microbiological assay. Moreover, relatively few figures are available for the B_{12} potency of individual milk products as estimated by any method.

The usefulness of animal assays both for comparison with other methods of determination and as some indication of the physiological vitamin value of foods is generally recognized. In the present paper values are given for the vitamin B_{12} potency of milk and some milk products as determined by rat assay. Possible effects of breed and ration of the cows and of various methods of handling the milk are also considered. The method used employed non-hyperthyroid B_{12}-deficient rats (Cary et al., '46; Hartman, '46). The basal ration, containing lactose and casein, was designed especially for determining the vitamin B_{12} content of dairy products. Comparison of this procedure with other rat-growth methods, as applied to milk products, will be considered in another communication.

ASSAY METHOD

The rats used were 28-day-old males weaned from stock colony[1] mothers fed a B_{12}-deficient ration during lactation.

[1] For composition of stock ration, see Hartman et al. ('51).

Throughout the assay, they were housed in individual cages provided with raised screen floors. Weight gain over a 4-week period was used to measure potency. The percentage compositions of ration no. 260, fed during lactation, and of ration no. 262, fed during the assay period, were respectively: dextrin,[2] 30.50, 40.38; lactose (U.S.P.), 15.00, 15.00; casein (10 × hot-alcohol extracted),[3] 20.00, 20.00; yeast (dried brewers',[4] 20.00, 10.00; salts (Hawk and Oser, '31), 4.50, 4.50; cottonseed oil, 9.85, 9.85; fish liver oil,[5] 0.15, 0.15; vitamins,[6] 0.00, 0.12. These rations and distilled water were supplied ad libitum.

Littermates were used in all comparisons within an assay, since litter variance was found to be a highly significant ($P < 0.01$) factor in experiments with B_{12}-deficient rats in this laboratory. Generally 10 litters were started in an assay. The results for approximately 18% of all litters started in these assays were eliminated from the final data for one of the following reasons: death of one or more members of the litter (7.7%); excessive intestinal synthesis of vitamin B_{12}-active material in the litter as indicated by unusual rapid growth of the negative control rat (Hartman et al., '51) (6.5%); failure of a test animal to consume at least 90% of a separately fed supplement of the test food (3.2%); or miscellaneous (0.6%).

Crystalline vitamin B_{12} [7] was used as the reference standard. In assaying fluid milk, the B_{12} and the milk were fed as

[2] "Amidex," a dextrinized corn starch manufactured by the Corn Products Refining Co., New York, N. Y.

[3] For method of preparation, see Hartman et al. ('51).

[4] Strain G, Anheuser-Busch Co., St. Louis, Mo.

[5] "Navitol with Viosterol," E. R. Squibb and Sons, New York, N. Y., containing 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D per gram.

[6] Contains in milligrams per 100 gm of ration: thiamine hydrochloride, 0.5; riboflavin, 0.5; pyridoxine hydrochloride, 0.5; calcium pantothenate, 3.1; choline chloride, 75.0; nicotinic acid, 3.1; inositol, 3.1; para-aminobenzoic acid, 18.8; biotin, 0.006; pteroylglutamic acid, 0.06; ascorbic acid, 3.08; alpha-tocopherol acetate, 6.25; 2-methyl, 1,4-naphthoquinone, 0.16. The pteroylglutamic acid was kindly supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[7] Kindly supplied by Merck and Company, Rahway, N. J.

separate supplements, the B_{12} by syringe and the milk in small glass dishes. For the assay of milk products, the reference B_{12} and the test supplement were incorporated at given levels in the B_{12}-deficient assay ration; the test material replaced equal amounts of protein (casein), lactose, salts and fat. The resulting diets were fed ad libitum.

To ascertain the character of the dose-growth response curve, results obtained with the reference B_{12} were utilized.

TABLE 1

Dose-growth regression lines [1] *with crystalline vitamin* B_{12}

TYPE OF REGRESSION LINE	SERIES 1 B_{12} FED AS SEPARATE DOSES [2]			SERIES 2 B_{12} FED AT GIVEN LEVELS IN RATION [3]		
	Av. slope	Coeff. of variation of av. slope	Combined slope	Av. slope	Coeff. of variation of av. slope	Combined slope
		%			%	
Growth [4]-log dose	47.6	5.6	46.1 [5]	53.6	29.0	.....
Log growth [4]-log dose	0.601	34.6	...	0.706	17.3	0.681

[1] The intercept of the regression line of any given assay was calculated from the internal data of that assay.

[2] Five assays; per assay, 8 to 19 litters, 2 to 4 doses; range of doses over all assays, 0.01 to 0.50 μg/day.

[3] Seven assays; per assay, 7 to 14 litters, 2 to 3 levels; range of levels over all assays, 0.005 to 0.06 μg/10 gm ration.

[4] Growth = av. 4 weeks weight gain of dosed rats minus av. 4 weeks weight gain of negative controls; range of these growths: 9 to 85 gm in the separate dose assays, 11 to 70 gm in the level-in-ration assays; range of av. weight gain of negative controls, 52 to 82 gm.

[5] Standard error: $\pm$ 2.18.

The assays in which the supplements were fed separately from the ration have been designated as series 1. Within individual assays as well as over this whole series (table 1), the results were found to fit quite well a linear regression of weight gain on log dose. Those assays in which the supplements were incorporated at given levels in the ration have been designated as series 2. In contrast to series 1, the data from series 2 fitted better a log growth-log dose linear re-

gression when the growth response was taken as the average difference in weight gain between the dosed rats and their littermate negative controls. The constants of the regression equations were determined by the method of least squares.

Examination of the data of series 1 revealed that both the slopes and the error variances of the 5 assays were statistically homogeneous (Bliss, '51). Therefore, a combined or laboratory slope, i.e., an average of the individual assay slopes each weighted according to the total number of dosed rats in the assay, was computed. This combined slope, together with that of series 2, is recorded in table 1. In series 2, calculations based upon actual intakes of the vitamin rather than upon the levels incorporated in the ration did not alter the type of the regression line or increase the precision of the assays.

Further examination of the results indicated that use of the combined slope would not, with either series, decrease the precision obtainable with individual slopes. Thus with series 1, application of a combined slope, even under conditions simulating the use of only a single dose of the unknown and a single dose of the standard, gave an average deviation of calculated from fed dose of 12% as compared to 20% when individual slopes involving multiple doses of the standard were used. The corresponding values for series 2 were 13 and 20%, respectively.

The combined slopes developed from the data obtained with the vitamin B_{12} standard (table 1) have been used in computing potencies in the assays of milk and milk products. The combined slope for fluid whole milk (5 assays) was found to be not significantly different from that for vitamin B_{12} (5 assays); moreover, the error variances of these two groups of assays were found to be statistically homogeneous. Furthermore, supplements of whole milk fed in addition to maximally effective doses of the reference B_{12} brought about no increase in weight gain over that obtained with the B_{12} alone. Thus the assay method appeared to be specific for the vitamin B_{12} activity of milk. Supplements of such milk products as

crude casein and dried skim milk also failed to give additional growth over that obtained with maximally effective levels of vitamin B_{12}.

In each assay one to three doses or levels of the reference B_{12} and one or two of the unknown were used. Separately fed supplements of the milk and of the vitamin B_{12} were given 4 or 5 times a week but the amounts have been expressed as the quantities received per day for a 6-day week.

RESULTS AND DISCUSSION

Considerable variation has been found (Collins et al., '51, '53) in the vitamin B_{12} level in milk between cows of the same breed and between samples from the same animal taken at different times. Therefore it should be emphasized here that the various lots of milk used in the present work represent herd milk. Moreover, a given lot consisted of a series of samples collected three, 4 or 5 times per week throughout the assay, each sample representing mixed milk from a number of cows in various stages of lactation.[8]

A previous report from this laboratory (Hartman and Dryden, '52) showed that cobalt, added to cow rations already containing amounts of this element adequate for normal health and functioning, failed to increase the vitamin B_{12} content of the milk as determined by the present rat assay method. The results of studies on the possible effects of other factors on the vitamin B_{12} potency of milk are summarized in table 2.

It can be seen (table 2, experiment 1) that the vitamin B_{12} potency of raw Jersey and Holstein milk produced by cows on pasture was not significantly different from that produced by cows fed only barn rations. This result is in accord with findings of preliminary comparative rat growth studies (Hartman et al., '49) carried out before crystalline vitamin B_{12}

[8] These samples were collected from groups of cows in the dairy herd at the Beltsville Agricultural Research Center. The average number of cows per sample ranged from 4 to approximately 60, while the total number of cows used per assay varied from 8 to more than 60.

TABLE 2

Effect of various factors on the vitamin B_{12} content of cow's milk as determined by rat assay

EXP.	NO. OF ASSAYS	AV. NO. OF SAMPLES PER ASSAY	TYPE OF MILK	VITAMIN B_{12} CONTENT [1]
				μg/l
1	1	28	Raw; mixed Jersey and Holstein:	
			Cows on barn feeds [2]	8.0
			Cows on pasture [3]	8.2
2	2	26	Raw; cows on barn feeds or pasture:	
			Jersey	7.1
			Holstein	7.5
3	1	29	Raw; cows on barn feeds; mixed Jersey and Holstein:	
			Fresh	5.8
			1 day old at 0°C.	5.3
			2 days old at 0°C.	5.7
			3 days old at 0°C.	5.1
4	5	23	Cows on barn feeds or pasture; Jersey or Holstein:	
			Raw	7.0
			Pasteurized, holding method [4]	8.5
5	1	18	Cows on barn feeds; mixed Jersey and Holstein:	
			Raw	6.2
			Pasteurized, holding method [4]	9.5
			Pasteurized, flash method [5]	7.2

[1] *F* values for differences: experiments 1–3, treatment mean square less than error mean square, therefore differences not significant; experiment 4, 3.2 (required for 5% significance, 4.0); experiment 5, 1.4 (required for 5% significance, 3.7).

[2] The barn-fed cows used in the experiments received a ration of alfalfa hay, grain mixture, corn silage and dried beet pulp.

[3] The pasture-fed cows used in the experiments received blue grass or mixed orchard grass and ladino clover pasture, a grain mixture, and occasional hay supplements. Milk collections were made in the interval between one and one-half and 4 months after the cows were put on pasture.

[4] 143–154°F. for 30 minutes.

[5] 160–162°F. for 23 seconds.

became available as an assay standard. These earlier studies indicated no marked difference, if any, in the vitamin B_{12} potency of milk from cows on pasture, from cows on barn feeds or from cows which had been for several years on a ration in which no vitamin B_{12} activity could be detected. On the other hand, de Heus and de Man ('51) and van Koetsveld ('53) found the vitamin B_{12} content to be about twice as much early in the pasture feeding period as it was previously during indoor feeding; the former workers also observed a gradual decline to the indoor level. The results obtained in the present studies are not necessarily in conflict with those obtained by the above workers since the cows used here had been on pasture for at least one and a half months before collection of the first sample.

Milk of the Jersey and Holstein breeds was compared in two assays (table 2, experiment 2). In one case, the cows of both breeds were on pasture. In the other, they were on barn feeds. In neither instance was there a significant difference in vitamin B_{12} potency. These findings are at variance with the conclusion of Anthony et al. ('51) that Holstein milk showed a greater concentration of B_{12} than Jersey milk. They are, however, in accord with the observations of the Wisconsin workers (Collins et al., '51) who found no noticeable difference between Jersey, Holstein and Guernsey milk and who came to no contrary conclusion in further studies (Collins et al., '53). Sreenivasamurthy et al. ('50, '53) apparently found no marked differences in the vitamin B_{12} activity of milk from several breeds of Indian cows.

In some of the above assays, portions of samples were, where necessary, stored at about 0°C. for feeding on days intervening between collections. From experiment 3, it can be seen that storage of raw milk in a household-type refrigerator at this temperature for one, two or three days brought about no detectable change in vitamin B_{12} potency.

Direct comparison was made in 5 assays between raw and pasteurized fractions of each sample collected (experiment 4). Although in every assay the pasteurized milk was on the

average slightly higher in B_{12} potency than the raw milk, analysis of variance applied either within individual assays or to the combined data of all 5 showed that this difference was not statistically significant. A similarly conducted comparison between holding and flash pasteurization revealed no significant difference between the two methods of heat treatment. Collins et al. ('53), from average values for raw and pasteurized milk, concluded that the pasteurization process did not appear to inactivate the vitamin B_{12} and cited unpublished results at Wisconsin to the effect that pasteurization does not alter the content of this vitamin in cow's milk.

It thus appears that, provided adequate dietary cobalt is supplied, the vitamin B_{12} content of cow's milk is, for the most part at least, little influenced by the ration. This finding can no doubt be accounted for by the microbiological synthesis of vitamin B_{12} in the rumen (Kon and Porter, '54). Moreover, the B_{12} content does not seem to vary significantly with breed of animal. Ordinary methods of handling milk likewise appear to be without effect. The results obtained in this laboratory by rat assay and those obtained by other workers using microbiological methods both appear to support these conclusions.

The average vitamin B_{12} potency of 10 lots of raw whole milk determined by rat assay in this laboratory was 7.1 ± 0.39 [9] μg/l. The individual lots ranged from 5.5 to 9.4 μg/l. A number of values for raw whole mixed or herd milk determined by other methods of assay have been reported in the literature. Using an assay employing the hyperthyroid rat, Lewis et al. ('49) reported only a trace of vitamin B_{12} in this product. With *L. lactis* Dörner as the test organism, Tastaldi ('50) and Sreenivasamurthy et al. ('50) found average values of 1.8 and 1.4 μg/l respectively. The latter authors, however, subsequently (Sreenivasamurthy et al., '53) found a higher B_{12} content (average about 5.8 μg/l [10]) when they treated their samples with cyanide. Employing three differ-

[9] Standard error.

[10] Calculated from data given by the authors.

ent organisms, *B. coli*, *Ochromonas malhamensis* and *L. leichmannii* ATCC 4797, Gregory ('54) obtained approximately the same results (1.8, 2.3 and 2.9 μg/l, respectively) for the vitamin B_{12} content of a single sample of milk. Most of the literature values have been determined with *L. leichmannii*

TABLE 3

Vitamin B_{12} content of certain milk products

PRODUCT	NO. OF SAMPLES	NO. OF BRANDS	TYPE OF ASSAY	VITAMIN B_{12} CONTENT	REFERENCE
				μg/kg	
Dried whole milk	2	2	Normal rat	36,39	Present studies
	1		Hyperthyroid rat	25	Lewis et al. ('49)
	1?		*L. leichmannii*	20	Elvehjem ('50)
Dried skim milk	3	2	Normal rat	37,39,42	Present studies
	1		Chick	60	Lillie et al. ('54)
	1 [1]		*L. leichmannii* 4797	30	Lillie et al. ('54)
	1?		*L. leichmannii* 4797	30	de Heus and de Man ('51)
Casein, crude	2	1	Normal rat	64,70	Present studies
	1		Hyperthyroid rat	30	Lewis et al. ('49)
	2?		*L. leichmannii*	30,70	Elvehjem ('50)
	11		*L. leichmannii* 4797	90 [2]	de Heus and de Man ('51)
	1?		*L. leichmannii* 4797	104	Peeler et al. ('51)
Dried whey	2 [3]	2	Normal rat	11,33	Present studies
	3		Chick	20,30,30	Lillie et al. ('54)
	2 [3]		*L. leichmannii* 4797	10,30	Lillie et al. ('54)

[1] Same sample as assayed with the chick.

[2] According to the authors, calculated from average value for casein fraction prepared from fluid whole milk.

[3] From Cheddar cheese.

[4] Same samples which yielded 30 and 20, respectively, by chick assay.

ATCC 4797. The following average figures have been obtained (μg/l): 5.6, 5.9 and 7.6 (Anthony et al., '51); 3.1,[10] 3.4, 4.1 [10] and 6.6 (Collins et al., '51, '53); 3 (Ford et al., '53); 4 (Gregory et al., '52); 3.8 (de Heus and de Man, '51); 4.3 [10] (Karlin, '54); 6.2 (van Koetsveld, '53); 1.8, 1.7 and 4.1 (Rusoff and Haq, '54). Although comparative data are not presented,

Collins et al. ('51) state that the values observed using *L. leichmannii* agreed well with the value for cow's milk obtained by an assay using the hyperthyroid rat. It can be seen from the above data that the average value for the vitamin B_{12} potency of raw cow's milk obtained in this laboratory by assay with the normal rat is of the order of the highest values obtained by other workers using microbiological methods.

The milk products, except for one lot of dried whole milk,[11] were obtained from commercial sources. Values obtained for the vitamin B_{12} potency of these products together with literature values for the same materials are shown in table 3.

SUMMARY

A vitamin B_{12} assay method using growth of the depleted normal rat as the criterion of potency has been described and utilized to assay milk and milk products. Assays were made of Jersey and Holstein milk collected under different conditions of feeding and subjected to different conditions of handling.

No differences in potency were observed between the two breeds or between barn-fed and pasture-fed cows. The average vitamin B_{12} content of 10 lots of raw whole milk was found to be 7.1 μg/l. This figure corresponds to the highest values obtained by other workers using microbiological methods of assay.

Neither pasteurization by the holding or flash methods nor storage of raw milk at 0°C. for as much as three days had a significant effect on the B_{12} potency.

Values are given for the vitamin B_{12} content of a few samples of several milk products.

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COMPARATIVE ASSAY FOR VITAMIN B_{12} IN CERTAIN MILK PRODUCTS BY VARIOUS RAT GROWTH METHODS

LESLIE P. DRYDEN, GEORGE H. RIEDEL
AND ARTHUR M. HARTMAN
Dairy Husbandry Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

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Rat growth assay methods for the determination of the vitamin B_{12} potency of natural materials involve the use of either the normal rat or the hyperthyroid rat. In the first instance, weanling young, which have been depleted by feeding their mothers a B_{12}-deficient diet, are themselves fed a similar diet during the assay period (Cary et al., '46; Hartman, '46; Zucker et al., '48, '50; Cuthbertson and Thornton, '52; Sherman et al., '55; Hartman et al., '56). In the second type of assay, stock colony young are made B_{12}-deficient subsequent to weaning by inclusion of a thyroactive substance in a B_{12}-deficient ration and are then continued on the same ration during the assay period (Register et al., '49a, b; Emerson, '49; Frost et al., '49, '53; Tappan et al., '50; Cheng and Thomas, '51; Scheid et al., '52).

Assay methods also vary in the manner of substitution of the test material in the assay ration and in the composition of the assay ration itself in respects other than the presence or absence of thyroactive material. The more important of such variations in the assay ration concern the sources of carbohydrate and of protein. The protein may be furnished by animal protein in the form of casein or by plant protein from such sources as soybean meal, corn meal, cottonseed meal, or the like.

Comparison of vitamin B_{12} potencies of dried whole milk and crude casein obtained by assay with a normal rat method (Hartman et al., '56) with those obtained by other workers (Lewis et al., '49) by use of the hyperthyroid rat suggested that these two assay methods might not be yielding comparable results with these substances. In the present paper, these two types of assays are considered as applied to the determination of the vitamin B_{12} content of certain milk products. Modifications of these methods have also been tried in an effort to determine whether the apparent conflict in the results obtained was due to some of the points of difference between the methods used other than the presence or absence of thyroactive material.

EXPERIMENTAL PROCEDURE

Except as otherwise indicated, the test animals consisted of 28-day-old male young weaned from stock colony mothers fed purified rations during lactation. All comparisons within an experiment or assay were made between littermates.

The customary assay procedure used in this laboratory has been described in a previous paper (Hartman et al., '56). Rations 260 and 262, used in the assays made by our regular method, contained hot-alcohol-extracted casein as the source of protein and dextrin and lactose as the sources of carbohydrate. Several different plant protein rations were used. The soy protein ration used in experiments in tables 1 and 2 had the following percentage composition: sucrose, 46.84; soy protein,[1] 38.09; DL-methionine, 0.20; salt mixture (Hawk and Oser, '31), 4.50; cottonseed oil, 9.85; fish liver oil,[2] 0.15; and added vitamins,[3] 0.37. The cottonseed meal ration used in experiments in table 1 had the following percentage compo-

[1] Soybean Protein no. 220, The Drackett Co., Cincinnati, Ohio.

[2] Containing, per gram, 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

[3] Contains in milligrams per 100 gm of ration: thiamine hydrochloride, 1.6; riboflavin, 1.6; pyridoxine hydrochloride, 1.6 calcium pantothenate, 10.0; choline chloride, 240.0; nicotinic acid, 10.0; inositol, 10.0; para-aminobenzoic acid, 60.0; biotin, 0.02; folic acid, 0.20; ascorbic acid, 10.0; alpha-tocopherol acetate, 20.0; 2-methyl-1, 4-naphthoquinone, 0.5. The folic acid was kindly furnished by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

sition: sucrose, 19.12; cottonseed meal,[4] 69.46; DL-methionine, 0.40; lysine, 0.40; salt mixture,[5] 2.95; cottonseed oil, 7.19; fish liver oil,[2] 0.11; and added vitamins,[3] 0.37. The corn-soy ration used in experiments in table 2 and for some of the assays in table 3 had the following percentage composition: yellow corn meal, 42.42; soybean meal, 42.42; DL-methionine, 0.30; salt mixture (Hawk and Oser, '31), 4.50; cottonseed oil, 9.85; fish liver oil,[2] 0.15; and added vitamins,[3] 0.37. Modifications of these basal rations are indicated in the appropriate places in the paper.

Vitamin B_{12}[6] and iodinated casein,[7] where fed, were incorporated in the basal rations in the amounts indicated in the tables.

RESULTS AND DISCUSSION

Tests of the suitability of certain assay rations for measuring the vitamin B_{12} activity of milk products

Before carrying out comparative assays of the vitamin B_{12} activity in milk products, preliminary experiments were run to determine whether rats fed the various rations under consideration would respond only to this vitamin. It seemed possible that the assay rations might be deficient in unidentified nutrients contained in the test substances or, on the other hand, that some component of the test materials might exert a depressing effect on growth. As an example of the latter effect, Ott ('51) found that substituting dried whey or vitamin-free casein for cerelose in a B_{12}-assay ration for chicks led to a

[4] Screw-pressed meal of low free gossypol content; kindly furnished by the Engineering and Development Division, Southern Regional Research Laboratory, U. S. Department of Agriculture, New Orleans, La.

[5] Hawk and Oser, '31, modified to allow for the ash content of cottonseed meal. Such modification consisted of removing the potassium phosphate, magnesium carbonate, and magnesium sulphate, decreasing by one half the relative amounts of potassium chloride and calcium carbonate and increasing by about 64% the sodium chloride.

[6] Kindly supplied by Merck and Co., Inc., Rahway, N. J.

[7] Kindly supplied by Cerophyl Laboratories, Inc., Kansas City, Mo.

growth depression which could be at least partially overcome by adding vitamin B_{12} to the ration, although the data presented did not indicate whether it was possible to attain maximal growth under these conditions.

A number of B_{12}-active substances, when fed in conjunction with or incorporated in the animal protein assay ration customarily used in this laboratory (no. 262), had no significant effect on growth under conditions where a maximally effective amount of vitamin B_{12} was already included in the ration. Among such substances were liver extract, whole liver powder, whole milk (5 ml/day) and Cheddar cheese (2 gm/day). On the other hand, dried skim milk, when substituted in this ration for dextrin, tended to have a slightly depressing effect on growth (experiment 1, table 1). Increasing the amount of vitamin B_{12} in the ration or substituting the skim milk for equivalent amounts of lactose, salts and B_{12}-deficient casein instead of dextrin appeared to counteract this tendency. The depression was likewise observed when thyroprotein was included in the ration (experiment 2) and in this instance, it was intensified rather than alleviated by increasing the level of B_{12} in the ration. Substitution of the skim milk for equivalent amounts of its components again appeared to prevent the depression. Crude casein, when substituted for B_{12}-deficient casein, had no effect on growth (experiment 3).

Somewhat similar experiments were carried out with plant protein rations containing maximally effective amounts of vitamin B_{12}. Two basal rations were used. The protein in one of these rations was supplied by a purified plant product (soy protein) while in the other, it was supplied by a crude source (cottonseed meal).

With the soy protein rations, the inclusion of dried skim milk at a 25% level produced a significant depression even though the dried milk was substituted for equivalent components (experiment 4). When thyroprotein was included, this depression became greatly intensified (experiment 5).

A similar tendency was found with the cottonseed meal rations when either dried whole milk (experiment 6) or crude

TABLE 1

Effect of incorporating certain milk products [1] into rations containing a maximally effective amount of vitamin B_{12}

EXP.	NO. OF LITTERS	IODINATED CASEIN	VITAMIN B_{12}	AVERAGE WEIGHT GAIN IN FOUR WEEKS			F [4]	Least signif. diff.
				Without milk product	With milk product			
					Not adjusted [2]	Adjusted [3]		
		%	*μg/10 gm ration*	*gm*	*gm*	*gm*		*gm*
Basal ration containing alcohol-extracted casein:								
Dried skim milk:								
			0.5	165	157	165		
1	20	0.00	1.5	164	165	...	1.2	...
			0.5	121	114	121		
2	6	0.15	1.5	134	110	...	3.9*	14.1
Crude casein:								
3	6	0.00	0.5	165	...	164	... [5]	...
Basal ration containing soy protein [6,7]:								
Dried skim milk:								
4	16	0.00	1.0	195	...	184	4.6*	10.8
5	4	0.20	1.0	160	...	122	15.1*	30.3
Basal ration containing cottonseed meal [7,8]:								
Dried skim milk:								
6 [9]	8	0.00	1.0	145	138	...	1.3	...
7	7	0.25	2.0	161	135	...	17.4**	15.0
Crude casein:								
8	7	0.00	2.0	167	154	...	2.9	...
9	7	0.25	2.0	161	143	...	22.2**	9.1

[1] Fed at level of 10% in experiments 1, 2, 3, 6 and 7, 19% in exprirments 8 and 9, and 25% in experiments 4 and 5.

[2] Replaced an equal amount of carbohydrate (dextrin in experiments 1 and 2), except in experiment 6, where it replaced an equal amount of the whole ration.

[3] Replaced an equivalent amount of carbohydrate (lactose in experiments 1 and 2), salts, fat and B_{12}-deficient casein.

[4] The symbol ** adjacent to or in connection with a F value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference.

[5] Treatment mean square less than error mean square; therefore not significant.

[6] Modified to contain half the quantity of added vitamins. Half the litters in experiment 4 received rations containing 1% sulfasuxidine.

[7] Mothers of experimental rats were fed similar rations during lactation. All mothers received 1 μg vitamin B_{12} per 10 gm ration.

[8] In experiment 6, the ration was modified at the expense of sucrose to contain 80.86% cottonseed meal and 6.7% cottonseed oil.

[9] Dried whole milk fed instead of dried skim milk.

casein (experiment 8) was incorporated at somewhat lower levels in the ration without adjustment of the ration for the components of the test products. Again the incorporation of iodinated casein led to a significant depression with dried skim milk (experiment 7) or crude casein (experiment 9).

Thus with plant protein rations also, the inclusion of certain milk products in addition to a maximally effective amount of vitamin B_{12} led under certain conditions to a growth depression which could be intensified by the addition of thyroprotein. However, as shown in table 2, when a shorter assay period of three weeks was used, a tendency towards depressed growth on plant protein rations containing thyroprotein did not always occur (experiments 3 and 7) when milk products were added to rations containing ample vitamin B_{12}, even though these rations were not adjusted for the components of the milk product. This does not, of course, preclude the operation of such growth-depressing factors under assay conditions where suboptimal amounts of vitamin B_{12} are fed.

The experiments in table 2 show the effect of adding certain milk products to plant protein rations in the absence of vitamin B_{12}. Again two types of rations were used — a purified ration containing soy protein and a cruder ration containing corn meal and soybean meal.

Iodinated casein was included in all except one of these experiments. With some rations containing thyroprotein, the rat has been shown to respond to unidentified nutrients contained in crude food substances (Ershoff, '49a; Petheil and Lardy, '49) and this response has been shown to interfere with the assay for vitamin B_{12} (Lewis et al., '50). This has been more frequently the case with casein-sucrose rations than with plant protein rations, since such vegetable products as soybean meal, cottonseed meal, corn meal and even soy protein contain at least some of these unidentified nutrients (Ershoff, '49b; Tappan et al., '53a; Dryden et al., '56 [8]).

In experiment 1, the addition of dried skim milk to the B_{12}-deficient soy protein ration failed to give a positive response,

[8] Dryden, L. P., G. H. Riedel and A. M. Hartman, Unpublished data.

even though the lot of skim milk powder which was used had been found by our regular assay method to contain considerable vitamin B_{12}. Part of the failure of the rats to respond with increased growth was evidently due to the lactose content of the skim milk powder (experiment 3). In three experiments, B_{12}-active crude casein gave somewhat variable re-

TABLE 2

Effect of incorporating certain milk products [1] into B_{12}-deficient plant protein rations

EXP.	NO. OF LITTERS	TYPE OF BASAL RATION [2]	AVERAGE WEIGHT GAIN IN THREE WEEKS					
			No vitamin B_{12}		Vitamin B_{12} [3]		F [4]	Least signif. diff.
			Without milk product	With milk product	Without milk product	With milk product		
			gm	*gm*	*gm*	*gm*		*gm*
Dried skim milk:								
1	5	Soy protein	54	54	77	73	6.0**	15.4
2	8	Corn-soy	41	61	93	86	25.4**	13.5
Lactose:								
3	8	Soy protein	66	44	91	94	34.9**	11.8
Crude casein:								
4	8	Soy protein	60	68	104	101	33.7**	11.5
5	6	Soy protein [5]	65	74	114	106	12.0**	20.7
6	8	Soy protein	65,54 [6],56 [7]	83	...	...	19.1**	9.1
7	6	Corn-soy	60	77	77	84	4.2*	15.0
8	7	Corn-soy	86	113	121	122	12.0**	14.2

[1] Ten per cent included in ration. With soy protein rations, replaced an equal amount of carbohydrate; with corn-soy rations, replaced 5% yellow corn meal and 5% soybean meal.

[2] Soy protein ration modified here to contain 53.23% sucrose and 31.70% soy protein. The rations in experiment 8 contained no iodinated casein, whereas all the others contained 0.15% of this product during the exprimental period. Mothers of experimental rats were fed a B_{12}-deficient purified casein-sucrose ration during lactation.

[3] One microgram in 10 gm ration.

[4] See footnote 4, table 1.

[5] Ration modified to contain dextrin in place of sucrose.

[6] Sufficient additional soy protein substituted for an equal amount of sucrose so that protein content of ration was same as that of test ration containing crude casein.

[7] B_{12}-deficient extracted casein and sucrose substituted for soy protein. Protein content (25%) maintained the same as in soy protein basal ration.

sults — a rather poor response in experiments 4 and 5 and a somewhat greater response in experiment 6. At least part of the low response may be attributed to the higher protein level of the crude casein ration. As shown in experiment 6, raising the protein level of the basal ration to that of the crude casein test ration, either with an increased amount of soy protein or with B_{12}-deficient extracted casein, led to depressed growth of the negative controls and thus to a relatively greater response with the crude casein. Although Lewis et al. ('50) found that the substitution of dextrin for sucrose in a thyroactive casein ration led to an increased growth response to vitamin B_{12}, in this instance using dextrin as the primary carbohydrate source in place of sucrose did not appear to change the nature of the results (experiment 5).

When the corn-soy ration, either with or without iodinated casein, was used (experiments 2, 7, 8), a much better response was obtained. It is evident that B_{12}-active milk products elicited a greater growth response when added to this corn-soy ration than when included in a more purified plant protein ration. It is likewise apparent that the growth-depressing effects noted above upon the addition of these milk products were not pronounced, if present at all, with this ration. This corn-soy ration, moreover, is closely similar in composition to a ration which has been commonly used for the assay of vitamin B_{12} in natural food materials. Thus for comparative assays between the rat assay method regularly used in this laboratory (Hartman et al., '56) and a hyperthyroid rat assay method, it was decided to use the corn-soy ration for the hyperthyroid rat.

Results of comparative assays of dried skim milk and of crude casein

For these assays, one commercial lot of dried skim milk and one commercial lot of crude casein were used throughout. Variations of each type of assay were made in order to test the influence of the various points of difference in the two methods. Such factors included the use of animal or vegetable

protein, the presence or absence of iodinated casein, the manner of substitution of the test products in the basal ration and the pre-treatment of assay animals. The results of the assays are given in table 3.

TABLE 3

Vitamin B_{12} potency of one lot of dried skim milk and one lot of crude casein, as determined by various methods of rat growth assay

GROUP	TYPE OF RATION FED	IODINATED CASEIN	DRIED SKIM MILK			CRUDE CASEIN		
			Assay no.	No. of litters	Vit. B_{12} potency	Assay no.	No. of litters	Vit. B_{12} potency
		%			$\mu g/kg$			$\mu g/kg$
1	Alcohol-extracted casein basal ration (no. 262):							
	Not adjusted [1]	0.00	1	7	5	12	10	38
	Adjusted [2]	0.00	2	7	38	13	10	71,57 [3]
		0.00	3	9	44	..	..	..
		0.04	4	6	51	14	6	53
		0.04	5	8	38	..	..	..
2	Corn-soy basal ration:							
	Not adjusted [4]	0.05	6 [5]	10	0	15 [5]	10	37
		0.05	7	9	14	16	9	49
		0.15	8	9	10	17	7	36
		0.15	9	5	5	..	..	..
	Adjusted [6]	0.05	10	9	27	18	10	77
		0.05	..	..	..	19	9	61
		0.15	11	3	12			

[1] Milk product replaced an equal amount of dextrin.

[2] Skim milk replaced an equivalent amount of lactose, salts and B_{12}-deficient casein. Crude casein replaced an equal amount of B_{12}-deficient casein.

[3] Values for two different levels.

[4] Half of milk product replaced an equal amount of corn; half replaced an equal amount of soybean meal.

[5] Mothers continued on stock ration after parturition; weanling young depleted of B_{12} by feeding them the corn-soy basal ration (with iodinated casein) for two weeks before they were placed on assay. In all other assays, assay rats were weanling young from mothers placed at parturition on a non-thyroactive B_{12}-deficient ration (no. 260 in group 1 and a purified casein-sucrose ration in group 2).

[6] In these assays, the basal assay ration fed to the negative controls and to the rats administered the reference standard B_{12} was modified as follows: in assays 10 and 11, to contain lactose, salts and B_{12}-deficient casein equivalent to that in the test ration; in assay 18, to contain an amount of B_{12}-deficient casein equivalent to the amount of crude casein in the test ration; in assay 19, by adjusting the proportions of corn and soybean meal, to have the same protein content as the test ration.

Most of these assays were carried out with rats that had been depleted of their vitamin B_{12} reserves by feeding their mothers a B_{12}-deficient diet during the lactation period. The assay rations were fed to the weanling young for a total period of 4 weeks when the basal ration that contained animal protein was used and for three weeks when the basal ration that contained vegetable protein was employed. One assay each, however, was carried out with dried skim milk (assay No. 6) and crude casein (assay No. 15) by use of a method of depletion similar to that used by many other workers, that is, by feeding stock weanling young the thyroactive basal ration for two weeks beyond weaning. An assay period of two weeks was used in this instance. With crude casein, the two methods of depletion gave essentially the same assay values. With dried skim milk, however, no activity was indicated by the latter method, whereas by the former method, at least some activity was found in all cases.

The effect of iodinated casein *per se* was tested with the animal protein assay ration. Its presence or absence appeared to have no effect on assay values, either with dried skim milk or with crude casein. With the vegetable protein assay ration, two different levels of iodinated casein were tried. The higher level gave somewhat lower assay values with the dried skim milk but made little, if any, difference with the crude casein.

Comparing the animal protein basal ration (group 1) and vegetable protein ration (group 2) directly, the assay values obtained with crude casein were essentially the same. With dried skim milk, however, when the rations were adjusted for the components of the milk, the animal protein ration still gave somewhat higher values for B_{12} potency of the skim milk than did the vegetable protein basal ration. Whether this difference in assay values was caused by a difference between animal and vegetable protein as such or to some other difference in the components or in the action of the two rations cannot be definitely stated.

Of all the factors tested, the most important seemed to be the method of substitution of the test products in the basal ration. Thus in every instance, higher assay values were obtained both for dried skim milk and for crude casein when the assay rations were adjusted for the components of the test product than when substitution was made for dextrin or for corn meal and soybean meal. With the animal protein assay ration, such adjustment was easily accomplished with the test ration, since the basal ration already contained lactose and casein. With the vegetable protein ration, it was necessary to adjust the basal ration itself (including, obviously, the rations containing the B_{12} standard) rather than the test ration. In the case of crude casein, adjustment was made in one case (assay no. 18) by including an equal amount of B_{12}-deficient casein in the basal ration and in the other case (assay no. 19) by equating the protein level of both basal and test rations by adjustment of the proportions of corn and soybean meal. The lowered assay values observed when such adjustments were not made can no doubt be attributed primarily, if not altogether, to the depressing effect of lactose or an increased protein level or both upon growth of rats fed B_{12}-deficient diets (Hartman et al., '49a, b). It may be pointed out that in the hyperthyroid rat assay method whereby certain literature values for the B_{12}-content of milk products were obtained (Lewis et al., '49), substitution of the test product was made by a method not very different from the one used here that gave low assay values.

Thus in these comparative assays, essentially the same values were obtained with crude casein in all cases when the rations were adjusted so as to all have the same protein level. With dried skim milk, on the other hand, assay values were affected not only by the method of substitution of the milk but also by the type of ration used, by the method of depletion of the young and by the amount, although not by the presence, of iodinated casein in the ration.

SUMMARY

Factors affecting the values obtained by various rat growth assay methods for vitamin B_{12} have been studied.

In the presence of maximally effective levels of vitamin B_{12}, certain milk products frequently tended to give depressed growth whether included in casein or plant protein rations. This depression was intensified by including iodinated casein in the ration. It could be alleviated in some instances by increasing the level of B_{12} in the ration or by altering the method of substitution of the test material in the ration.

With plant protein rations containing soy protein and deficient in vitamin B_{12}, either a low response or none at all was observed when dried skim milk or crude casein was included in the ration. These results were at least partially attributable to the lactose or protein content of these milk products or to both. With corn-soy rations, a better response was obtained.

Comparative assays of one commercial lot each of dried skim milk and crude casein were made using extracted casein and corn-soy assay rations. With crude casein, essentially the same assay values were obtained in all cases when the rations were adjusted to have the same protein level. With dried skim milk assay values were affected not only by the method of substitution of the milk but also by the type of ration used, the method of depletion of the young and the amount, although not the presence, of iodinated casein in the ration.

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NUTRITIONAL STUDIES WITH THE GUINEA PIG

IV. FOLIC ACID

MARY ELIZABETH REID, MARY G. MARTIN
AND GEORGE M. BRIGGS

Laboratory of Biochemistry and Nutrition, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland

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Folic acid was shown to be a dietary essential for the guinea pig by Woolley and Sprince ('45), a finding which has since been confirmed by other workers (Mannering, '49; Woodruff, Clark and Bridgeforth, '53; Reid, '53). A marked folic acid deficiency, indicated not only by growth failure and short survival time but also by profoundly changed values for erythrocytes, hemoglobin, hematocrit, and total leucocytes, was produced (Reid, '54) in very young animals without the use of an antimetabolite. Presumably the animals employed in the studies of Woolley and Sprince were also quite young. Woodruff, Clark and Bridgeforth ('53), using 8-week-old animals weighing 250 to 300 gm, produced a deficiency as evidenced by a disturbed blood picture either by the omission of para-aminobenzoic acid (PABA) from a purified diet deficient in folic acid or by the inclusion of 1% sulfasuxidine with or without PABA. Wichmann, Salminen and Roine ('54), using a diet containing PABA, concluded that a dietary supply of folic acid was not essential because intestinal synthesis was thought to be sufficient to satisfy the folic acid requirement. Their animals had an average weight of 198 gm at the start and presumably were about three weeks old. The variations in the findings of these investigators have probably resulted from both the differences in age at which

the animals were placed on the experimental diets and the presence (Woodruff et al., '53; Wichmann et al., '54) or absence (Woolley and Sprince, '45; Reid, '53) of PABA in the diets.

The present study is concerned with (1) the guinea pig's requirement of folic acid for growth and survival, (2) the blood values of animals reared on different dietary levels of folic acid, (3) the rate of onset of folic acid deficiency symptoms in the blood, (4) the interrelations between folic acid and PABA on growth and survival, and (5) the effect of variations in the ascorbic acid level in influencing the response to different dietary levels of folic acid.

METHODS

Equal numbers of male and female guinea pigs of the Hartley strain [1] with a range in weight of 95 to 115 gm were placed on the experimental diets at two to 5 days of age. In the last experiment, however, the starting age ranged from two to 8 days. Except when otherwise indicated, 8 animals were used in each experimental group. The basal diet (diet 13) and the procedures for the care of the animals have been previously described (Reid and Briggs, '53). The ration consisted in percentage amounts of the following: casein 30, corn oil 7.3, sucrose 10.3, cellophane spangles 15, corn starch 20, cerelose 7.8, potassium acetate 2.5, magnesium oxide 0.5, salts (Briggs et al., '52) 6, and 0.2% each of choline chloride, ascorbic acid, and inositol, and, with the exception of folic acid, liberal amounts of the known vitamins but no added PABA. In different experiments folic acid [2] was added in the amounts indicated. The diets were refrigerated until used. No greens of any kind were fed. A few extra animals were placed on the deficient diets in each experiment for use as substitutes for animals which failed to make a start on the experimental regime. Such substitutions were made

[1] In one experiment animals of the Beltsville strain were used.

[2] Pteroylglutamic acid obtained from Lederle Laboratories, Pearl River, New York.

before the 5th day on the diets. Between the 5th and 21st day an occasional animal (an average of two out of 100) succumbed for reasons other than the type of diet (e.g., broken leg, protruding intestine) This accounts for some of the groups reported in the tables having less than 6 or 8 animals.

For the collection of blood samples a modification of the method of Vallejo-Freire ('51) was used. A foot was held in warm water (45 to 47°C.) for 20 seconds to dilate the vessels and promote the blood flow after which a cut was made in the soft tissue near the insertion of the nail. Bleeding was stopped by tying off the toe above the cut.

RESULTS

Growth and survival with different dietary levels of folic acid

The average weights [3] at successive periods and the number of survivors, with levels of folic acid ranging from none to 2000 mg/kg of diet, are shown in table 1. With no folic acid in the diet there were no survivors in some experiments, whereas in others there were one or two at the end of the 6-week experimental period. With 1 mg of the vitamin per kilogram of diet, one-fourth of the animals survived. With 2 or 3 mg, survival was much improved but maximum growth was not attained. With 6 mg of folic acid growth was maximal and all of the animals survived. Growth at the 10 and 15 mg levels was not greater than at 6 mg. One animal in the 15 mg series died after a short illness, apparently of pneumonia. Since the tests with the 2000 mg level were made with the Beltsville strain of guinea pig, a somewhat smaller type than the Hartley strain, the growth rate of this group is not directly comparable with that of the Hartley strain. However, the average weights of the Beltsville group at the 2000 mg level slightly exceeded, though not significantly so, those of the

[3] Since the variations in weight from test to test were no larger than the variations among animals on the same diet on the same test the results from all tests on the same diet were pooled.

10 mg level with the Beltsville animals in the same experiment (data not given).

Symptoms of folic acid deficiency

In the absence of dietary folic acid growth tended to be somewhat retarded by the end of the second week. Thereafter some of the animals continued to gain slowly whereas others remained stationary for a time, then lost weight rapidly.

TABLE 1

Growth and survival with different dietary levels of folic acid

FOLIC ACID	NO. OF ANIMALS	AVERAGE WEIGHTS AT SUCCESSIVE PERIODS [1]			
		Weeks 2	4	6	
mg/kg					
none	40	143 (37)[2]	177 (19)	223 (4)	± 15
0.22	8	154 (8)	185 (3)	302 (1)	
0.66	8	158 (8)	158 (3)	...	
1.00	24	141 (24)	184 (18)	276 (6)	± 19
1.50	8	159 (8)	200 (6)	264 (5)	± 12
2.00	16	153 (16)	224 (16)	284 (15)	± 18
3.00	30	159 (30)	241 (29)	311 (28)	± 7
6.00	38	165 (38)	263 (38)	337 (38)	± 8
10.00	111	160 (111)	269 (111)	339 (111)	± 8
15.00	14	171 (14)	251 (13)	324 (13)	± 9
2000.00	8 [3]	141 (8)	217 (8)	297 (8)	± 13

[1] Initial weights: 95 to 115 gm.

[2] Number of survivors at end of period given within parentheses.

[3] Different (lighter weight) strain (Beltsville) of guinea pigs.

As the deficiency progressed, the animals became less active, lost appetite, became weak, tended to develop diarrhea and salivation was profuse. Low food containers were supplied so that the food could be reached with a minimum of effort. The terminal stage of the deficiency was much like that described by Woolley and Sprince ('45); i.e., there was "a convulsion in which the animal would fall on its side and twitch its head and legs spasmodically." At autopsy, a tendency to fatty infiltration of the liver and adrenal hemorrhages was observed. An almost completely aplastic condition of the

bone marrow was detected in histological studies by Dr. G. L. Fite.[4] The most outstanding signs of the deficiency were found to be in the blood picture as described in the following section.

Blood studies of animals maintained on different levels of folic acid

The results of these investigations are summarized in table 2. Insofar as was possible the blood studies were made near the end of the 6th week of the dietary regime. However, because of the short survival time of the unsupplemented controls and of the group receiving 1 mg of folic acid per kilogram of diet, blood studies of these animals had to be made considerably earlier. Since there were only 4 survivors in the no folic acid group, the values here shown may not be representative of the average picture. Other results for this group are shown later. The values found for the two groups (receiving no folic acid or 1 mg/kg) suggest a very poor condition with respect to both the red and white cells. With 2 mg of folic acid, the hematocrit was much improved and the leucocyte count was twice that found at the 1 mg level. With 3 mg/kg the values for the hematocrit, hemoglobin and erythrocyte counts appeared to be close to normal but the leucocyte number was not more than half that found at the higher levels of folic acid. The 6 mg level appeared to be adequate for producing normal blood values. No significant differences were found in either the red or white cell picture at folic acid levels of 6, 10, 15 and 40 mg.

Rate of development of folic acid deficiency symptoms in the blood

To obtain better information as to the rate of onset of the deficiency symptoms blood studies were made after varying periods of time on groups (6 to 8 animals) receiving no folic acid or 1, 2, 3 and 6 mg/kg of diet. The first determinations

[4] Laboratory of Pathology and Histochemistry, National Institutes of Health.

TABLE 2

Average blood values for animals maintained on different levels of dietary folic acid

Folic acid, mg/kg	0	1	2	3	6	10	15	40
No. of animals	4	16	6	13	16	41	19	8
Days on diet at time of tests	33	26–31	41	40–45	38–50	37–49	32–40	51
Average wt., gm	196 ± 13 [1]	198 ± 10	298 ± 20	308 ± 12	316 ± 16	316 ± 12	304 ± 14	376 [2] ± 16
Hematocrit, %	33.8 ± 2.5	33.6 ± 5.1	37.8 ± 1.6	42.1 ± 1.7	42.6 ± 0.5	42.2 ± 0.5	41.0 ± 1.1	40.0 ± 0.9
Hemoglobin, gm/100 ml	12.1 ± 0.4	..	11.98 ± 0.7	13.8 ± 0.7	14.2 ± 0.2	14.4 ± 0.2	14.1 [3] ± 0.2	14.4 ± 0.3
Erythrocytes, cells × 10^6/mm^3	..	..	5.07 ± 0.3	5.57 ± 0.4	5.57 ± 0.2	5.74 [4] ± 0.5	5.49 [5] ± 0.3	..
Mean corpuscular volume, μ^3	..	..	74.8 ± 2.1	77.0 ± 4.6	77.5 ± 2.1	73.1 [4] ± 1.7	69.5 [5] ± 3.2	..
Total leucocytes, cells/mm^3	962 ± 106	1334 ± 217	2167 ± 367	3092 ± 553	4881 ± 376	4827 ± 190	5261 ± 337	5050 ± 315
Granulocytes, cells/mm^3	187 ± 94	..	758 ± 242	994 ± 212	1522 ± 164	1440 ± 156	1862 ± 315	1600 ± 167

[1] Standard error.

[2] Greater weight due to fact that the animals, when tested, were older than those of the other groups.

[3] Hemoglobin determinations on only 8 of the animals.

[4] Blood counts on 25 animals.

[5] Blood counts on only 5 animals.

were made on the no folic acid, 1, and 2 mg groups after approximately two weeks on the diets and on the 3 and 6 mg groups after three weeks. The results (table 3) show little difference between the groups in the blood picture at this time but by the end of the third week the blood condition of the no folic acid group had deteriorated markedly, so much so that further tests were not possible. By the end of the

TABLE 3

Changes in weight and in blood picture in relation to time and the folic acid content of the diet (6–8 animals per group)

		FOLIC ACID (*mg/kg*)				
		0	1	2	3	6
Days on diet	(a)	13	14–17	14–17	21–24	21–24
	(b)	21	27–30	27–30	35–38	35–38
	(c)			42	49	49
Weight, gm	(a)[1]	146	156	159	207	219
	(b)	174	220	258	295	304
	(c)			339[2]	376	386
Hematocrit, %	(a)	41.9	42.3	41.3	42.5	45.4
	(b)	35.7	30.4	35.9	41.1	44.9
	(c)			39.3	43.1	44.5
Hemoglobin, gm/100 ml	(a)	14.37	13.95	13.63	14.43	14.50
	(b)	13.98	10.18	11.76	13.15	14.52
	(c)			12.99	13.77	14.16
Erythrocytes, cells $\times 10^6$/cmm	(a)	6.27	6.00	5.55	6.25	6.13
	(b)	5.56	4.07	4.73	5.74	5.70
	(c)			5.66	6.14	5.57
Mean corpuscular volume, μ^3	(a)	66.9	70.3	74.0	68.2	75.4
	(b)	65.1	77.5	75.6	72.1	79.4
	(c)			69.7	70.1	77.5
Total leucocytes, cells/mm^3	(a)	3225	3877	4410	3475	3508
	(b)	1722	2015	2190	2433	4400
	(c)			2800	2840	4350
Granulocytes, cells/mm^3	(a)	1587	1627	1830	1375	1117
	(b)	364	641	530	642	1717
	(c)			1010	940	1817

[1] Weight after days on diet shown in (a) above, etc.

[2] Increase in weight and improvement in blood picture resulted from coprophagy.

4th week the blood values of the 1 and 2 mg groups also showed a striking deterioration whereas those of the 3 mg group exhibited only a slight regression. No change was seen in the 6 mg group other than the lowering of the erythrocyte count, apparently a normal occurrence in the early growth of the guinea pig. An unexpected improvement both in the blood picture and in growth and general appearance of the animals was observed in the 2 mg group at the end of the 6th week. This change was apparently a consequence of an increased availability of folic acid associated with coprophagy, a practice which was observed directly and was also indicated

TABLE 4

Interrelations between folic acid and para-aminobenzoic acid on growth and survival

FOLIC ACID	PABA	SURVIVAL	AVERAGE WEIGHTS		
			Weeks		
			2	4	6
mg/kg	*mg/kg*		*gm*	*gm*	*gm*
0	0	3/8	151	175	213 ± 17 [1]
0	100	7/8	156	216	262 ± 17
2	0	8/8	152	216	300 ± 11
2	100	8/8	163	232	298 ± 9
10	0	7/7	167	262	345 ± 7
10	100	7/7	170	253	330 ± 20

[1] Standard error.

by the accumulation of only a small amount of feces in the litter under the screens. Because of failure to devise a successful method to prevent this practice, these animals, which had been deteriorating rapidly, showed a slow but steady improvement. Further blood tests were not made. However, in spite of the difficulty encountered in continuing the experiment, the results demonstrate clearly its intended purpose.

Interrelations between folic acid and PABA on growth and survival

Duplicate groups of 8 animals each were placed on diets containing no folic acid, 2 mg and 10 mg/kg of folic acid. To

the diets of one set of each of these three groups 100 mg of PABA were added. With no folic acid in the diet the addition of PABA was beneficial both as to growth and survival as is shown in table 4. With 2 mg of folic acid per kilogram, the addition of PABA appeared to have a slight beneficial effect on growth up to the 4th week but thereafter the difference faded out. With the 10 mg level of folic acid no beneficial effect of the PABA was seen during any part of the experimental period.

Effect of variations in the ascorbic acid level in influencing the response to different levels of folic acid

Growth and survival. Four experiments were conducted with ascorbic acid levels ranging from none to 5 gm/kg of diet with folic acid levels varying from none to 10 mg/kg. The general plan of the experiments and the results with respect to growth and survival are shown in table 5. Deficiency of both vitamins had a greater retarding effect on growth than the deficiency of either vitamin alone. The double deficiency shortened the average survival time to 19.5 days as compared to 24 days for a deficiency of folic acid alone and 26 days for a deficiency of ascorbic acid alone. In agreement with the results of Silverman and Mackler ('51), folic acid did not have an antiscorbutic effect. With a diet lacking folic acid, the addition of as much as 2 gm of ascorbic acid per kilogram of diet resulted in no apparent improvement over that obtained with only 50 mg, an amount known to be sufficient to prevent macroscopic symptoms of scurvy but insufficient to permit the development of a normal tooth structure (Reid, '54). With 1 mg of dietary folic acid the survival time, with only 50 mg of ascorbic acid, was slightly less than with higher ascorbic acid levels. At this folic acid level no differences in growth and survival were observed in the 1, 2, 2.5 and 5 gm levels of ascorbic acid. With 6 and 10 mg/kg of folic acid and 1 and 5 gm levels of ascorbic acid the growth

rate was satisfactory and all of the animals survived. In these tests there was more rapid growth at the 5 gm ascorbic acid level but the apparent increase over that at lower levels is probably not significant since similar differences were not observed in additional tests.

The blood picture with varying ascorbic acid and folic acid levels. Blood studies were made in further tests with low

TABLE 5

Lack of effect of variations in ascorbic acid level in influencing the response to different levels of folic acid

ASCORBIC ACID	FOLIC ACID	NO. OF ANIMALS	AVERAGE WEIGHT AT SUCCESSIVE PERIODS				AVERAGE SURVIVAL TIME
			Weeks				
			2	3	4	6	
mg/kg	*mg/kg*		*gm*	*gm*	*gm*	*gm*	*days*
none	none	8	139	118 (3)[1]	all dead	...	19.5
0.05	none	8	151	144 (7)	144 (1)	...	23.6
0.20	none	8	146	158 (5)	all dead	...	23.5
2.00	none	8	148	156 (6)	all dead	...	24.1
0.50	1	6	151	190	186 (4)	277 (1)	28.0[2]
1.00	1	6	141	172 (5)	180 (5)	all dead	31.7
2.50	1	6	139	177	185 (4)	287 (1)	31.0[2]
5.00	1	6	145	185	171 (5)	all dead	32.0
none	6	6	163	150	123 (3)	...	26
1.00	6	6	136	167	207	311	all lived
5.00	6	6	156	201	243	346	all lived
1.00	10	6	159	194	239	336	all lived
5.00	10	6	181	232	282	399	all lived

[1] Number of survivors given within parentheses.

[2] Average survival time of the 5 animals which succumbed.

levels of ascorbic acid (100 and 200 mg/kg) and with 2, 4 and 6 mg levels of folic acid. No significant differences between the groups were observed in the red cell picture (hemoglobin, hematocrit and cell count, all of which were within the normal range) but differences were seen in the leucocyte count at the 2 mg folic acid level. The results of these studies are shown in table 6. At the higher folic acid levels there was little

difference, if any, between the counts at the two ascorbic acid levels. Since the number of determinations under any one age and set of conditions was small (2 to 5), the leucocyte values for the entire period were averaged.

TABLE 6

Total number of leucocytes as affected by the amounts of folic acid and ascorbic acid supplied

(8 animals per group)

FOLIC ACID AND ASCORBIC ACID SUPPLIED	LEUCOCYTES (cells/mm³)					
	Days on diet					
	28	35	42	51–61	66–68	Average
Folic acid, *2 mg/kg* and Ascorbic acid						
100 mg/kg	2875	2725	3400	3025	3125	3030
200 mg/kg	3675	5025	4300	3700	4900	4320
Folic acid, *4 mg/kg* and Ascorbic acid						
100 mg/kg	4275	4475	4175	4725	4000	4330
200 mg/kg	4525	3725	5125	4533	5475	4677
Folic acid, *6 mg/kg* and Ascorbic acid						
100 mg/kg	4500	5100	5400	4812	4600	4882
200 mg/kg	5025	6900	4337	5200	5725	5437

DISCUSSION

The young guinea pig quickly develops folic acid deficiency by the mere exclusion of the vitamin from the diet. The possibility of avoiding the use of antimetabolites, with the danger of resultant complications, should make the guinea pig a useful animal for studying the physiological action of this vitamin.

The young guinea pig appears to have a higher requirement for folic acid than any other animal thus far studied. During the present investigations which have extended over a period

of three years, the minimum requirement for maximum growth has been found to be between 3 and 6 mg per kilogram of diet. This represents a daily intake of 100 to 200 μg. Woolley and Sprince ('45) prevented the deficiency by a daily feeding of only 6.5 μg of the vitamin. The probable explanation of this low requirement is the slow rate of growth (3.3 gm/day) of their animals. Mannering ('49, unpublished data of Mannering and Brown) stated that a daily intake of 100 μg was essential for the production of the maximum growth response. The latter findings are in closer agreement with those of the present studies. In our later studies the requirement has tended to be somewhat less than that found in the earlier studies, the results of which are shown in tables 1 to 5. The difference in part may result from the fact that in the more recent experiments the animals were two to 5 days older when the experiments were started. During the longer period of association with the mother presumably the gastrointestinal tract acquired a greater variety of types of gastrointestinal flora, including types capable of synthesizing folic acid.

In contrast to the guinea pig, the rat fed normal protein levels (Kornberg, Daft and Sebrell, '46) requires no dietary folic acid for maximum growth. The pig also has no dietary requirement of the vitamin (Stokstad, '54). For maximum growth the chick is reported (Hogan, '49–'50) to require from 0.25 to 1.5 mg per kilogram of diet. The minimum requirement for the monkey has not been determined but normal growth has been obtained with a daily intake of 120 μg (Day and Totter, '48) and 100 μg (Smith and Elvehjem, '51). In relation to body weight the requirement of the monkey appears to be considerably less than that of the young guinea pig. The minimum requirement of the young guinea pig for the production of the normal erythrocyte picture appears to be no higher than that for growth but, for producing the normal leucocyte picture, the requirement is definitely higher. The lowest intake at which the normal white cell picture was

obtained was 6 mg/kg of diet. However, the minimum requirement may be somewhat less than this amount since in the early phases of these investigations no levels were run between 3 and 6 mg. This high requirement of folic acid for the production of the normal leucocyte picture is similar to the findings of Campbell, Brown and Bennett ('44) for the chick. However, they found a 10-fold higher requirement for normal leucocyte production than for growth. The difference between the folic acid requirement for growth and for leucocyte production in the guinea pig is much less but may be as much as two-fold. More study is necessary before a more exact quantitative relationship can be stated. Although the growth rate was close to maximum at the 3 mg level, there usually were some fatalities in these groups. Presumably the leucocyte number and possibly antibody production also were not sufficient to give adequate protection against spontaneous infection. That folic acid plays an important role in protecting the animal against bacterial infection has been well demonstrated (Little, Oleson and Roesch, '50; Ludovici and Axelrod, '51; Wertman, Crisley and Sarandria, '52; Asenjo, '54).

The only other study on the relation of folic acid to growth and the maintenance of a normal blood picture in the guinea pig is that of Woodruff et al. ('53) in which they found that 3 mg/kg of diet were adequate to prevent blood changes for at least 8 weeks. However, it did not permit a daily growth rate equal to that of a commercial stock diet. Two factors contribute to the apparent discrepancy between their results and ours; namely, they started their tests with much heavier and older animals and their basal diet contained PABA. An additional third factor may be that of a difference in genetic strain of the experimental animals. In comparison with the blood values found in our animals, theirs had lower erythrocyte counts, slightly lower hemoglobin values, higher hematocrits and greater volume of the red cells.

In common with other animals (Bethell, '54), the guinea pig appeared to have a high tolerance for folic acid (2000

mg/kg of diet) when the vitamin was incorporated in the diet. This is in marked contrast to a toxic effect as indicated by renal lesions when the vitamin was administered by subcutaneous injections in the amount of 8 mg/day for periods of 6 to 10 days (Clark, Dodgen and Darby, '53). Harned et al. ('46), using intravenous injections, reported the order of susceptibility to the compound in different small laboratory animals was guinea pig, rabbit, rat, mouse.

The present findings with PABA are in agreement with the results of Woodruff et al. ('54); namely, that a dietary supply of PABA is beneficial if the dietary folic acid is inadequate. In addition, the present results show definitely that added dietary PABA is not essential if the dietary folic acid is adequate.

A consideration of age is essential in evaluating the effect of folic acid on the leucocyte picture in the guinea pig since a correlation is known to exist between the age of the guinea pig and its total leucocyte count, the number increasing with age (Corsy, '11; Bender and DeWitt, '23; Scarborough, '30). On the other hand, in the erythrocyte picture of the normal animal no variation with age has been reported. The present results, however, show that there may be a drop in the number of erythrocytes following the stage of infancy.

No benefit was derived from increasing the amount of ascorbic acid when the intake of folic acid was below the minimum level for growth except when the ascorbic acid level was very low, down almost to the point at which slight damage to the odontoblasts might be expected to occur (Reid, '54). With 2 mg of folic acid per kilogram of diet, 200 mg of ascorbic acid had a definitely more favorable effect on the leucocyte count than did 100 mg. This may be a consequence of an effect of ascorbic acid on the ability of the animal to convert folic acid to a more physiologically active form (Nichol and Welch, '50). If ascorbic acid functions in this way, the guinea pig would appear to be similar to the monkey (May,

Hamilton and Stewart, '53) in not requiring an abundance of ascorbic acid to effect the conversion.

SUMMARY

Folic acid deficiency can be produced in the young guinea pig by the mere exclusion of the vitamin from the diet.

Folic acid deficiency is characterized by retardation of growth, gradual loss of appetite and activity, weakness, tendency to diarrhea, profuse salivation in the late stages, tendency to fatty infiltration of the liver and adrenal hemorrhages, an aplastic condition of the bone marrow, leucopenia and anemia.

The young guinea pig has an unusually high requirement for folic acid. From 3 to 6 mg of the vitamin per kilogram of diet is the minimum requirement for growth and the production of the normal red blood cell picture. The requirement is higher (6 mg or more) for producing and maintaining a normal leucocyte count. In the presence of 2 mg or less per kilogram of dietary folic acid, 200 mg of ascorbic acid per kilogram stimulates the production of leucocytes. With respect to growth and survival, ascorbic acid does not spare folic acid.

Added PABA is not a dietary essential for the guinea pig but it has an important supplementary value if the dietary supply of folic acid is inadequate.

At the age of 6 weeks the Hartley strain guinea pig reared on a complete diet had the following average blood values: hematocrit, 42.4; hemoglobin, 14.3; erythrocytes, 5.65; mean corpuscular volume, 75; total leucocytes, 4800; granulocytes, 1400.

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PHYSIOLOGICAL AVAILABILITY OF THIAMINE FROM POTATOES AND FROM BROWN RICE [1]

KATHLEEN P. WARNICK,[2] MARY V. ZAEHRINGER, SHIRLEY V. BRING AND ELLA WOODS [3]

Department of Home Economics Research, University of Idaho, Moscow

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TWO FIGURES

The concentration of thiamine in a food does not necessarily indicate how well that food will serve as a dietary source of this essential nutrient. The physiological availability of the thiamine in a particular food may be influenced by factors such as other components of the diet, a change in the intestinal microflora or the absence of some factor needed for utilization of thiamine. Parsons et al. ('45) reported that live yeast cells ingested by human subjects competed with the host for dietary thiamine. Green and co-workers ('41) found that foxes died of thiamine deficiency when certain raw fish were included in an otherwise thiamine-adequate diet. Melnick et al. ('45) found that thiamine was destroyed in the intestinal tract when raw clams were eaten. Later work (Sealock and Davis, '49) showed that raw fish and clams contain the enzyme thiaminase which catalyzes the hydrolytic splitting of thiamine into the pyrimidine and thiamine moieties. Ensminger et al. ('45) found that pigs fed natural rations deposited thiamine in the tissues more efficiently than pigs on purified rations; they believed that certain factors present in the natural rations but not supplied in the purified rations were needed for optimal utilization and deposition of thiamine in the animal tissues.

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[2] Present address: 809 East 8th Street, Moscow, Idaho.

[3] Present address: 1416 North 13th Street, Boise, Idaho.

Melnick et al. ('39, '42) studied the urinary excretion of thiamine in normal individuals and reported that the "percentage of available thiamine which is excreted in the urine is a function of how great an excess is present." Urinary thiamine excretion by subjects on a controlled diet has been used as a measure of the availability of the dietary thiamine, a decrease in thiamine excretion being interpreted as reflecting a decreased availability of the vitamin from the test food.

A project was undertaken at this station to determine the physiological availability of the thiamine in some common foods when used as the source of a significant amount of the daily thiamine in a mixed diet. The foods chosen for study first were lamb and potatoes, using urinary excretion of thiamine by human subjects as the measure of availability.

Because of the rather unexpected results obtained with potatoes, a second series of tests was run using weanling pigs as the experimental animals. Miller et al. ('43) and Ensminger et al. ('45) reported that the pig has a marked ability to store thiamine in the tissues. The use of this animal, which has a digestive system similar to man's, provided an opportunity to determine the amount of thiamine from the test food which was stored in the tissues. This paper reports the results of both series of experiments in this study.

HUMAN STUDIES

Experimental

These experiments consisted of thiamine excretion studies with human subjects on known intakes of thiamine.

The subjects were senior Home Economics students and young University staff members, from 21 to 28 years of age. They consumed a weighed mixed diet planned to supply the amount of thiamine to which the subjects were accustomed and all other known nutrients in adequate amounts. The customary thiamine intake was estimated from food records kept by three staff members and from records of foods served in the University dining rooms.

The test foods replaced comparable foods in the control diet — potatoes replaced brown rice and whole wheat bread, lamb replaced meat loaf and peanut butter — so that approximately one-third of the day's thiamine intake came from the test food. The potato test diet contained 200 gm of baked potatoes and the lamb test diet 120 gm of roast lamb at each of two meals. The lamb-and-potato test diet contained 200 gm of potato and 120 gm of lamb at one meal. The control diet contained, by analysis, 0.88 mg of thiamine, the potato test diet, 1.04 mg, the lamb test diet, 0.79 mg, and the lamb-and-potato test diet, 0.85 mg. Each test diet was eaten for a two-day period. This short test period was used to give a measure of the availability of the thiamine in the test foods as they are used in an ordinary diet. The test-diet periods were preceded, separated and followed by two-day periods on the control diet. The series was concluded with a period in which unenriched white bread and white rice replaced the whole wheat bread and brown rice in the control diet, and pure thiamine was given to make the total thiamine intake equal to that on the control diet.

Complete 24-hour urine collections were made throughout the study. The collection bottles contained a preservative of acetic acid and ethyl alcohol; aliquots for assay were refrigerated under toluene. Thiamine determinations on the foods and the urine samples were done by a modification of the thiochrome procedure of Hennessy and Cerecedo ('39) using the Coleman photofluorometer.

Results

The data for thiamine intake and excretion of each subject during the periods on the test diets and the control diet are given in table 1. The percentage excretion is shown graphically in figure 1.

For all subjects in series A, less thiamine was found in the 24-hour urine samples when potatoes were the important source of thiamine than when any of the other experimental

diets were eaten, although the potato diet contained somewhat more thiamine. Statistical analysis of the data showed that this difference was significant at the 1% level. The average percentage of the thiamine intake excreted by the 6 subjects was one-fourth lower for the potato test-period than the average for the control periods preceding and following it (21.8% for the control periods and 15.1% for the potato test-period). The thiamine excreted during the periods on the lamb, lamb-and-potato and pure thiamine test diets was

TABLE 1

Thiamine intake and urinary thiamine excretion by human subjects on various test diets

DIET	THIAMINE INTAKE	THIAMINE EXCRETION					
		Subjects					
		BF	GH	JH	KP	LF	HW
	mg	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*
Series A							
Control	0.88	169	261	145	133	235	224
	0.88	158	234	166	157	244	232
Potato	1.04	158	192	127	92	198	217
	1.04	126	191	121	76	206	190
Control	0.88	163	245	166	102	248	215
	0.88	173	246	127	101	224	220
Lamb	0.79	157	231	129	114	218	223
	0.79	160	210	133	110	203	210
Control	0.88	135	195	104	94	188	219
	0.88	136	168	117	91	188	244
Lamb and potato	0.85	155	210	135	119	194	222
	0.85	148	195	124	101	201	216
Control	0.88	132	168	129	105	170	216
	0.88	127	153	95	99	136	218
Pure thiamine	0.88	150	205	109	98	182	243
	0.88	185	201	105	110	194	206
		MC	SD	CM	KP		
		μg	*μg*	*μg*	*μg*		
Series B							
Control	0.81	255	82	100	193		
	0.80	263	82	115	168		
Potato	0.80	215	75	75	161		
	0.80	205	45	77	123		

not significantly different from that excreted during the corresponding periods on the control diet.

The first part of this study was repeated. The 4 subjects in series B also excreted less thiamine on the potato test-diet than on the control diet (an average of 19.5% for the control periods and 15.2% for the potato test-period). Statistical analysis of the data showed that these differences were highly significant.

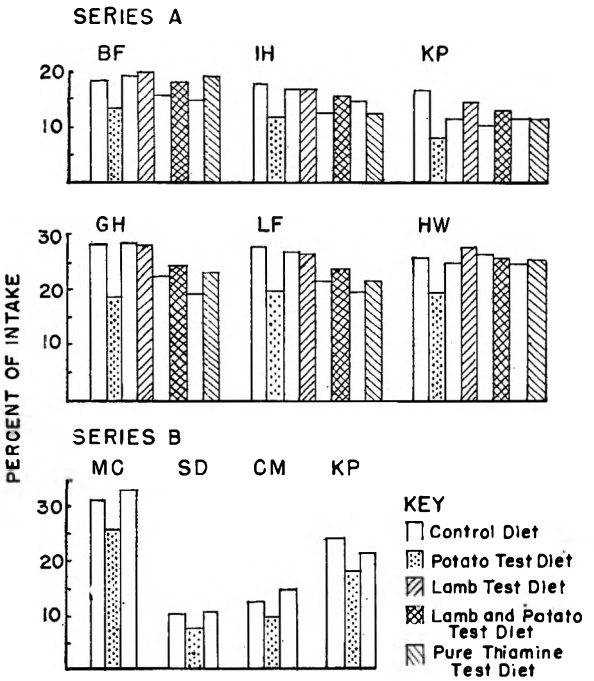

Fig. 1 Percentages of the thiamine intake excreted in the urine by human subjects on 5 test diets.

Because potatoes yield an alkaline residue, it was postulated that the potato test-diet might have changed the pH of the urine sufficiently to cause destruction of thiamine while the urine was in the bladder. Solutions of thiamine in Na_2HPO_4 — KH_2PO_4 buffers at pH 6.79 and lower retained 96% of the thiamine after one hour at 37°C., but the solution at pH 6.83 retained only 80%. A urine sample with a pH of

6.8 retained 97% of the thiamine after one hour of incubation but portions of the same urine sample with NaOH added to change the pH to 7.0 retained only 82%. Some destruction of thiamine could be expected, therefore, at a pH above 6.8.

The pH and the thiamine concentration were determined for urine samples collected at one and one-half-hour intervals from 6:00 A.M. to 10:30 P.M. by subject KP on one day of basal diet and one on the potato diet. Only one of these samples had a pH above 6.8 — the 3:00 to 4:30 P.M. collection for the day on the potato diet. This sample had a pH of 7.06 and contained 5.1 μg of thiamine, compared with the corresponding sample from the day on the control diet which had a pH of 5.33 and contained 8.9 μg of thiamine. Greater differences in thiamine content of some corresponding samples were found, however, when the pH of each was definitely acidic — e.g. the 6:00 to 7:30 P.M. collection on the control day had a pH of 5.01 and contained 12.0 μg of thiamine; the corresponding sample on the potato day had a pH of 5.25 but contained only 7.3 μg of thiamine. The 24-hour excretion for the day on the control diet was 17% of the intake and for the day on the potato diet it was 12%. The lower excretion of thiamine during the period of the potato test-diet could not be explained entirely as the result of destruction of thiamine in the bladder urine because the pH of the urine was above 6.8 for only a short time.

In order to determine whether the lower excretion of thiamine during the periods of the potato test-diet was due to a low availability of the thiamine in potatoes or to increased utilization of the vitamin in the tissues, pig feeding studies were carried out followed by thiamine assays of the animal tissues.

PIG STUDIES

Experimental

Two groups of 7 weanling pigs each were fed diets in which potatoes or brown rice replaced a portion of the grain in the control diet so that approximately one-half of the total thiamine intake was supplied by the test food.

The dry feed in the control diet had the following composition in pounds: barley, 30; oats, 30; wheat, 25; sun-cured alfalfa, meat meal and fish meal, 3.3 each; bone meal, 0.5; skimmilk powder, 50. For the dry feed in the test diets, the amounts of barley, oats and wheat were reduced by one-half, the alfalfa, meat meal and fish meal were increased to 5.0 lb. each and the bone meal and skimmilk powder remained the same. The control-diet dry feed, which all pigs received for an adjustment period of 7 days, was mixed with water in a 1:2 ratio. The test diets were fed in the ratio of one part of test-diet dry feed, two parts of cooked test-food (potatoes or brown rice) and three parts of water. One pound of the control-diet dry feed contained essentially the same concentration of nutrients as ¾ lb. of the test-diet dry feed plus 1½ lb. of cooked test-food. The concentration of nutrients in the three diets was calculated to exceed the nutrient allowances for swine recommended by the National Research Council ('50). The potatoes and rice were cooked daily and refrigerated until used. Thiamine losses during 24-hour storage were negligible for the rice and did not exceed 10% for the potatoes.

The pigs were housed in the individual metabolism cages described by Lehrer and Wiese ('53). They were fed 4 times a day at 4-hour intervals; water was offered after each feeding. Special feeding pans, designed to minimize feed losses from spilling, consisted of loaf pans mounted in shallow drip pans on heavy boards. The dry feed, test food and water were thoroughly mixed in the pans, in amounts based on the quantity of food each pig had eaten at the previous meal. The pans were weighed before and after each feeding to determine food intake. The pigs were weighed weekly.

In the first group of pigs, 5 were 4-week-old Poland China litter-mates and two were 5½-week-old Duroc litter-mates.[4] The 7 pigs in group 2 were 4-week-old Durocs from a single litter.

[4] A high mortality rate in the Poland China litter reserved for this study made the substitution of the two Durocs necessary.

Twenty-four-hour urine collections were attempted during the latter study. The urine samples which were collected successfully were assayed for thiamine content.

Because the length of feeding period which would be needed in order to show a difference in thiamine storage in the tissues was not known, the larger and older Durocs in group 1 were sacrificed after 11 days on the test diets but the Poland Chinas were continued on the test diets for 21 days. All of the group 2 animals were kept on the test diets for 21 days.

After slaughter, the left ham, loin and shoulder cuts of all the animals and the liver, heart and kidneys of the animals of group 1 were removed from the carcasses and frozen. Each cut was thawed, deboned, separated from all visible fat, ground and thoroughly mixed before aliquots were taken for analysis. Corresponding cuts fom all animals in the group were assayed at the same time.

RESULTS

No report could be found of feeding tests with 4- to 6-week-old pigs using the type of diet and feeding methods used in this study. The weight gains of these animals are summarized in table 2. The growth rates, which compared well with the normal growth curves of Ittner and Hughes ('38), demonstrated the practicability of using pigs of this age as experimental animals in nutrition studies.

The thiamine intake and the concentration of thiamine in the tissues of each pig are given in table 3. The average concentration of thiamine in the muscles and organs of the pigs on each diet is shown in figure 2.

The thiamine content of the organs assayed — heart, liver and kidney — did not vary with the diet as did the thiamine content of the muscle tissues. This difference between skeletal muscles and the organ tissues in response to dietary thiamine has been reported by Miller et al. ('43) and Ensminger et al. ('45).

In group 1, the Poland China pigs fed the potato diet had an intake of thiamine 11% higher than those fed the rice

TABLE 2

Weight gain of pigs on experimental diets

GROUP	PIG NUMBER	SEX	DIET	WEIGHT		WEIGHT GAIN ON TEST DIET
				When received	Final	
				lb.	*lb.*	*lb.*
Group 1						
Poland China	4	F	Control	8	20	11
	1	M	Rice	12¼	25½	12
	3	F	Rice	12	23	11
	5	M	Potato	12	24	11
	6	F	Potato	13	28	14
Duroc	2	M	Rice	27	45½	15¼
	7	F	Potato	25	41½	14
Group 2						
Duroc	11	F	Control	17¼	40¾	24¼
	12	M	Rice	14	41¾	25¾
	13	F	Rice	16¾	38¼	22¾
	17	F	Rice	17	37½	19¼
	14	F	Potato	14¾	37	21
	15	M	Potato	18½	41½	21¼
	16	F	Potato	19¼	45¾	23½

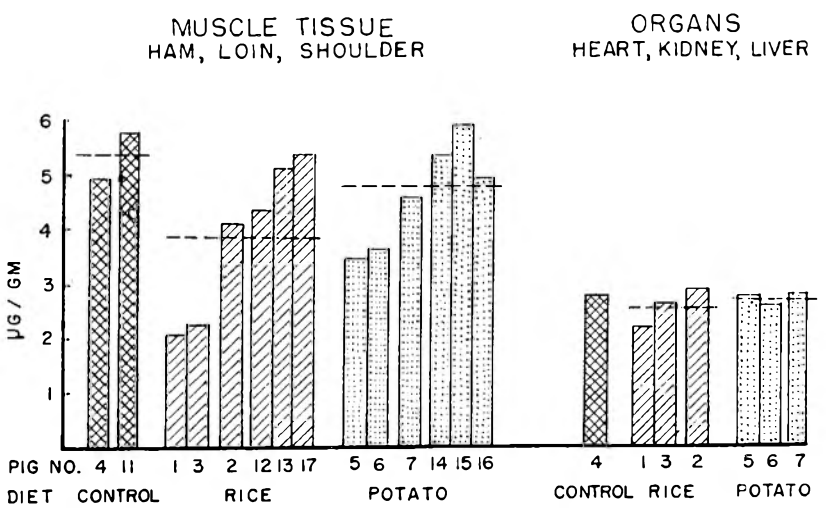

Fig. 2 Average concentration of thiamine in the muscle tissues and the organs of weanling pigs on three experimental diets.

TABLE 3

Thiamine intake and concentration of thiamine in the tissues of pigs on three experimental diets

GROUP	PIG NUMBER	DIET	THIAMINE INTAKE DURING TEST PERIOD	THIAMINE CONCENTRATION OF TISSUES					
				Muscle tissues			Organs		
				Ham	Loin	Shoulder	Liver	Heart	Kidneys
			mg	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*
Group 1									
Poland China	4	Control	26	5.29	5.12	4.48	2.58	3.64	2.20
	1	Rice	25	2.19	2.21	1.94	1.94	2.96	1.88
	3	Rice	24	2.57	2.24	2.08	2.51	3.50	2.01
	5	Potato	25	3.86	3.46	3.22	2.76	3.41	2.25
	6	Potato	30	3.86	3.91	3.32	2.29	3.32	2.35
Duroc	2	Rice	28	4.33	4.48	3.82	2.27	3.86	2.66
	7	Potato	23	4.82	4.66	4.30	2.16	3.77	2.55
Group 2									
Duroc	11	Control	65	6.25	5.92	5.36	...	...	...
	12	Rice	63	4.70	4.60	3.85	...	...	...
	13	Rice	54	5.55	5.25	4.79	...	...	...
	17	Rice	51	5.87	5.46	5.00	...	...	...
	14	Potato	52	6.16	5.18	4.90	...	...	...
	15	Potato	54	6.70	5.93	5.22	...	...	...
	16	Potato	65	5.40	5.07	4.58	...	...	...

diet, but the concentration of thiamine in the muscle tissues of the pigs fed the potato diet was 64% higher. The Duroc in group 1 which was fed the rice diet received one-fourth more thiamine than the one fed the potato diet, but again the concentration of thiamine in the muscle tissues was higher for the pig fed the potato diet (9% higher).

In group 2, although there was considerable variation in intake and thiamine storage among individual animals receiving the same treatment, the average intake of thiamine was approximately the same for the pigs on both test diets, but the average thiamine concentration in the muscle tissues of the pigs fed the potato diet was 9% higher than that of the pigs fed the rice diet. Statistical analysis of the pooled data showed that the differences in thiamine concentration in the muscle tissues of the pigs on the two test diets were significant at the 1% level. There might have been greater differences in the thiamine storage on the two test diets if the experimental feeding period had been shorter. The large Durocs ate much more food than the Poland Chinas in group 1, and may have received sufficient thiamine from either diet so that the slower storage on the rice diet may have caught up with the more rapid storage of thiamine from potatoes.

The thiamine content of the urine samples collected in the latter study was low. The pigs fed the potato diet excreted an average of 3.1% of their intake (based on 12 observations) and the pigs fed the rice diet excreted an average of 4.2% (based on 17 observations). The pH of several samples of urine as voided was around 6.5, so the low thiamine content was probably not the result of destruction of the thiamine in the bladder due to alkalinity.

The data from these studies on young pigs suggest that the lower excretion of thiamine in the urine of the human subjects receiving important amounts of thiamine from potatoes may have been due to increased accumulation of the thiamine in the tissues. It is possible that both the deposition of dietary thiamine and the urinary excretion of the vitamin are in-

fluenced by certain dietary factors which may vary from food to food.

SUMMARY

Human subjects excreted less thiamine when potatoes furnished approximately one-third of the dietary thiamine than when the same amount was furnished by brown rice, lamb, lamb and potatoes together or pure thiamine. To determine whether the lower excretion of thiamine during the potato test-periods was due to low availability of the thiamine in potatoes or to increased utilization of the vitamin in the tissues, pig feeding studies were carried out followed by thiamine assays of the animal tissues.

Two groups of weanling pigs were fed diets in which either potatoes or brown rice replaced a portion of the grain in the control diet so that the test food supplied approximately one-half of the total thiamine intake. Thiamine assays of ham, loin and shoulder cuts indicated that the pigs fed potatoes had a higher concentration of thiamine in the muscle tissues than did the pigs fed the rice diet. Thiamine concentration in the heart, liver and kidney did not vary with the type of diet eaten.

The decrease in urinary excretion and the increase in tissue storage of thiamine when potatoes were the important source of this vitamin in the diet suggest that unidentified factors associated with the source of dietary thiamine may influence the utilization and deposition of thiamine in the tissues. This study indicates that there is need for further investigation of the physiological availability of nutrients from different food sources and from different food combinations.

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REQUIREMENTS OF RATS FOR VITAMIN B_{12} DURING GROWTH, REPRODUCTION AND LACTATION

WERNER G. JAFFÉ
Instituto Nacional de Nutrición, Caracas, Venezuela

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Large sectors of the human populations live on a more or less exclusively vegetarian diet. This fact induced us several years ago to initiate studies on the effect of such a type of feeding on experimental animals kept under similar conditions for many generations. Preliminary experiments showed the existence of some factor (Jaffé, '46), later identified as vitamin B_{12} (Jaffé, '48) lacking in this kind of ration. Because vegetarian diets are low in this vitamin, it seemed interesting to conduct some long-range experiments on their effect on experimental animals. Moreover, an attempt has been made to determine the minimum vitamin B_{12} requirements for growth, reproduction and lactation of the rat under conditions of uniform intake during more than one generation.

EXPERIMENTAL

The animals were descendants of the "Sprague Dawley" strain. All the rats of the experimental series were from a stock kept on a soybean oil meal-corn ration since 1948, while a control group was always fed a commercial stock diet. The animals were housed in screen bottom cages in a room without air conditioning. Large litters were always reduced to 6 within 48 hours after birth, weaned at 21 or 28 days of age and kept together in a common cage to permit brother and sister mating for the following generation. In special cases,

the females of one litter were bred with males of another of the same experimental group. Pregnant females were weighed and put in single cages. Females were bred only once except in a few cases when they were bred a second time.

The composition of the experimental basal diet in parts by weight was as follows: soybean oil meal, 46; whole yellow corn meal, 46; USP Salt Mixture no. 2, 2; sesame oil containing 0.2% of percomorphum oil and 0.2% of wheat germ oil, 5; and the following vitamins per kilogram of diet: thiamine hydrochloride, 3.0 mg; riboflavin, 3.0 mg; pyridoxine hydrochloride, 2.0 mg; Ca-pantothenate, 2.0 mg; niacin, 20 mg; folic acid, 0.25 mg; biotin, 0.10 mg; *p*-amino benzoic acid, 250 mg; inositol, 100 mg; choline chloride, 1 gm.

This diet had only traces of vitamin B_{12} activity, as determined with *L. leichmannii*. The control diet was a commercial pelleted rat ration,[1] which according to the manufacturer, contains about half animal and half vegetable proteins. Its content of vitamin B_{12} was about 30 μg/kg as determined with *L. leichmannii*. This and the experimental rations contained about 24% of crude protein. Ad libitum feeding was used throughout.

The rats in the series fed the diets supplemented with vitamin B_{12} were descendants of animals kept for 10 or more generations on the basal experimental diet and the females were transferred to the supplemented rations when they were transferred to individual cages, one to 6 days before parturition. The litters were bred continuously on the same supplemented diet. The experiments were set up so as to avoid any carry-over or repletion of diminished reserves of the vitamin. Therefore, only animals held on the respective diets at least through the second generation are included.

The animals were weighed individually and the mean weaning weight of each litter calculated. For the tabulation, these mean weights, rather than individual weights, were used in order to avoid a possible bias due to the tendency of rats in larger litters to be lighter.

[1] Ratarina, produced locally.

For the determinations of soluble, reduced nonprotein sulfhydryl compounds in the livers, a modification (Jaffé and Budowski, '54) of the Grunert and Phillips ('51) method for glutathione was used. Vitamin B_{12} was determined with a modification of the USP method.

RESULTS

In table 1 data are presented on the reproduction of rats kept for 7 years on the soybean oil meal-corn ration. The enumeration of the generations starting with the 5th is only approximate as the groups were selected according to the date of the birth of their litters and the corresponding generation calculated only with the first and last animals of each group. Therefore, some overlapping may exist among the last 4 groups.

The litters in the first generation showed distinctly better performance than later ones with respect to the number of animals born in each litter, number of surviving animals, and weaning weights. This was to be expected, as the complete stock ration was fed during gestation. The performance in this group is virtually the same as for the animals kept on the stock ration for the whole lactation period. Starting with the second generation, a very considerable deterioration in the performance of the litters may be observed. The number of animals weaned per litter was only about half that in the first generation. As the number of young which died between the second day after birth and the weaning age was always small, it is clear that this is due mostly to the higher number of stillbirths or to deaths in the first two days of life. There was a tendency for all of the young in litters of certain mothers to die in contrast to a low death rate in the litters of others.

No difference between the first and succeeding generations could be observed with respect to the weight gain of the mothers during the 4 weeks of lactation or to the mean weight of the young at birth.

TABLE 1

Reproduction and lactation performance of rats kept for several generations on a vegetable diet low in vitamin B_{12}

GENERATION	LITTERS BORN	LITTERS WITH NO SURVIVORS	YOUNG BORN PER LITTER	AV. WT. OF YOUNG AT BIRTH	YOUNG WEANED PER LITTER [1]	AV. WEANING WTS. (4 WKS.)	YOUNG DEAD PER LITTER BETWEEN DAY 2 AND WEANING	WT. GAIN OF MOTHER DURING LACTATION
				gm		*gm*		*gm*
1	9	0	8.4	5.7	5.3	63.1	0.3	9.0
2–3	24	8	6.0	5.8	2.1	41.4	1.0	6.2
3–4	19	6	7.1	5.6	3.0	47.9	0.1	10.5
5–7	32	11	7.7	6.0	2.3	38.9	1.1	2.1
8–12	32	8	6.9	5.6	4.2	43.1	0.8	10.3
13–15	30	9	7.6	5.3	3.6	45.7	0.6	7.6
16–18	36	10	7.0	5.3	3.1	42.9	0.7	0

[1] Larger litters were reduced to 6.

TABLE 2

Reproduction and lactation performance of rats kept on different experimental diets

GROUP NO.	DIET AND SUPPLEMENTS	LITTERS BORN	LITTERS DEAD	YOUNG BORN PER LITTER	AV. WT. OF YOUNG AT BIRTH	YOUNG WEANED PER LITTER	YOUNG DEAD PER LITTER BETWEEN DAY 2 AND WEANING	AV. WEANING WTS. (4 WKS.)	WT. GAIN OF MOTHER DURING LACTATION	AV. WT. OF MOTHERS AT BIRTH OF FIRST LITTER	AV. AGE OF MOTHERS AT BIRTH OF FIRST LITTER
					gm			*gm*	*gm*	*gm*	*days*
1	Basal	30	9	7.6 ± 0.37 [1]	5.4 ± 0.19	3.6	0.5	46.7 ± 3.1	9.1 ± 3.0	184 ± 3.9	147 ± 2.5
2	+ 3 μg of vit. B_{12}	15	1	8.0 ± 0.62	5.8 ± 0.13	5.3	0.1	57.7 ± 2.2	21.4 ± 3.0	197 ± 5.5	95 ± 4.4
3	+ 5 μg of vit. B_{12}	26	3	8.2 ± 0.43	5.7 ± 0.19	4.8	0.4	66.6 ± 1.0	17.1 ± 3.7	196 ± 5.5	111 ± 3.8
4	+ 30 μg of vit. B_{12} + 0.2% methionine	20	1	7.5 ± 0.50	5.5 ± 0.22	5.2	0.7	62.8 ± 3.1	7.6 ± 5.6	..	..
5	Stock	103	2	9.3 ± 0.29	6.0 ± 0.05	5.5	0.3	66.6 ± 1.0	0.8 ± 2.1	215 ± 5.6	96.5 ± 3.7

[1] Standard error of the mean.

The results of various supplements of vitamin B_{12} on reproduction and lactation performance are summarized in table 2. The performance of the animals on the basal diet was poor in comparison with the B_{12}-supplemented groups with respect to the survival of litters, number of animals weaned per total litters born, weaning weights at 4 weeks and the age of the mothers at the birth of their first litters. The results of group 2 show that the addition of 3 μg of vitamin B_{12} per kilogram of diet did not result in a similar weight gain of the young as

TABLE 3

Post weaning growth of rats bred on different experimental diets

DIET AND SUPPLEMENTS	NO. OF ANIMALS	SEX	WEIGHT AT 3 WEEKS	WEIGHT AT 7 WEEKS
			gm	*gm*
Basal	23	M	27.9 ± 0.9 [1]	119.2 ± 4.9 [1]
Basal	21	F	29.3 ± 2.1	109.2 ± 3.6
Basal + 3 μg/kg vit. B_{12}	25	M	34.5 ± 1.1	154.5 ± 3.1
Basal + 3 μg/kg vit. B_{12}	28	F	34.4 ± 1.0	123.5 ± 1.7
Basal + 5 μg/kg vit. B_{12}	30	M	41.8 ± 0.6	170.2 ± 3.3
Basal + 5 μg/kg vit. B_{12}	30	F	41.5 ± 0.7	137.0 ± 1.9
Stock	24	M	42.1 ± 0.9	163.6 ± 3.4
Stock	28	F	42.3 ± 1.8	134.4 ± 2.1

[1] Standard error of the mean.

that observed with larger supplements, although the performance was nearly the same in all other respects.

Supplements of 5 μg/kg of vitamin B_{12}, and 30 μg/kg together with 0.2% of methionine gave identical results. A comparison between the group of rats fed the complete stock diet and the two latter supplemented groups shows that there were no significant differences in all those aspects studied in our experiments with the only exception of the litter size, the birth weight, and possibly the number of litters in which all animals died before weaning time. In experiments not in-

cluded in the present paper there was no consistent difference in birth weight between stock and experimental groups.

Observations on post-weaning growth of young from different groups are included in table 3. These animals were weaned at three weeks. It can be seen from the results that growth during the 4-week period was about 75% of the normal

TABLE 4

Reduced liver glutathione in adult male rats bred on different diets

DIET AND SUPPLEMENT	NO. OF ANIMALS	LIVER GLUTATHIONE
		mg/100 gm
Basal	17	211 ± 6 [1]
Basal + 3 μg/kg of vitamin B_{12}	11	222 ± 5
Basal + 5 μg/kg of vitamin B_{12}	6	214 ± 5
Basal + 30 μg/kg of vitamin B_{12}	12	202 ± 5
Basal + 30 μg/kg of vitamin B_{12} and 0.2% of methionine	6	245 ± 11
Stock	20	269 ± 6

[1] Standard error of the mean.

TABLE 5

Content of vitamin B_{12} in the livers and kidneys of adult male rats kept on different diets

DIET	AVERAGE VITAMIN B_{12}	
	Liver	Kidney
	μg/kg	*μg/kg*
Basal	0.013 ± 0.0013 [1]	0.101 ± 0.031
Basal + 5 μg B_{12} per kg	0.033 ± 0.0057	0.181 ± 0.028
Stock	0.108 ± 0.0060	2.25 ± 0.25

[1] Standard error of the mean.

rate in the vitamin B_{12}-deficient group kept on the basal diet, and that there were no significant differences in this respect between the groups receiving supplements of 3 or 5 μg/kg or the stock ration. No post-weaning death was observed in this experiment.

The results of determinations of soluble, reduced liver nonprotein sulfhydryl compounds are presented in table 4. There were no significant differences between the unsupple-

mented and vitamin B_{12}-supplemented groups; the stock animals however, had higher levels. Hemoglobin, hematocrit, and urea determinations as well as red and differential white blood cell counts were made on groups of adult male rats from groups 1, 3, and 5. All the values found were within the range accepted as normal. Vitamin B_{12}-deficient animals had slightly higher values of blood urea than stock animals (0.27 $\pm$ 0.017 gm/l vs. 0.24 $\pm$ 0.016 mg/l).

In table 5, the values for vitamin B_{12} in livers and kidneys of adult male rats, kept on one of three different diets, are presented. It can be seen that the diet containing 5 µg/kg of vitamin B_{12} did not cause as high tissue levels of this vitamin as the stock diets although the values were higher than they were with the basal ration.

DISCUSSION

In confirmation of Dryden et al. ('52), the data in tables 1 and 2 show that in mother rats fed a diet low in vitamin B_{12} there is a tendency for the entire litters of certain mothers to die; this is in contrast to a relatively low mortality rate for the litters produced by other individuals. This suggests that by applying the principles of selective breeding it might be possible to develop a strain of rats that is relatively resistant to vitamin B_{12} deficiency. We used mostly brother and sister matings in order to accentuate any such tendency.

Nevertheless, the differences observed between the different deficient groups in subsequent generations are small and of doubtful significance. The first deficient group (second to third generation) showed the poorest overall performance, but the observed differences are not impressive in view of the fluctuations between the following experimental groups. Between the third to 4th and 16th to 18th generations, no such selective trend was observable.

The lack of a significant difference between the first groups and the last, which were observed after 6 to 7 years of almost continued brother-sister breeding on the vitamin B_{12}-low diet, is contrary to what we had expected to find. It may be related

possibly to the fact that the performance of our rats on the soybean-corn ration was much better than that described by several other investigators (Schultze, '53; Perdue and Phillips, '54, and Sherman, Schilt and Schaeffer, '55). These authors have reported results showing poor performance and especially a very high post-weaning mortality rate in rats on a diet low in vitamin B_{12} even after supplementation with this vitamin. We were unable to make similar observations. Usually, the mortality was near zero after weaning. There were some instances in the course of these experiments when the young of several litters in the group fed the B_{12}-deficient diet died one to 6 weeks after weaning and in one case, several litters of the stock animals died at the same time under very similar circumstances. In all cases, vitamin B_{12}-supplemented litters showed the same mortality as those reared on the unsupplemented ration. There were usually no gross abnormalities observable at autopsy. These cases were very infrequent, the total not reaching even 5% of those weaned. No gross congenital malformations have been observed in the course of the present experiments. It seems possible that the high mortality observed by other authors was due to infection or to changes in the intestinal flora. As our colony has been kept very isolated — no new animals have been brought in from outside since the start of these experiments — it may be in an unusally healthy condition. The lack of growth-stimulating action of aureomycin in our vitamin B_{12}-low animals (Jaffé, '51) may possibly be interpreted in a similar manner.

As Schultze ('53) has shown, there may be differences between lots of soybean protein with respect to their nutritional value for rats. As the soybean oil meal used during the years of experimentation came from several sources, there may have been differences which could have obscured the outcome of our experiment. There are other deficiency symptoms such as sterility, which were not studied in the present experiments and which may have had some influence on the results.

Although we were unable to detect the operation of a genetic selection of animals with better performance on a diet low in vitamin B_{12}, the results presented in table 1 show that it is possible to breed rats for at least 18 and probably many more generations on a fortified soybean-corn ration not supplemented with vitamin B_{12}.

The lack of vitamin B_{12} in the diet caused a high mortality rate of the litters, a low birth weight and weaning weight, and the deficient females were older when they gave birth to their first litters. The first two deficiency symptoms were at least partially, and the latter two completely overcome by a supplement of only 5 µg/kg of vitamin B_{12}, while the dose of 3 µg/kg was insufficient to secure normal weaning weight. As there was no difference observable between the effect of supplements of 5 or 30 µg/kg, the minimum dose must be somewhere between 3 and 5 µg/kg of vitamin B_{12} in a soybean-corn diet under the present conditions.

The tendency of vitamin B_{12} supplements to cause greater birth weights in rats, observed by other authors (Daniel et al., '53; Dryden et al., '51) can also be detected in our results, although the young born of mothers fed the stock ration were still heavier at birth notwithstanding the larger litter sizes in this group. The replacement of soybean oil meal by full-fat soy flour causes the differences in litter size between the experimental and control groups to disappear (Jaffé, '55). They are therefore probably not related to vitamin B_{12}.

The females on the deficient diet were significantly older when their first litters were born than those on the diet supplemented with vitamin B_{12}. This is in accordance with observations of Dryden et al. ('54) on sexual maturation, which was found to be delayed in vitamin B_{12}-deficient animals.

It can be concluded from the data of table 3, that the difference in growth, for the 4 weeks after weaning, between rats receiving vitamin B_{12} supplements of 3 or 5 µg/kg of diet respectively, was small in both males and females (8.4 gm for males and 6.4 gm for females) as compared with the differ-

ences in weaning weights (difference of three-weeks and 7-weeks weights of groups receiving the 3 μg/kg supplements of vitamin B_{12} subtracted from corresponding difference of groups receiving 5 μg/kg of the vitamin).

This and the fact that females kept on the diet supplemented with 3 μg/kg of vitamin B_{12} gave birth to their first litters at practically the same age as the rats receiving the larger amount of this vitamin, and that they reached the same weight at this time (table 2) can be taken as evidence that the former dosage is nearly sufficient for growth.

A difference with respect to the level of reduced glutathione of livers between vitamin B_{12}-deficient and supplemented animals as described by Register ('54) could not be detected in the present experiments (table 4) in agreement with work on mice (Jaffé, '54). The higher levels in animals fed a diet supplemented with methionine are probably to be explained on the basis of observations of Leaf and Neuberger ('47) on the influence of sulfur-containing amino acids on tissue glutathione.

The levels of vitamin B_{12} found in the livers in our animals are in reasonably good agreement with values reported by Moinuddin and Bentley ('55), considering the different experimental conditions used, and show that tissue saturation has not been achieved with the addition of 5 μg/kg of this vitamin to the basal diet. The kidney values are higher than those reported by the aforementioned authors.

The present results may be interpreted as indicating that the need of the rat for vitamin B_{12} is greater for lactation than it is for growth and reproduction. Inasmuch as supplements of 5 or 30 μg/kg of diet gave identical results, the former dose may be considered sufficient for the whole reproductive cycle of the rat under the present conditions.

The present results should not be applied to other diets than that used during this study. Experiments now under way with diets in which the carbohydrate source is sucrose are

giving results which would indicate a somewhat higher requirement for vitamin B_{12}.

The observations on rats presented in this paper are very similar to those made earlior on mice (Jaffé, '54), with the difference that in the latter species a supplement of 3 µg/kg of vitamin B_{12} to the corn-soybean oil ration was as effective as the higher doses.

SUMMARY

A rat colony was kept on a fortified soybean oil meal-corn ration, low in vitamin B_{12}, for 18 generations, using mostly brother and sister matings.

Litters starting with the second generation showed high mortality, low birth weights, low weaning weights, slow post weaning growth, and low liver and kidney vitamin B_{12} levels. Females of this group were older, when giving birth to their first litters, then the controls. Blood characteristics and liver glutathione levels were normal.

No significant difference between succeeding generations could be detected and therefore no indication for a genetic selection toward resistence to vitamin B_{12} deficiency could be found.

The addition of 3 µg of vitamin B_{12} per kilogram of diet eliminated most of the deficiency symptoms, but did not result in optimal weaning weights and post-weaning growth, while supplements of 5 µg of vitamin B_{12} per kilogram of diet, or 30 µg of this vitamin together with 0.2% of methionine, gave identical results in overcoming these deficiency signs. All of the animals used had been kept for at least one generation on the respective experimental diets previous to the experiments presented.

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BENEFICIAL EFFECTS OF ALFALFA ON THE OVARIAN DEVELOPMENT OF IMMATURE RATS FED MASSIVE DOSES OF ALPHA-ESTRADIOL [1]

B. H. ERSHOFF, H. J. HERNANDEZ AND J. H. MATTHEWS
Department of Biochemistry and Nutrition, University of Southern California, Los Angeles, and Nutrilite Products, Inc., Buena Park, California

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It is well established that the prolonged administration of massive doses of estrogens inhibits ovarian development in the immature rat (Zondek, '41). In the present communication data are presented indicating that the deleterious effects of massive doses of alpha-estradiol on ovarian development in the immature rat can be largely counteracted by the concurrent feeding of dried alfalfa. The protective factor (or factors) in alfalfa is apparently distinct from any of the known nutrients.

EXPERIMENTAL

Experiment 1. Comparative effects of dried alfalfa and supplements of the known nutrients on ovarian weight and morphology in immature rats fed massive doses of alpha-estradiol

The basal ration employed in the present experiment consisted of sucrose, 66%; casein,[2] 24%; salt mixture,[3] 5% and

[1] Communication no. 401 from the Department of Biochemistry and Nutrition, University of Southern California.

[2] Vitamin-free test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbell, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

corn oil,[4] 5%. To each kilogram of the above diet were added 10 mg of alpha-estradiol and the following crystalline vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2 methyl-naphthoquinone, 5 mg; choline chloride, 2 gm; vitamin A, 5,000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. In addition to the basal ration the following diets were also employed, consisting of the basal ration plus each of the following supplements: (a) 20% oven-dried alfalfa[5] (b) 20% sun-dried alfalfa[5] (c) 20% vacuum-dried alfalfa[5] (d) 5% casein[2] (e) 5% corn oil[4] (f) 2.5% salt mixture[3] (g) supplements of the following vitamins per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2 methyl-naphthoquinone, 5 mg; vitamin A, 5,000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 300 mg. Supplements were added in place of an equal amount of sucrose. In addition to the above rations a control diet was also tested which consisted of the basal ration with alpha-estradiol omitted. One hundred and ten female rats of the Wistar strain were selected at 21 to 23 days of age and an average weight of 42.4 gm (range 36 to 48 gm) for the present experiment. Animals were placed in metal cages with raised screen bottoms and were fed the above diets ad libitum (20 rats on the basal ration; 10 animals per group on the remaining diets). Diets were made up weekly and stored under refrigeration when not in use. Rats were fed daily, and all food not consumed 24 hours after feeding was

[4] Mazola.

[5] The oven-dried, sun-dried and vacuum-dried alfalfa were all prepared from the same batch of freshly cut alfalfa.

discarded. These measures were employed to minimize the oxidative changes in the diet. Feeding was continued for 8 weeks. Animals were autopsied on the 56th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

TABLE 1

Comparative effects of dried alfalfa, alfalfa fractions and supplements of the known nutrients on the ovarian weight of rats fed massive doses of alpha-estradiol

SUPPLEMENTS FED WITH BASAL RATION	ALPHA-ESTRADIOL IN RATION	NUMBER OF ANIMALS	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	AVERAGE OVARIAN WEIGHT [1]
	mg/kg of diet		*gm*	*gm*	*mg*
Experiment 1					
None	10	20	42.8	177	18.6 ± 1.0
B vitamins, C and K	10	10	42.7	175	18.8 ± 0.9
Vitamins A, D and E	10	10	42.5	182	18.2 ± 1.6
5% Casein	10	10	42.4	199	19.1 ± 1.8
5% Corn oil	10	10	42.6	172	17.4 ± 1.2
2.5% salt mixture	10	10	42.3	176	19.3 ± 1.3
20% oven-dried alfalfa	10	10	42.2	184	34.2 ± 4.2
20% sun-dried alfalfa	10	10	42.2	175	41.6 ± 2.5
20% vacuum-dried alfalfa	10	10	42.1	175	40.8 ± 4.2
Basal ration without alpha-estradiol	0	10	41.9	209	53.4 ± 3.0
Experiment 2					
None	10	10	41.4	170	17.3 ± 1.1
20% alfalfa meal 1	10	10	41.2	185	19.4 ± 1.8
20% alfalfa meal 2	10	10	41.0	179	28.1 ± 1.7
20% alfalfa meal 3	10	10	41.2	184	34.2 ± 3.3
20% alfalfa meal 4	10	10	41.4	184	38.3 ± 3.9
20% alfalfa meal 5	10	10	41.1	185	38.1 ± 4.0
20% alfalfa meal 6	10	10	41.1	189	43.2 ± 2.1
Basal ration without alpha-estradiol	0	10	41.0	202	50.6 ± 1.8
Experiment 3					
None	10	10	41.0	155	14.5 ± 0.9
13% alfalfa residue	10	10	40.8	173	36.0 ± 4.0
7% dried alfalfa juice	10	10	40.9	175	33.7 ± 3.8
Basal ration without alpha-estradiol	0	10	41.0	185	46.5 ± 2.8

[1] Including standard error of the mean calculated as follows $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

Results are summarized in table 1. The findings indicate that the ovaries of rats fed the basal ration remained immature both in weight and microscopic appearance; only two of the 20 rats in this group exhibited mature follicles and corpora lutea. Similar results were obtained in rats administered the vitamin supplements or supplements of casein, corn oil or salt mixture. In contrast to the above animals, 5 out of the 10 rats fed the oven-dried alfalfa supplement and 9 out of the 10 rats each fed the sun-dried or vacuum-dried alfalfa supplements exhibited mature follicles and corpora lutea with ovarian weights averaging approximately twice those of animals fed the unsupplemented basal ration. Mature follicles and corpora lutea were also present in all 10 of the rats fed the basal ration with alpha-estradiol omitted. The weight increment of rats fed the rations containing alpha-estradiol was approximately 15% less than that of animals on the basal ration with alpha-estradiol omitted. Rats fed the alfalfa supplements, however, did not differ significantly in weight from animals on the unsupplemented basal ration.

Experiment 2. The variation in activity of different batches of alfalfa meal in counteracting the deleterious effects of alpha-estradiol on the ovaries of immature rats

Tests were conducted with 6 different batches of commercial oven-dried alfalfa meal. Grossly the alfalfa samples were indistinguishable in appearance. Eighty female Wistar rats comparable in age and weight to those employed in the first experiment were divided into 8 groups of 10 rats each and were fed the following rations: (a) basal ration with alpha-estradiol omitted (b) basal alpha-estradiol-containing ration and (c to h) basal ration plus 20% oven-dried alfalfa meal, samples 1 to 6 inclusive.[6] The alfalfa supplements were

[6] The alfalfa meal employed in this experiment was prepared from alfalfa harvested during the months of July to November, 1954. No correlation was observed between the month of cutting and the effectiveness of the various lots of alfalfa in counteracting the deleterious effects of alpha-estradiol feeding on the ovaries of immature rats.

incorporated in the basal ration in place of an equal amount of sucrose. Animals were autopsied after 8 weeks of ad libitum feeding and ovarian weights determined. Results are summarized in table 1. In agreement with earlier findings the ovaries of rats fed the basal ration appeared immature both in weight and appearance. Considerable differences were observed, however, in the ovarian weight and appearance of rats fed the various alfalfa samples. One of the lots was virtually devoid of activity with corpora lutea present in only two of the 10 rats in the group. Three of the lots had moderate activity with corpora lutea present in 4 to 6 of the 10 rats in each group. The remaining two lots had high activity with all rats in each group exhibiting corpora lutea formation. In general the average ovarian weight of rats fed the various alfalfa fractions was directly correlated with the number of animals in each group with corpora lutea present.

Experiment 3. Effects of dried alfalfa juice and alfalfa residue on ovarian weight and morphology of immature rats fed massive doses of alpha-estradiol

Tests were conducted to determine the comparative effects of dried alfalfa juice and desiccated alfalfa residue [7] on the ovaries of immature rats fed alpha-estradiol. The experimental procedure was similar to that employed previously. Forty female Wistar rats comparable in age and weight to those employed above were divided into 4 groups of 10 rats each and were fed the following rations: (a) basal ration with alpha-estradiol omitted (b) basal ration containing alpha-estradiol (c) basal ration plus 13% of desiccated alfalfa residue and (d) basal ration plus 7% of dried alfalfa juice. Supplements were added in place of equal amounts of sucrose. Animals were autopsied after 8 weeks of ad libitum feeding and ovarian weights determined. Results are summarized in table 1. As in the previous tests the ovaries of rats fed the basal ration were immature both in weight and appearance.

[7] The water-washed alfalfa pulp remaining after the extraction of the juice.

Considerable activity was exhibited, however, by both alfalfa fractions. Five of the 10 rats fed the alfalfa juice and 6 of the 10 animals fed the alfalfa residue exhibited extensive corpora lutea formation with ovarian weights averaging more than twice those of animals on the basal ration.

DISCUSSION

The findings indicate that the deleterious effects of massive doses of alpha-estradiol on ovarian development in the immature rat can be largely counteracted by the concurrent feeding of dried alfalfa. The protective factor (or factors) in alfalfa is apparently distinct from any of the known nutrients inasmuch as supplementing the basal ration with increased amounts of all the known vitamins, salt mixture, protein in the form of casein or fat in the form of corn oil in amounts equal to or exceeding the amount of such nutrients in alfalfa meal was without protective effect. Both the dried alfalfa juice and the water washed alfalfa pulp remaining after the extraction of the juice were potent sources of the protective factor (or factors). Considerable variation has been observed, however, in the activity of different batches of alfalfa meal in promoting ovarian development in rats fed alpha-estradiol. Commercial samples of oven-dried alfalfa meal which appeared identical in appearance ranged from markedly active to virtually inert in activity. No data are available to account for this varation in activity. Preliminary findings suggest that differences in processing procedure may be responsible, at least in part, for this variation. Thus in experiment 1, the three samples of alfalfa tested (oven-dried, sun-dried and vacuum-dried) were all prepared from the same batch of freshly cut alfalfa. The oven-dried alfalfa in this test appeared to be less active than the sun-dried or vacuum-dried material. Other samples of oven-dried alfalfa (lots 5 and 6, table 1), however, had marked activity.

Considerable data are available indicating that alfalfa and other types of green forage contain unidentified factors with gonadotropic activity. As far back as 1934 Friedman and

Friedman reported the presence of a gonad-stimulating substance in extracts from alfalfa meal. This work was confirmed and extended in subsequent investigations by the same authors (Friedman and Friedman, '39). As was true in the present investigation, a great deal of variability was observed in the activity of different lots of test material (Friedman and Mitchell, '41). An unidentified water-soluble factor in cereal grass has been reported which is effective in promoting ovulation of rabbits sensitized with estrogen. This factor which differs in stability and in other properties from gonadotropins obtained from animal sources apparently exerted its effect by causing the release of pituitary gonadotropin (Borasky and Bradbury, '42; Bradbury, '44; Bradbury and Hodgson, '42). An unidentified factor in grass has been reported which produces early vaginal opening and stimulates early ovarian activity in immature rats. This material which has been referred to as the "sex maturity factor" was orally active, water soluble and was concentrated by alcohol precipitation of grass juice (Gomez et al., '41).

It has been demonstrated that the ovarian inhibition caused by the prolonged administration of estrogens in the immature rat is due to an impaired secretion of pituitary gonadotropins (Zondek, '41). The inhibition of ovarian development that occurs under these conditions can be counteracted by the concurrent administration of pituitary or chorionic gonadotropins. Inasmuch as desiccated alfalfa under conditions of the present experiment was also effective in counteracting the ovarian inhibition caused by the prolonged administration of an estrogen, the possibility exists that the protective effect of alfalfa was due to the presence of an orally active gonadotropin in this material, possibly comparable to the factor or factors indicated above. Another possibility is that alfalfa contains a factor (or factors) which was effective in promoting the synthesis and secretion of pituitary gonadotropin under conditions of the present experiment. An alternate interpretation is that alfalfa contains an unknown nutrient (or nutrients) which may be part of an enzyme system concerned

with estrogen inactivation. The prolonged feeding of massive doses of alpha-estradiol may have increased requirements for this factor to the point that a relative deficiency was produced in rats fed the basal ration. This deficiency may have manifested itself in impaired estrogen inactivation, estrogen excess and inhibition of pituitary gonadotropin secretion. Further studies are needed to determine which of these possibilities, if any, are responsible for the observed results.

SUMMARY

The ovaries of immature rats fed a purified ration containing massive doses of alpha-estradiol remain immature both in weight and in microscopic appearance. The deleterious effects of alpha-estradiol feeding on ovarian development in the immature rat can be largely counteracted by the concurrent feeding of dried alfalfa. The protective factor (s) in alfalfa is apparently distinct from any of the known nutrients. Both the dried alfalfa juice and the water-washed alfalfa pulp remaining after the extraction of the juice were potent sources of the protective factor (s). Commercial batches of dried alfalfa meal vary markedly in their content of the protective factor (s).

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THE INFLUENCE OF VITAMIN B_{12} AND AUREOMYCIN UPON THE GROWTH OF PROTEIN-DEFICIENT CHILDREN

IAN F. S. MACKAY, S. J. PATRICK, DOREEN STAFFORD
The Department of Physiology, University College of the West Indies, Jamaica, B.W.I.
AND
FRED S. CLEVELAND, JR.[1]
Merck and Co., Rahway, New Jersey

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The purpose of this study was to examine the influence of vitamin B_{12} and aureomycin upon the growth of protein-deficient children and at the same time to make an assessment of their nutritional status. A brief preliminary report has been made (Mackay et al., '54).

There is disagreement as to the effect of dietary supplementation with vitamin B_{12} on human growth and conflicting results have been reported (see for example Wetzel et al., '49, '52; Benjamin and Pirrie, '52; Scrimshaw and Guzman, '54). Studies with aureomycin have shown positive results (Snelling and Johnson, '52; Robinson, '52; Carter, '53). If such supplements should have a positive influence upon growth, their administration might make an important contribution towards alleviating the widespread malnutrition that exists in the tropics and which is present in the Caribbean area. It was for these reasons that the present study was undertaken.

The children upon whom the observations were made in the present study lived in small rural communities in the southern part of the parishes of St. Thomas and St. Andrews in Jamaica, B.W.I. The people of these districts earn a rather poor subsistence by agriculture. Some fishing is done but this

[1] Present address: Stevens Institute of Technology, Hoboken, New Jersey.

provides not so much food as currency. A group of children in an orphanage was also included in the study.

The study extended over a period of two years (1951-1953). In the first half of the study, the growth of 2012 children was measured. As a result of these measurements, 955 children were selected for supplementation on grounds of regular attendance at school. During the second half of the study supplements were administered to 4 groups which contained approximately equal numbers of children of each sex and growth type (failure or normality according to the Wetzel standard).

In order to meet criticisms that might be made of previous studies, an attempt was made to ensure the adequacy of the present study by (1) including a large number of subjects, (2) assessing the nutritional status of the group and (3) including a suitable control group. The dietary supplements were administered in the form of lime flavoured troches which contained either (a) no growth factor or (b) 100 μg of vitamin B_{12} or (c) 50 mg of aureomycin or (d) 100 μg of vitamin B_{12} and 50 mg of aureomycin. An attempt was made to supply each child with one troche each day but due to non-attendance at school the average actual consumption of the dietary supplements was below this and is given in table 1. The following observations were made: (1) Growth measurements involving determination of height, weight and age; (2) Clinical studies, including reports on malaria and parasitic infestation; (3) An assessment of the concentration of various blood constituents; (4) A direct dietary survey.

RESULTS

Growth. The average heights and weights of the children before any dietary supplementation, are shown in table 2. It is apparent that the Jamaican children are lighter in weight than English children of the same age (Daley, '49). Reference to the Wetzel grid shows that the Jamaican children are considerably behind American children in developmental age (Wetzel, '44).

The initial selection of the children to be used in the work on the evaluation of aureomycin and vitamin B_{12} as growth promoters was based upon the use of the Wetzel grid. However, for the calculation of the final results, the assessment of growth was based upon increments of height and weight separately.

TABLE 1

The average daily consumption per capita of the dietary supplements by Jamaican children

GROUP	ROCHE	AV. DAILY CONSUMPTION	
		Vitamin B_{12}	Aureomycin
		μg	*mg*
I	Placebo	0	0
II	Vitamin B_{12}	65.3	0
III	Aureomycin	0	31.7
IV	Vitamin B_{12} + aureomycin	64.0	32.0

TABLE 2

The heights and weights of Jamaican children

AGE	BOYS			GIRLS		
		Height	Weight		Height	Weight
years	NO.	*inches*	*pounds*	NO.	*inches*	*pounds*
4–5	6	41.8 ± 1.7 [1]	36.3 ± 3.1 [1]	9	40.6 ± 4.8 [1]	36.6 ± 8.9 [1]
5–6	15	44.6 ± 2.2	41.8 ± 6.3	15	42.9 ± 2.8	37.6 ± 4.3
6–7	17	46.1 ± 2.0	43.6 ± 3.9	22	45.0 ± 2.4	41.8 ± 6.1
7–8	101	46.5 ± 2.5	45.1 ± 7.1	98	47.2 ± 2.2	46.2 ± 7.1
8–9	157	48.5 ± 2.6	49.4 ± 7.1	159	48.9 ± 2.4	50.9 ± 6.9
9–10	161	50.2 ± 2.2	54.6 ± 8.2	180	50.9 ± 3.2	55.5 ± 8.3
10–11	146	51.8 ± 2.6	59.5 ± 7.4	173	52.2 ± 2.8	59.3 ± 9.3
11–12	144	53.7 ± 2.6	64.2 ± 8.3	137	54.3 ± 3.2	65.7 ± 11.2
12–13	128	55.7 ± 2.5	71.3 ± 4.1	122	56.8 ± 3.2	75.5 ± 13.7
13–14	107	57.4 ± 3.0	78.8 ± 11.8	111	58.6 ± 2.8	83.7 ± 14.6
14–15	85	58.5 ± 3.2	84.7 ± 13.4	68	59.4 ± 10.1	92.3 ± 14.1
15–16	14	60.5 ± 2.0	94.4 ± 12.5	14	61.1 ± 2.8	100.3 ± 14.7

[1] Mean and standard deviation.

The weight of the children was measured on a beam balance. As there is little seasonal variation in the warm climate of Jamaica, the clothes of the children were light in weight throughout the year, so the children were measured clothed, but without their socks and shoes.

The height was measured with the children standing erect without shoes and with their heels together and their backs and heads touching a vertical scale.

All those children over 12 years of age at the start of the treatment were excluded from the analysis, in order to avoid the effect of the increased growth rate that occurs with puberty. With these restrictions, there were 523 individuals

TABLE 3

Distribution of individuals by sex, town and treatment

DISTRICT	CONTROL		VITAMIN B_{12}		AUREOMYCIN		AUREOMYCIN + VITAMIN B_{12}		TOTAL	
	M	F	M	F	M	F	M	F	M	F
Yallahs	18	27	21	21	18	22	18	23	75	93
	(45)[1]		(42)		(40)		(41)		(168)	
Maxfield Park	23	12	25	15	24	14	25	13	97	54
	(35)		(40)		(38)		(38)		(151)	
Acolus	10	10	9	10	8	6	7	8	34	34
	(20)		(19)		(14)		(15)		(68)	
St. Benedict	8	11	7	9	5	10	6	10	26	40
	(19)		(16)		(15)		(16)		(66)	
Grove	5	4	6	3	9	4	6	3	26	14
	(9)		(9)		(13)		(9)		(40)	
Bull Bay	3	3	3	4	3	5	5	4	14	16
	(6)		(7)		(8)		(9)		(30)	
Total	67	67	71	62	67	61	67	61	272	251
	(134)		(133)		(128)		(128)		(523)	

[1] Total male and female children shown in parentheses.

left whose records were studied. The distribution by treatment, town and sex is shown in table 3. The number of children in each area varies widely, but the number of children in each treatment group is reasonably even.

The criteria for judging the effectiveness of the treatments were: (1) rate of weight gain and (2) rate of height gain. The treatment periods were not of equal length in all cases, nor were measurements always made at the same intervals, there-

fore the gross weight and height gains would not be an adequate measure of the response to the treatment.

For each individual, the rate of weight gain in pounds per month and the rate of height in inches per month were calculated by the method of least squares. An analysis of variance (Snedecor, '46) of these data was made for each of the two variables.

The conclusions which were indicated by the variance analysis are: (1) the rate of weight gain differs among the treatments; (2) The rate of height gain is not significantly

TABLE 4

Growth measurements of Jamaican children

	AV. RATE OF WEIGHT GAIN POUNDS PER MONTH	AV. RATE OF HEIGHT GAIN INCHES PER MONTH
Control	0.60 ± 0.031 [1]	0.184 ± 0.0049 [1]
Vitamin B_{12}	0.56 ± 0.031	0.175 ± 0.0049
Aureomycin	0.67 ± 0.032	0.178 ± 0.0050
Vitamin B_{12} + aureomycin	0.66 ± 0.032	0.176 ± 0.0050
Town		
Yallahs	0.66	0.178
Maxfield Park	0.58	0.201
Aeolus Valley	0.69	0.168
St. Benedict	0.56	0.165
Grove	0.46	0.151
Bull Bay	0.75	0.146

[1] Standard error.

different from treatment to treatment; (3) Both the rate of weight gain and the rate of height gain differ among the towns; (4) The differences (or non-difference in the case of height) among the treatments are not affected by the differences among the towns.

The averages for the treatments and towns are shown in table 4.

Since *both* groups of children who received aureomycin showed greater rates of weight gain than the other groups, it seems probable that aureomycin had a significant positive effect. It must be pointed out, however, that the effect was

small and if the weight gain of only one aureomycin group is compared with that of the control group the difference is not significant at the 95% level.

In addition to the measurements made during the treatment period, the weight and height measurements during the pre-treatment period, the age and the sex of each individual were known. The use of such added information about co-variables is often of value in improving the discriminating power of the statistical analysis. The co-variables which it was expected might be of value were: (1) Rate of weight gain in the pre-treatment period; (2) Rate of height gain in the pre-treatment period; (3) Weight at the start of the treatment; (4) Height at the start of the treatment; (5) Age at the start of the treament.

A preliminary comparison of sex differences with the area-treatment groups showed that this was a negligible source of variation, and it was not considered further. All this co-variable information proved to be of little value in assessing the effects of the treatments. Including the information of all 5 of the co-variables results in only 12% improvement in the estimate of the experimental error. None of the conclusions from the original analysis was affected by including the co-variable data.

The average age at the start of the treatment of the 523 children who were included in the analysis was 116 months. Although a variance analysis shows statistically significant differences among the towns and among the treatments, these differences were not considered to be practically meaningful. The fact that age as a co-variable has no effect on the results confirms this opinion.

Clinical examination. The children were examined before and after the supplementation period and data were collected on the incidence of "Brown hair," conjunctivitis, "Excess tissue," cheilosis, angular stomatitis, perlèche, "Swollen gums," caries, odontoclasia, dyssebacia, depigmentation of the skin, dry skin on arms and legs, "Permanent goose flesh," follicular keratosis, "Glazed legs," "Crackled skin on legs"

and palpable liver. The definition of these signs in general followed that of Abbott ('50), Burgess and Laidin ('50), and Jelliffe and Williams ('54). The picture which emerged from this work was that of marginal malnutrition; for example chelosis was found in 5% of the population, dry rough skin on the legs in 40%, tongue abnormalities in 20% and a palpable liver in 10%. There appeared to be no important differences in the clinical signs following the administration of the supplements. Particular attention was paid to evidence of toxicity resulting from the administration of the vitamin B_{12} or aureomycin, but no such evidence was observed.

Parasitic infestations. Through the courtesy of Dr. Oscar Felsenfeld and his group from Chicago an assessment was obtained for the incidence of parasitological infestation of these children.

A low incidence of infestation with hookworm (1.5%) and ascaris (2.6%) was found. The incidence of Trichuris trichura (4%) and Guardia lamblia (14%) was greater, although, in comparison with African communities, it is very low indeed (McGregor and Smith, '52). This is probably due to the efficient and continuous regular treatment by the Public Health Authorities. The children in Jamaican school receive at regular intervals treatment for intestinal parasites. This treatment by the Public Health Authorities was a continuous and constant factor and was not altered during the period of this study. No antibiotics were given by the Public Health Authorities. There were no important differences in the infestation rates amongst the 4 groups of children. It is interesting to note that in 39% of the cases, the stools contained large amounts of undigested food.

Malaria officers of the Island Medical Service kindly examined the schools in our area during the time of our survey. The children were examined for enlargement of the spleen. The blood smears were taken from all of those with enlarged spleens and random samples of others. Of 698 children examined, 18.2% showed splenic enlargement and 27.0% of all blood smears taken were positive.

Biochemical measurements. Estimations of the concentrations of total serum protein, serum albumin, haemoglobin and serum cholinesterase were carried out on blood obtained from a random selection of children from each group.

These estimations were carried out on blood obtained from the finger tips of the children 9 to 12 months after the beginning of the supplementation period. The total serum protein was estimated in two ways on each sample of serum; by the gradient-tube method of Lowry and Hunter ('45) and by the spectrophotometric estimation of the intensity of the colour produced by the biuret reaction as described by Reinhold.[2] The latter reaction was also used to estimate serum albumin after precipitation of globulin by bringing the serum to a final concentration of 19.5% sodium sulphate and 6.5% sodium sulphite, and saturating the solution with ether. Haemoglobin was measured by the direct photometric method described in Hawk et al. ('47). Serum cholinesterase was measured by a micro modification of the method of Michel ('49), whereby all volumes were reduced by a factor of 10 so that the estimations were carried out using only 10 microliters of serum. The release of acetic acid from acetyl choline by the cholinesterase caused a change in colour of phenol red which was estimated by the decrease in optical density at 540 mμ. From this measurement was calculated the amount of acetic acid liberated and the amount of carbon dioxide which would have been liberated in a system containing a bicarbonate buffer. The values for cholinesterase could thus be expressed in units of QCO_2 as in conventional gasometric methods for cholinesterase. Small volumes of reagents were measured by micro-pipettes constructed according to the directions of Bessey, and optical density measurements for all the methods used here were carried out by means of a Beckmann spectrophotometer adapted for micro work as described by Bessey ('50).

The results of these determinations are shown in table 5. The total serum protein agrees well with the values obtained

[2] Reinhold, J. G. Personal communication.

from North American subjects but there appears to be comparatively less albumin and more globulin in the Jamaican children. A group of 31 normal adults, not West Indians, but living in Jamaica, was found to have an average concentration of serum protein of 7.12 gm % and an average concentration of serum albumin of 4.25 gm %. The hemoglobin concentration appears to be definitely below average values for children from other areas of the world (Hawkins and

TABLE 5

Blood constituents in the groups of children after dietary supplementation

CONSTITUENT		CONTROL (Group I)	VITAMIN B_{12} (Group II)	AUREOMYCIN (Group III)	AUREOMYCIN + VITAMIN B_{12} (Group IV)
Total serum protein, gm % (Gradient tube)	No.	52	47	50	49
	Mean	7.40	7.35	7.29	7.37
	S.D.	0.32	0.41	0.51	0.40
Total serum protein, gm % (Colorimetric)	No.	52	47	50	49
	Mean	7.39	7.33	7.24	7.33
	S.D.	0.32	0.39	0.43	0.41
Serum albumin, gm % (Colorimetric)	No.	52	47	50	49
	Mean	3.85	3.71	3.62	3.76
	S.D.	0.36	0.39	0.32	0.34
Haemoglobin gm %	No.	52	47	50	49
	Mean	11.44	11.69	11.91	11.29
	S.D.	1.7	1.8	1.4	1.5
Serum cholinesterase, Q_{CO_2}	No.	16	20	16	15
	Mean	51	53	50	49
	S.D.	8	9	11	9

Kline, '50). The non-West Indian group of adults (15 males and 16 females) had an average of 14.4 gm % hemoglobin.

Measurements of the concentration of vitamin B_{12} after administration of a test dose of this substance have been reported elsewhere (Patrick, '55). It was found that 19 of the children from group I had an average serum concentration of vitamin B_{12} of 0.21 mμg/ml, in close agreement with the values quoted for healthy North American adults (Rosenthal and Sarett, '52). Nineteen of the children from group II had

an average serum concentration of B_{12} of 0.33 mμg/ml, suggesting that the administration of the dietary supplements did in fact result in a substantial increase in the level of vitamin B_{12} in serum.

In the absence of biochemical measurements made on a normally well-nourished group of children of the same racial and environmental status, it is difficult to evaluate nutritional status from these measurements. The data obtained do not indicate that the administration of aureomycin or vitamin B_{12} had any effect on the concentration of the blood proteins.

Dietary survey. Measurements were made throughout the year on the dietary intake of 165 children, selected at random. The period during which each individual child's intake was measured was 7 days. In addition, a separate survey was made of the diet at the Maxfield Park Orphanage.

The purpose of the dietary survey was to obtain a representative estimate of the dietary intake of the population as a whole. The survey was carried out over a period of one year in order to observe whether seasonal variations were important. It was decided that it would give a better overall estimate of the population's dietary intake if as large a number as practical was observed for weekly intervals rather than a smaller number of the children for a longer period.

As in the majority of the children investigated no marked variation in individual diets was observed, and as no marked seasonal variation was observed, we feel that the results obtained from the 165 children are reasonably representative of the whole population that was studied.

The diets were studied by an investigator "living in" with the family. Two to three families were covered in each 7-day period.

The cooking methods were closely observed, recipes for composite dishes were obtained from the mother and the ingredients checked as they went into the pot. Each child's portion was weighed immediately before it was eaten, any waste being weighed at the end of the meal. School meals were observed and checked and the amounts of "bought

TABLE 6

Average daily per capita intake of various nutrients by Jamaican children

SCHOOL	NUMBER STUDIED	AVERAGE AGE		CALORIES	PROTEIN	FAT	CARBOHYDRATE	CALCIUM	IRON	VITAMIN A	THIAMINE	RIBOFLAVIN	NIACIN	ASCORBIC ACID	PHOSPHORUS	RATIO OF ANIMAL TO VEGETABLE PROTEIN
		yrs.	*mos.*		*gm*	*gm*	*gm*	*mg*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*	*mg*	
Aeolus Valley	43	10	11	1952	44.8	21.3	236	265	13.1	1848	0.730	0.528	6.09	142	606	1:2.5
August Town	7	10	8	1733	49.8	43.6	286	390	16.9	1063	0.953	0.829	7.77	73	637	1:1.7
Bull Bay	17	10	10	1621	45.8	29.9	289	242	12.5	1396	0.932	0.599	8.52	131	628	1:2.8
St. Benedicts	31	12	11	1591	46.5	32	279	215	9.9	1352	0.963	0.639	7.95	46	568	1:2.3
Grove	21	11	6	1671	49	37	289	286	14.1	2781	0.962	0.768	9.71	172	639	1:1.9
Yallahs	47	11	1	1546	46	32	276	308	14.7	1447	0.961	0.706	7.99	151	621	1:1.8
Average of above		11	4	1686	47	32	276	284	13.5	1648	0.917	0.678	8.01	119	617	1:2.16
NRC recommended allowance (Modified for tropical use)		10–12 yrs 78 lbs		2313	70			1100	12	4500	1.20	1.800	14.7	75		1:1.0
Maxfield Park 4–7 years				1561	47.5	46.5	240.5	747	10.5	2048	0.908	1.356	6.43	56	860	
7–11 years				2006	60.5	65	297.5	613.5	13.67	2218	1.179	1.312	8.57	68	889	

lunches'' and extras were weighed whenever possible, but reliance had to be placed on accurate histories where extras were consumed. Consumption of these extras was negligible.

Results were calculated to ''edible portion per head per day'' and food tables (Platt, '45; United States Department of Agriculture, '50 and '52; and the Institute of Nutrition of Central America and Panama, '52) were consulted to obtain the composition of the foods.

A small amount of experimental work was done on the moisture content of various foods to enable composite recipes to be calculated back to raw ingredients. The averages of the results of the dietary survey are shown in table 6. A full description of the dietary survey is in preparation.

The intakes of all the nutrients, with the exception of iron and vitamin C, were definitely lower than the National Research Council standards (1953, adapted for tropical use). The most severe deficiencies were in vitamin A and riboflavin. The low figures observed for calcium intake were obtained from the food intake alone and no allowance was made for the water intake, which, as it comes from a limestone area, may make up the inadequacy of the calcium.

Serum calcium estimations done on a group of 10 children gave the following result which is within the normal range. Serum calcium 10.2 ± 0.74 mg per 100 ml of serum.

The calorie intake of the children was approximately 27% less than the recommended allowance by the National Research Council. The quality of the protein could be described as poor. Instead of the normal ratio of 1 part animal to 1 part vegetable protein, the protein consumed by these children was 1 part animal to 2.16 parts vegetable. The only dietary factor in excess of the recommended allowance was that of ascorbic acid, which is not surprising in a citrus growing country.

DISCUSSION

It is well known that antibiotics act as growth factors in animals. Therefore it is reasonable that an investigation of

the influence of these factors in human subjects should be undertaken as, should a positive effect be observed upon growth it might provide us with a means of alleviating malnutrition. The obvious way to alleviate malnutrition is to remedy all of the dietary deficiencies but this is not always a practical possibility in tropical areas such as the Caribbean, and therefore the importance of examining any suggestions for a solution to nutritional problems cannot be over-emphasized. For this reason this study was undertaken.

The results obtained here suggest that aureomycin has a slight positive effect on weight gain, but no effect on height gain in these children. There was no positive effect observed from the administration of vitamin B_{12}, but it should be emphasized that for the practical purpose of alleviating malnutrition these results are disappointing and it is unlikely that such factors would be of any practical value in dealing with the problem of growth failure where marginal malnutrition exists.

It might be that more obvious effects would be observed in a population which was more chronically and severely undernourished than that in Jamaica. The low intake of animal protein at first suggested that the children may have been deficient in vitamin B_{12} but the estimations of serum B_{12} concentration failed to confirm this.

The estimation of nutritional status where the malnutrition is only marginal is a difficult assessment and we have yet to find a satisfactory index for this measurement. We had thought that growth measurement might be most helpful, and this may be the case in large groups of children, but in the individual case it is probably of little value.

The study is self-consistent; that is, it was run in several towns and the results are essentially the same in all the towns. Since there is this high degree of internal consistency we believe that the explanation of the disagreement of the results here with other studies must be due to factors which distinguish the children in Jamaica from the children who were subjects of the other studies.

SUMMARY

1. A study was made to examine the influence of vitamin B_{12} and aureomycin upon the rate of growth in protein-deficient children.

2. The results of this study present evidence for a slight positive effect of aureomycin upon the rate of weight increase, but no effect upon the rate of height increase.

3. The results show that vitamin B_{12} has no positive effect upon weight or height increments.

4. The nutritional status was observed to be low. Among the observations made were clinical examinations, growth measurements, biochemical measurements and a direct dietary survey.

5. There is no evidence to suggest that either aureomycin or vitamin B_{12} would be of practical value in alleviating the malnutrition found in such areas as the Caribbean.

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