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ALFRED FABIAN HESS

Alfred Fabian Hess
— *A Biographical Sketch*

(October 9, 1875 — December 5, 1933)

The father of brain chemistry, J. L. W. Thudichum, wrote that *"Work, work, and again work* were the three main features" of the success of science (Drabkin, '58). These might easily have been the words of Doctor Alfred Hess, a study of whose life reveals that several similarities existed between these versatile giants. Both derived the stimulus for investigation from clinical patients who furnished their problems, and patients ultimately benefited from their solution. They were both men with triple careers of physician, scientific investigator and teacher. Such a career, according to Thudichum, merely demands that one is to "do the work of three men." To neither came major academic appointments and both made their scientific contributions despite a busy practice of medicine. Drabkin notes that such an individual must be prepared at times to be lonely and misunderstood. Surely Hess and Thudichum shared these fates.

Too often in our present day world do we hear distinctions made between clinical and basic researches. Too often is it implied that the physician without a full-time academic appointment in a university or medical school has no opportunity of contributing to the understanding of disease, basic physiology, nutrition, or biochemistry. Reflection on Hess's career and contributions serves admirably to illustrate the falseness of this bigoted view. Such reflection serves further to illustrate the tremendous contribution to the science of nutrition made by physicians—contributions oft-times attributed to others by the enthusiastic, academically oriented laboratory researcher and teacher.

Accordingly, on this the 26th year after the death of Alfred Hess and the year after which his illustrious collaborator, Windaus, has died, it seems particularly fitting to review his life and works. Unfortunately, neither of the present biographers had the privilege of knowing Doctor Hess. Accordingly, they have drawn heavily on the published accounts of others for the details of his life. For much of the personal information concerning him they have had the privilege of communications and discussions from those who knew him, particularly Mrs. Alfred Hess, Mrs. Helen Benjamin, Dr. Samuel Karelitz, Dr. Edwards A. Park and many others. The appraisal of his work and of its importance is, however, our own.

Alfred F. Hess was born in New York City on October 9, 1875, the son of Selmar and Josephine Hess. Until his death on December 5, 1933, at the age of 58, New York was his home except for periods of study in Europe. His father was successful in business and afforded him every educational advantage that he desired. Following attendance at private preparatory schools in New York, he spent a year at Columbia University, then transferred to Harvard where he graduated in 1897. The next year he enrolled at the College of Physicians and Surgeons in New York City and from this institution received his M.D. degree in 1901. He served an internship at Mt. Sinai Hospital, New York City, where he remained for two and one-half years. Graduate studies at Prague, Vienna and Berlin occupied the next two years. On a brief visit home during this period he courted and married Miss Sara Strauss. They returned to Europe and spent their honeymoon in Prague.

Back in New York, Hess spent a short time at the Rockefeller Institute and then entered the private practice of pediatrics which had always been his chosen field. His practice was never allowed to interfere with his scientific activities, which were carried out in several laboratories over the course of 25 years. Fortunately

he was financially independent and was able to devote an increasing portion of his time to research, most of which he financed. He never held a full-time teaching position. A fortunate result of this was freedom from administrative tasks. This practicing pediatrician became one of the foremost medical investigators of his day. His contributions to the field of nutrition were numerous and often turning points in this rapidly developing field.

His marriage was an ideally happy one for both him and his wife (Flexner, '36). Mrs. Hess was perhaps the first person to recognize that "under a modest and more or less inarticulate exterior, he had great gifts of mind and heart. She herself had her own interests throughout life. Mrs. Hess loved people. Doctor Hess loved competent persons with whom he could discuss his interests—scientific, medical, artistic or what not." He counted among his close friends such men as Abraham Flexner and Edwards A. Park, whose aid Mrs. Hess enlisted in the posthumous publication of his collected works (Flexner, '36). Despite his enviable reputation in both the United States and Europe as a medical scientist, his innate reticence and meticulously analytical approach to persons and phenomena did not lead to great social popularity.

It has often been said that Hess did not desire recognition in the form of a university professorship or the sharing in the Nobel Prize. Abraham Flexner, in his biography ('40), states that this failure of academic recognition did not worry Hess, but left him all the more time for research. "He was quietly amused by the fact that he was passed over, but it did not sour or disappoint him." It is evident that Alfred Hess would have been less than human had he been able to view such disregard with complete indifference. It is more probable that his equanimity was such as to mask his disappointment.

In a letter to a friend he wrote, "Work, work, work. Success comes perhaps too late but always lasts too long. Men live on their reputations." In the judgment of Flexner ('36), "it could be said (of Hess), if it could be said of any man, that he lived in the spirit of the injunction: work while it is still day for the night cometh wherein no man can work."

Throughout his scientific interests he was extremely sensitive to human suffering. One of his earliest ('14) professional concerns resulted in a paper entitled "The neglect to provide for the infant in the anti-tuberculosis program." With his characteristic thoroughness in pursuing matters another paper appeared 4 years later entitled "A tuberculosis preventorium for infants." It is said that he limited his practice to infants under the age of 5. However, his personal warmth as a physician and his unstinting giving of all of his talents led to the care of children of close friends until they had more than reached adulthood.

Doctor Samuel Karelitz of New York recalls asking Doctor Hess in consultation concerning an infant extremely ill with what is now recognized as erythroblastosis fetalis. Doctor Hess came to the bedside from a dinner party and proffered wise advice. He became engrossed in the problem and over the next few weeks, in his zeal to do all that he possibly could to help, repeatedly called or sent Doctor Karelitz references or some new fact concerning the disease.

As a scientist, Hess proceeded slowly and deliberately to analyze problems which usually came to his attention through such clinical experiences, and then proceeded to reduce these to a critical question which could be studied either in infants or the laboratory. His studies on infants were made at the Hebrew Infant Asylum, which he transformed from a well-meaning but old-fashioned institution into a modern establishment with medical and nursing staffs. He always felt that results obtained in experimental animals must not be considered seriously for clinical application until they had been confirmed in the human. His observations in the human were oft-times uncanny on their quantitative aspects. For example, he compared the efficacy of the potato and fruit juices as anti-scorbutics, demonstrated that certain preparations of dried milk retain a significant degree of anti-scorbutic activity, and that pasteurization reduces the anti-scorbutic potency of fresh milk. Hess's recognition of the effect of oxidation on

destruction of ascorbic acid led him to point out in his Harvey lecture of 1921 (Hess, '21) that it should be "possible so to alter the process of manufacture or of the preservation of foods to increase their anti-scorbutic content, and render them more nearly equivalent of fresh food." Wide recognition is now given to this principle that food processing should be designed to preserve nutrient values.

In this same lecture Hess emphasized that deficiencies should not be associated exclusively in our minds with specific diseases such as scurvy or rickets. He spoke of latent nutritional deficiencies and the impairments which result therefrom. On the other hand, his nicety of judgment was such that he insisted upon objective evidence of therapeutic benefit in instances of latent deficiency before attributing symptoms to a lack of nutrients. He was never guilty of the exaggerated claims of the over-enthusiastic nutritionist for the benefits derived from additional vitamins. Indeed, in the Cutter lecture delivered at Harvard Medical School, February 14, 1922 (Hess, '22) he stated, "I should like to refer briefly to an aspect which is well known to the children specialists and to other clinicians, but is rarely considered by the laboratory investigator of nutrition. I refer to the opposite of disorders due to deficiency, to disorders resulting from an excess, from an over-supply of one or more dietary factors. It seems quite possible that experiments, which, under prevailing point of view, are judged solely by the criterion of an adequacy of the various food elements, may be more correctly interpreted as due in part to an over-supply of some factor." It is unfortunate, indeed, that this balanced judgment of nutrition so well displayed by Hess did not characterize all of the nutritional writing and thinking during the subsequent 30 years! It is only within the present decade that this sense of balance seems to have been restored to much of nutritional thought.

Abraham Flexner has described Hess's method of work—a method which it would be well for more to emulate ('36), "He began the day by disposing of his mail and then turned to some current journal. Almost everything he read suggested ideas, but before he began on any specific problem he would spend hours and days and weeks looking up the literature of the subject so as to avoid repetition and trimming of his conception down until it became a relatively simple statement of the end which he undertook to reach. In a very high degree he possessed the capacity to reduce in this way complex problems to a manageable 'Fragestellung.' He had a way of noting on small white cards items, ideas, and points of view which seemed spontaneously to germinate in his mind. In discussions with his subordinates complete frankness prevailed, and he was always ready to modify his problem when good reasons were given. Unexpected results did not frighten him, nor did he lightly discard them. On the contrary, they led to further investigation with a result that his problem often changed as he worked, but he never settled down to experimental work without a long and patient process of reasoning beforehand. He was one of those rare scientists who do not refrain from experiment because of lack of elaborate apparatus or material. On the contrary, simple apparatus and a relatively small number of experimental animals thoroughly and carefully studied usually sufficed to convince him whether he was right or wrong in his hypothesis. In dealing with students he was impatient if they began by using even so simple a mechanism as a stethoscope. He had a way of saying, 'Tell me what you *see*. Often the most important and accurate facts are obtained by observation.'

"His devotion to knowledge as such is perhaps best illustrated by his willingness to turn over his ideas to others whenever he reached the point at which his own fundamental training failed him. He did not stop an investigation. He did not endeavor to carry on clumsily. Having reached the point at which his own competency ceased—and he was an infallible judge in this respect—he turned over a definite problem to someone else. He had a certain prophetic vision which one of his associates describes as 'uncanny' as to the probable results which would be obtained, and he therefore did not hesitate to urge and stimulate those who, in his judgment,

could solve the problem which he had in the shape in which he was ready to turn over to them."

One may summarize Hess's experimental method as follows (Flexner, '36). He first defined clearly and precisely in words the problem itself. Secondly, he considered and often listed known facts on the subject. Thirdly, he listed those facts which were wanting but which were ncessary to solve the problem at hand. He then translated the problem into experimentation. The first probe was a simple, direct, crude experiment with a minimum of sophistication of method. Methodology might later be refined if necessary, but his aim was always to demonstrate a principle and not to become overly involved in the refinement of details which he left to others. He was never satisfied with a conclusion, no matter how logical, unless it had been subjected to experimental tests. He was ever aware of the cost of an experiment and weighed the expenditure of time, of effort or resources against the probable importance of the findings and then decided whether the work was worth the cost.

In the appraisal and interpretation of results Doctor Hess examined the data on the controls first, and only when these were satisfactory would he consider the experimental results. If unsatisfactory, the failure of the controls had to be examined if necessary by additional experimentation. Every effort was made to eliminate personal bias through the use of "blind technique." One experiment led to another in a logical sequence based upon the findings at hand. He did not plan more than one experiment ahead.

He read two to three hours each day in the literature of his subject and in his reading studied the facts or data presented in the paper and drew his own conclusion. He stated that by this method one is just as likely to obtain very valuable information from the poor article as from the good one.

One of his assistants of many years, Mrs. Helen Benjamin, writes, "One of Doctor Hess's most outstanding characteristics was his extraordinary preoccupation with his work. Indeed, it seemed at times as if he had no other interests or hobbies. (He once said that his wife was his only hobby.) I recall his coming to the laboratory in the morning every once in a while full of enthuiasm for some newly planned research which he had thought out in detail the night before—during a concert. The music, he said, hardly disturbed him at all.

"Another incident which demonstrates the same quality deals with a visit Doctor Hess made to the library for some reference material. The volume he asked for was a very old one and the librarian had great difficulty in finding it. She finally returned from the stacks about 20 minutes later covered with dust and full of apologies. 'Not at all,' said Doctor Hess, 'I was so busy thinking that I didn't notice that you took so long. Besides I've decided on a completely new approach. I think I won't bother with that reference at all.'

"Along with this kind of absent-mindedness, which sometimes gave the appearance of a disregard for the feelings of others, Doctor Hess had a sparkling but occasionally biting sense of humor. The combination earned him a few enemies. Yet without exception those who knew him well and understood his quirks held him in great affection and esteem. He was nevertheless a prodigious worker, willing to drive himself hard and often remarking that 'nothing comes easy.' Yet the most characteristic pose for a portrait would have been seated at his desk, apparently relaxed, his activity hidden from easy sight in his unusually scintillating mind."

His scientific approach to a clinical problem can be illustrated from a paper published with Victor C. Myers in the Journal of the American Medical Association for December 6, 1910, entitled "Carotinemia: a new clinical picture." This description of carotinemia followed his observation of two infants receiving a daily ration of carrots during the testing of the food value of dehydrated vegetables. In the course of the short space of the 4 pages of this report we can discern the evolution of this subject in his mind. He provided a complete clinical description, suspected on clinical grounds its pathogenesis and confirmed the hypothesis by feeding carrots to other children on the same ward. The pigment was detected in the plasma and its solubility in purified pe-

troleum benzin was noted, and it was distinguished from xanthophyll by solubility tests. The range of individual variation was predicted on the basis of observations in 4 patients and there is indicated a continuing interest in the preparation of an extract of carotene from carrots, its parenteral administration and its excretion in the urine. There is evidence of a thorough review of the previous clinical and experimental literature. Other than some more quantitative observations, the subsequent 49 years have added very little to the understanding of carotinemia in infants.

In everything which he undertook, Hess was equally as thorough in his observations, as imaginative in planning experiments, and as keen in his judgment of their clinical application as in this important but simple clinical observation which must have escaped the attention of physicians for many years previously.

Although usually thought of in relation to nutrition, the contributions of Alfred Hess to medical sicence cover many fields. He described the use of a simple duodenal catheter in infants only two years following its introduction for adults and made some of the first observations on the pancreatic enzymes in infancy. The technique that he described is still a valuable tool in the study of fibrocystic diseases of the pancreas. He had an early interest in infectious disease, particularly in tuberculosis. Much of this experimental work was carried out in the laboratory of Doctor William H. Parke in the New York City Health Department. His bibliography of 227 published papers includes such a diversity of non-nutritional subjects as "Fatal Obliterating Endophlebitis of the Hepatic Veins," "An Examination of Excised Tonsils," "Car Conductors as Dissimenators of Tuberculosis," "German Measles (Rubella): an Experimental Study," "Institutions as Foster Mothers for Infants." He was first to recommend splenectomy in idiopathic thrombocytopenic purpura. In the course of his early studies on the coagulation of the blood in scurvy he noted and described thromboplastin. Both observations were important discoveries in their own right.

The major nutritional interests of Doctor Hess are summarized in two classic monographs ('20, '29) which appeared in 1920 and 1929. The monograph on scurvy is comprehensive and was written after 7 years of intensive study both in man and with guinea pigs. It merits comparison as a mile post in our knowledge of scurvy with the treatise of Lind ('53) and remains the authoritative source book next in date after 1753. Although the chemical nature of ascorbic acid was unknown and analytical chemical methods were not to be established for another decade, there is little that one can now add to the definitive description of the clinical and pathologic picture, prevention and treatment of infantile scurvy given in Hess's monograph. One particular chemical contribution was Hess's demonstration of the catalytic action of minute amounts of copper in the destruction of the anti-scorbutic vitamin in milk, which was reported with Unger in 1921.

His most basic contributions to nutrition were made during the course of studies on rickets which occupied his entire attention during the last 14 years of his life. His first paper on the subject was published in 1917 and demonstrated the effectiveness of cod liver oil in the protection against rickets of Negro infants. He became interested in the seasonal and geographical variations in the incidence of rickets, related these observations to sunlight, and then proceeded to study systematically the influence of sunlight and other sources of irradiation (mercury vapor and carbon arc lamps) on both human and experimental rickets. This led methodically to observations on the ability of ultraviolet irradiation to impart anti-rachitic properties to foods and to cholesterol and its derivatives.

By the spring of 1925 it had been established that the anti-rachitic dietary factor was distinct from fat soluble vitamin A; that rickets could be prevented by either cod liver oil or by exposure to ultraviolet light; that foodstuffs developed anti-rachitic potency after exposure to ultraviolet rays; that cholesterol or a closely related sterol could be rendered anti-rachitic by

similar means; and that activation of cholesterol was attended by a change in its optical spectrum.

Hess was aware of all of this information and had confirmed most of the observations in his own laboratory. In March, 1925, he wrote to the German chemist, Adolph Windaus, who had devoted his life to studying and classifying compounds related to cholesterol. His request that Windaus join the fight against rickets by giving attention to his sterol problem received a courteous reply. However, it was not until more than a year later that further communications from Hess bore fruit. Hess obtained a whole series of cholesterol derivatives from Windaus and irradiated these with ultraviolet light and tested the products in rats. Initial studies established the fact that cholesterol itself was not the compound so activated. In the February, 1927, issue of the Proceedings of the Society for Experimental Biology and Medicine appeared a paper by Hess and Windaus entitled "Development of marked activity in ergosterol following ultraviolet irradiations." This reported that rickets had been cured in rats by as little as 0.003 mg per rat per day. This brief, succinct, classical paper contains but 206 words. It ends with the sentence, "This is a complete report."

The following year Windaus was Awarded the Nobel Prize in chemistry for the work that culminated in this report. Although Hess was not included in this honor, Windaus repeatedly gave him credit for his part in stimulating this research and he shared with Hess the monetary portion of the award as evidence of this indebtedness. (These funds were used to finance additional researches.) In a paper presented before the Prussian Academy of Science on July 1, 1937, Windaus stated that "his studies had always followed a systematic rate of development, namely, one study suggested the next, but in the question of the anti-rachitic vitamin it was not so—I indicate that the stimulus to participate in the vitamin studies was given by Alfred Hess of New York." Windaus died in June, 1959. The correspondence between these two collaborators is currently being prepared for publication by Doctor Samuel Karelitz.

Although many of Hess's friends felt that he should have received the Nobel Prize as well, as has been pointed out by Ashley Weech ('58) the acclaim received by Windaus was of great importance. "The experience of Hess in 1925 of being unable to persuade an organic chemist to collaborate actively in solving a medical problem was by no means unique. Research in the basic sciences in its effort to achieve the status of applied research was oriented toward industry rather than biology or medicine. The awarding of the prize to Windaus changed all this. In 1929 Hess himself complained at the number of communications appearing from laboratories of pure as well as of applied science had become so great as to make it almost impossible to keep pace with them." Perhaps the efforts of Hess to marshall the abilities of others to solve the clinical problem which occupied his attention may have been in large part responsible for the extensive collaboration which now exists between many sciences and medicine.

Many honors did come to Alfred Hess. His early endeavors earned him membership in the American Pediatric Society and later the Association of American Physicians. He delivered a Harvey lecture in New York, the Cutter lectures at Harvard, the Ingleby lectures at Birmingham, England, and was awarded the honorary degree of Doctor of Science by the University of Michigan. In 1927 he received the John Scott medal from the Franklin Society and, 4 years later, the John Mather Smith Award for his work on nutrition.

Hess continued to work under high pressure despite warnings by his physician that he should reduce his pace and give up public speaking because of hypertension and minor cardiac attacks. He disregarded this advice and insisted on speaking at a nurses' graduation exercise on December 5, 1933. He suddenly collapsed and died in his automobile on the way home.

Time does not dim, but highlights, the contributions of Hess. Perusal of his collected works 25 years following his death is more like reading current literature than history. His thinking was so sound and his judgment so fine that little that he

authored had less pertinence and validity today than when it was written. Not least among his contributions is the example which he set for nutritional scientists in his clinical curiosity, his method of work and pattern of investigation.

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The Enhancing Action of Certain Carbohydrates on the Intestinal Absorption of Calcium in the Rat

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It has been known for many years that dietary lactose exerts a stimulating effect on the retention of calcium in several species of animals. The metabolism and physiologic functions of lactose, including its effects on calcium utilization, have been reviewed by Duncan ('55) and Atkinson et al. ('57). Fournier ('54) and Wasserman and Comar ('59) have shown that certain other sugars are also capable of enhancing calcium retention in the rat, namely, cellobiose, raffinose, melibiose, glucosamine, mannitol and sorbitol. Glucose, galactose, fructose and sucrose have little or no effect on calcium utilization. Wasserman and Comar ('59) could find no correlation between effectiveness in promoting calcium or strontium absorption and any of the usual physical or chemical properties of the carbohydrate. However, the property of enhancing absorption of the alkaline earth metals appeared to be positively correlated with a prolonged residence time of the carbohydrate in the gut.

The relationship of lactose to calcium utilization has been studied extensively, and several theories attempting to explain the enhancing action of lactose have been proposed. One well-accepted assumption has been that lactose increases calcium absorption by lowering the pH of the gastrointestinal-tract contents through the action of lactobacilli, which utilize the slowly absorbed lactose in their own metabolism and release lactic acid. The extensive work of Fournier et al. ('55), however, indicated that intestinal pH is not a major factor in calcium utilization, i.e., some of the sugars that have the greatest effect on calcium metabolism are those attacked with difficulty by intestinal bacteria. These investigators suggested the idea that those sugars they termed "structural carbohydrates," lactose, galactose, xylose, arabinose, mannose, melibiose and raffinose, exert their effects in some manner by influencing the bone cell in the process of ossification.

Recent investigations by Lengemann et al. ('59) and Lengemann ('59) indicate that the primary action of lactose is not exerted in the bone cell, but rather in the intestine at the site of calcium absorption. Lengemann showed that calcium absorption from the ileum could be stimulated only if calcium and lactose were together in the same segment, and that rats fed for a two-week period a diet containing 10% of lactose prior to feeding of radiocalcium did not absorb a greater proportion of calcium than did rats fed a diet containing 10% of glucose.

The stimulation of calcium absorption by certain substances may be caused by stimulation of the flow of digestive juices which in turn produces the positive effect. Suggestive evidence was obtained by Lengemann and Dobbins ('58); they showed that bile has an enhancing effect on calcium absorption.

The experiments reported in this paper indicate that lactose and certain other sugars exert their positive influence on calcium absorption because they are slowly absorbed and are able to reach the lower intestinal tract. Sugars such as glucose, sucrose and fructose are shown to have an enhancing effect similar to that of lactose when they are injected directly into a ligated ileal segment along with Ca^{45}-labeled calcium chloride.

METHODS

The experimental techniques described in detail by Lengemann et al. ('59) were used with only slight modifications.

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Young male albino rats purchased from Holtzman and Company, weighing between 100 and 125 gm, were fasted 24 hours prior to the experiment. The rats were anesthetized with ether, their abdominal cavities opened, and the duodenum ligated approximately 8 cm distal to the pyloric sphincter. In other rats, the ileocecal junction and an area 12 to 15 cm proximal to the ileocecal junction were ligated to form closed sacs of uniform length. One-half milliliter of test solution was injected into each sac, and the incision was closed by suturing peritoneum, muscle and skin with silk. Preliminary experiments had established that it was not necessary to ligate the duodenum at the pyloric sphincter, since the rate of calcium absorption was the same with or without a ligature at this site.

The test solution consisted of 5 mg of $CaCl_2$, 10 μc of $Ca^{45}Cl_2$ and 100 mg of one of several sugars or bile per milliliter of solution. In the inhibition experiments, enough Mg^{++}, Sr^{++} or Na^{+} as the chloride or PO_4^{---} (Na salt, pH 7 was added to the test solution to make the molar concentration of Ca^{++} and inhibitor exactly equal.

Except in the time study, the rats were killed 4 hours after injection. The femurs were removed, ashed for 48 hours at 525°C and dissolved in 10 ml of 3 N HCl. Aliquots of the acid solution were spotted inside circles drawn on a large sheet of Whatman No. 1 filter paper. After the paper was allowed to dry at room temperature, the circles were cut out and counted for Ca^{45} activity with a Nuclear-Chicago D-47 gas-flow, micromil window counter and Model 186 scaler.

In the kinetic studies, the ligated intestinal segment including its contents, as well as the femurs, was removed. The segments were excised with the ligatures intact, placed in 5 ml of dilute NaF solution, and cut into small pieces. After standing in a refrigerator for 24 hours, aliquots of the aqueous solution were used for counting radioactivity and for glucose determinations by the method of Folin and Wu.

RESULTS

Since rapidly absorbed sugars such as glucose, fructose or sucrose ingested with a test dose of calcium do not increase utilization of the latter, even though much of the sugar would remain unabsorbed in the upper part of the gastrointestinal tract, it was doubted that they would influence calcium absorption from the duodenum. However, these sugars, as well as more slowly absorbed sugars and bile, were tested for their effect on calcium absorption from duodenal segments.

None of the substances tested, except possibly lactose and sorbitol, increased the rate of calcium absorption from the duodenal segment. A slight enhancing effect of lactose and sorbitol was not statistically significant.

The same substances used in the duodenal experiments were tested for their effect on calcium absorption from ligated ileal segments. Each of the sugars markedly enhanced calcium absorption (table 1). Bile did not affect calcium absorption from ligated ileal segments, although it has been shown to have an enhancing action when injected intraperitoneally (Lengemann and Dobbins, '58).

The absorption of Ca^{45} from the ileum in the presence of glucose was determined as a function of time after injection. The degree of Ca^{45} absorption and the femur content of Ca^{45} at various time intervals are shown graphically in figure 1. There was a positive glucose effect at 30 minutes, the shortest time interval recorded, on Ca^{45} deposition in the femur. The femur

TABLE 1

The influence of various sugars and bile on calcium absorption from the ileum[1]

Number of rats	Test substance	Percentage of administered dose in femur	Percentage increase in absorption
6	Control	0.9 ± 0.2[2]	—
5	Bile	1.1 ± 0.3	none
6	Lactose	2.5 ± 0.3	178
3	Sorbitol	2.8 ± 0.1	210
5	Glucose	2.3 ± 0.2	156
3	Sucrose	3.0 ± 0.3	233
3	Fructose	3.0 ± 0.2	233
3	Galactose	3.1 ± 0.2	244
3	Xylose	2.4 ± 0.4	167

[1] One-half milliliter of aqueous solutions containing 2.5 mg of $CaCl_2$, 5 μc of $Ca^{45}Cl_2$ and 50 mg of test substance were injected into ligated ileal segments. Femurs were removed 4 hours after dosing.

[2] Values represent mean ± standard error of the mean.

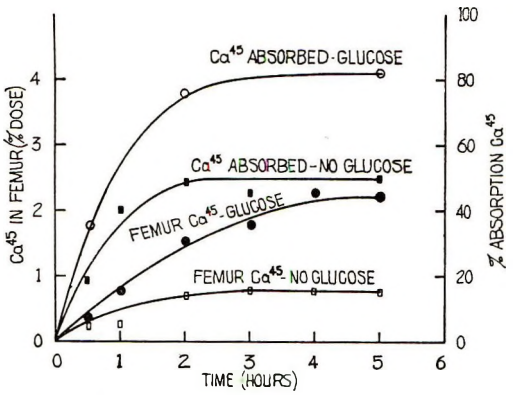

Fig. 1 The femur content of Ca^{45} and the percentage of dose absorbed at various time intervals after the injection of a test dose of Ca^{45}, with or without glucose, into ileal segments.

Ca^{45} content was increased over 100% at one hour, and was almost tripled at 4 hours as compared with the controls. These differences were highly significant ($P < 0.01$). The Ca^{45} absorbed from the ileal segments after each time interval was determined, by measuring the Ca^{45} remaining in the segment, and is expressed in figure 1 as percentage of dose absorbed. The amount of ileal Ca^{45} was measured in only two or three animals per treatment for each time interval. The difference between the control and test animals at one-half hour and at one hour was highly significant ($P < 0.01$), but variation within each group was much greater at longer periods of time ($P < 0.05$ at two hours and $P < 0.1$ at 5 hours). For the controls, 40% of the dose was absorbed during the first hour and approximately 48% had been absorbed by the end of the second hour. Little, if any, absorption occurred with longer periods of time. However, the Ca^{45} activity in the femurs of the control group continued to increase for another hour. In the presence of glucose, 60% of the injected dose was absorbed during the first hour. Rapid absorption continued during the second hour until almost 80% of the dose had been absorbed. Only a small quantity of Ca^{45} was removed from ileal segments in this group after time intervals greater than two hours, although the femurs continued to accumulate Ca^{45} until a maximal value was reached at 4 hours.

In addition to the radioactivity measurements, the glucose in the ileal segments of test animals and controls was measured at several time intervals. These quantities are presented in table 2. It is of interest that the glucose concentration approaches a minimum in the controls at about the same time as calcium absorption ceases (fig. 1). It is apparent from the data in table 2 that considerable glucose is still present in the ileum in the test group three hours after injection. This may account for the longer period of Ca^{45} absorption in this group as compared with the controls (fig. 1).

The effects of Mg^{++}, Sr^{++}, Na^{+} and PO_4^{---} on the enhancing action of lactose and glucose with respect to calcium utilization were studied, and the results are summarized in table 3. Mg^{++} or Sr^{++} decreased Ca^{45} absorption from the ileum in the presence of glucose or lactose, but neither of the divalent cations influenced absorption in the control group. The monovalent sodium ion had no effect on calcium absorption in any of the groups. The addition of phosphate to the test solutions reduced calcium utilization markedly in all three groups. The absorption of Ca^{45} was no greater with glucose or lactose, in the presence of PO_4^{---}, than it was in the controls in the absence of phosphate. The rate of calcium utilization over the 4-hour experimental period in each of the two test

TABLE 2

Glucose remaining in ileal segments at various time intervals after dosing[1]

Time interval	Test substance	Glucose/ segment	Glucose dose absorbed
hours		*mg*	*%*
½	Glucose	32.0	40
½	Control	3.2	
1	Glucose	28.0	50
1	Control	2.9	
3	Glucose	10.8	80
3	Control	0.9	
5	Glucose	0.8	100
5	Control	0.8	

[1] Fifty milligrams of glucose were injected into ligated ileal segments of experimental animals; 2.5 mg of $CaCl_2$ were injected into control and experimental animals. The glucose remaining in the segments after various intervals of time was estimated from pooled samples and is expressed as milligrams of glucose per segment. Three rats per treatment were used.

TABLE 3

The effect of various ions on calcium absorption from the ileum in the presence or absence of sugar[1]

Ion tested	Percentage of Ca^{45} dose in femur		
	No sugar	With glucose	With lactose
Control	0.8 ± 0.1[2]	2.3 ± 0.2	2.3 ± 0.2
Mg^{++}	0.7 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
Sr^{++}	0.8 ± 0.1	1.2 ± 0.1	1.5 ± 0.3
Na^{+}	0.9 ± 0.2	2.6 ± 0.6	2.4 ± 0.3
PO_4^{---}	0.3 ± 0.1	0.7 ± 0.1	0.8 ± 0.2

[1] One-half milliliter of aqueous solutions containing 0.045 moles of $CaCl_2$, 5 μc of $Ca^{45}Cl_2$, 50 mg of sugar and 0.045 moles of the ion being tested were injected into ligated ileal segments. Femurs were removed 4 hours after dosing. Three rats per treatment were used.

[2] Values represent mean ± standard error of the mean.

groups was only one-third as great as that of rats receiving no phosphate. Also, the phosphate ion exhibited a marked inhibitory action on calcium utilization in the control animals.

DISCUSSION

Our studies indicate that the absorption of calcium from the duodenum is little influenced by any of a variety of sugars. On the other hand, all the sugars tested increased greatly the utilization of calcium from ileal segments. Cramer and Copp ('59) have demonstrated that about 65% of a dose of Sr^{89} is absorbed from the ileum when food is ingested at the time of Sr^{89} administration. Much less absorption takes place from the duodenum and jejunum, principally because the alkaline earth metal moves rapidly through the intestines until it reaches the ileum. Lengemann et al., ('59) showed that lactose was most effective in its positive influence on Sr^{85} absorption in the ileum, although they reported an appreciable response in the duodenum and jejunum. From the present studies, it may be postulated that lactose owes its enhancing effect to the fact that it is slowly absorbed (i.e., lactose is slowly hydrolyzed to glucose and galactose which in turn are absorbed) and is available for its role in calcium absorption throughout the small intestine.

Glucose, fructose, galactose and sucrose are absorbed rapidly from the digestive tract by active mechanisms (Hawk et al., '54). Feeding these rapidly absorbed sugars along with a test dose of calcium does not enhance calcium absorption (Wasserman and Comar, '59). On the other hand, the carbohydrates (lactose, cellobiose, sorbose, ribose, xylose, raffinose, melibiose, glucosamine, mannitol and sorbitol), which have been shown to enhance alkaline earth absorption (Wasserman and Comar, '59), are probably all absorbed slowly from the intestinal tract. It is well known that lactose and the pentoses are absorbed slowly and reach the lower portion of the digestive tract, where they influence the microflora (Hawk et al., '54). Likewise, it has been shown (Verzar, '36) that sorbose and glucosamine are absorbed slowly by passive diffusion. The oligosaccharides, raffinose and melibiose, are probably removed slowly from the intestine, since they require hydrolysis by intestinal enzymes before significant absorption takes place. The sugar alcohols, mannitol and sorbitol, are probably absorbed slowly, for there is evidence suggesting that sorbitol exerts an effect on the growth of bacteria in the lower portion of the rat's intestinal tract (Morgan and Yudkin, '57). Since the experiments presented in this paper have demonstrated that a variety of carbohydrates, including glucose, fructose and galactose, have the ability to enhance calcium absorption from the ileum, it appears that ingested lactose and certain other sugars exert their positive influence simply because they are slowly absorbed and reach the lower regions of the intestinal tract where they play an active role in calcium absorption.

Magnesium and strontium are probably absorbed from the intestine by the same sugar-utilizing mechanism which is used in the absorption of calcium. Both of these divalent cations decreased the enhancing action of glucose or lactose on calcium absorption, but had no effect in the control animals in our inhibition study. The inhibitory action of Mg^{++} or Sr^{++} was not caused by increased osmosis or some other physical interference, since Na^{+} did not effect the rate of Ca^{45} utilization in any of the animals tested.

SUMMARY

1. The influence of various sugars and of bile on calcium absorption from ligated

duodenal and ileal segments in rats was studied, using a technique based on the determination of Ca^{45} in the femur following injection into intestinal segments.

2. Bile, lactose, sorbitol, glucose, sucrose, fructose, galactose and xylose did not significantly influence calcium absorption from the duodenum.

3. Each of the sugars above enhanced greatly the absorption of calcium from ileal segments. Bile had no effect.

4. The influence of glucose on calcium absorption from the ileum, and its utilization was studied as a function of time, and a positive glucose effect was apparent at 30 minutes. Glucose increased calcium utilization 100% at 1 hour and 300% at 4 hours.

5. Mg^{++} and Sr^{++} decreased Ca^{++} absorption in the presence of glucose or lactose; PO_4^{---} decreased absorption of Ca^{++} in the presence or absence of sugars; and Na^{+} had no effect in the test groups or controls.

6. It is concluded that lactose and certain other sugars owe their enhancing action on calcium utilization to the fact that they are slowly absorbed from the intestinal tract and are available for a role in calcium absorption throughout the small intestine.

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Anomalous Development of Ossification in the Inner Ear of Offspring of Manganese-Deficient Rats[1]

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It has been known since 1939 that a maternal dietary deficiency of manganese results in defective offspring which exhibit ataxia. Norris and Caskey ('39), working with chicks, were the first to report a congenital ataxia resulting from a maternal deficiency of this element. Ataxia has also been observed in the offspring of manganese-deficient rats (Shils and McCollum, '43; Hill et al., '50; Hurley et al., '58), swine (Plumlee et al., '56), and guinea pigs (Everson et al., '59). Incoordination, lack of equilibrium and retraction of the head are striking symptoms in these young. Since 1939, various investigations have been carried out in search of a lesion, either morphological or biochemical, which could account for this abnormal condition; but neither histological studies of the nervous system (Shils and McCollum, '43; Caskey et al., '44; Hill et al., '50; Hurley et al., '58) nor assays of various enzymes (Van Reen and Pearson, '55; Hurley et al., '58, '59; Everson et al., '59) have revealed the nature of the congenital defect resulting in ataxia.

A recent report from this laboratory showed that development of body-righting reflexes was markedly delayed in the offspring of manganese-deficient rats (Hurley and Everson, '59). Since the vestibular apparatus is involved in righting reflexes, the region of the inner ear has been examined in young of manganese-deficient and normal rats. The present communication reports the occurrence of an abnormal development of the otic capsule in newborn young of manganese-deficient rats.

METHODS

Weanling female rats of the Sprague-Dawley strain were purchased from commercial sources, and were maintained on a ration composed primarily of fresh, whole homogenized market milk, fortified with 400 I.U. of vitamin D per quart, and containing 13 μg of manganese per 100 ml, by spectrographic analysis. The milk was supplemented with the following nutrients per 100 ml of milk: copper (as copper sulfate), 1.3 mg; iron (as ferrous sulfate), 1.3 mg; iodine (as potassium iodide), 1.3 μg; pyridoxine, 100 μg; and corn oil, 0.3 ml. For the control groups, manganese (as manganous sulfate) was added in amounts to provide 600 μg for each 100 ml of milk.

A mixture of crystalline vitamins[2] in glucose,[3] to which was added cod liver oil, was given three times each week, in amounts to provide the following intake in micrograms, per day: Ca pantothenate 500, *p*-aminobenzoic acid and riboflavin, each 100; thiamine·HCl, pyridoxine and nicotinic acid, each 300; menadione, 250; folic acid, 6; biotin, 2.5; vitamin B_{12}, 0.3; and choline, 10 mg; inositol, 5 mg; α-tocopherol, 1.1 mg; ascorbic acid, 1 mg; vitamin A and vitamin D, 15 U.S.P. units each.

At maturity, the animals were mated with normal males receiving a stock diet, and the resulting young of the two groups were compared. Ossification of the inner ear was studied in cleared specimens stained with alizarin red S for visualization

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[3] Cerelose.

of the skeleton, according to the method of Wright et al. ('58). Most of the offspring examined were sacrificed on the day of birth (newborn), but a few animals were studied at one and two days of age.

RESULTS

Examination of the region of the inner ear revealed the following pattern of ossification, which appeared to be the same for both normal and manganese-deficient young. Ossification of the otic capsule begins in the vestibular region with the formation of two osseous arches. The first ossification center appears in the dorsal portion of the most anterior arch (fig. 1). When the dorsal curvature of the anterior arch becomes well-defined, ossification commences in the posterior arch, first at a single center (fig. 2), and subsequently in several centers which then fuse to form a diffuse but clearly-defined curvature (fig. 3). The next site of ossification is the posterior medial curvature of the cochlea which appears to develop from its attachment to the ventro-medial portion of the posterior arch (fig. 4). Shortly after, the lateral wall of the cochlea begins to ossify anterior to the fenestra rotunda; during the course of this ossification, the anterior half of the fenestra rotunda becomes osseous (fig. 5). Subsequent ossification of the cochlear shell proceeds anteriorly to form the first cochlear spiral (A, fig. 6) which contains the fenestra ovalis. At about the same time, the ventral portions of a third dorsally-positioned arch begin to form from the medial aspect of the ends of the anterior and posterior arches (B, fig. 6).

Twenty-three newborn offspring in 8 litters of manganese-supplemented females were studied. Of these, one animal was clearly abnormal, exhibiting various skeletal anomalies, and was therefore rejected for ear studies. (Its litter-mate showed normal development.) The remaining 22 animals showed a range of development varying from the stage of beginning ossification of the posterior medial curvature of the cochlea (fig. 4), to the stage of beginning ossification of the lateral wall of the cochlea and ossification around the fenestra rotunda (fig. 5). Thus, in all of the manganese-supplemented animals, ossification of the anterior and posterior arches was well-established in the newborn.

Offspring of manganese-deficient females showed a marked difference in the degree of ossification of the otic capsule, as compared with their controls. (These results are summarized in table 1.) However, as in the normal animals, a range of stages of development was displayed. In order to provide a basis for comparison of these two ranges, the normal animals were grouped into those showing the *least* development (fig. 4 and column "A" in table 1), and those more advanced. (Only 6 of the 22 normal young showed the minimal development, and stages less developed than this in controls could be found only in fetuses.)

Of the 39 deficient young examined, 22 (56%) showed less development of the otic capsule than was seen in any of the control young. These 22 young came from 11 of the 15 litters studied (73%). Ossification of the inner ear varied in these 22 young from no development at all to establishment of the anterior and posterior arches and appearance of an ossification center in the cochlea. Only two of the 22 animals showed an ossification center in the cochlea, and in 9 animals, ossification of the posterior arch was not accomplished.

TABLE 1

Ossification of the otic capsule in newborn young of manganese-deficient and normal rats

Group	Young examined		Stage of development					
	Animals	Litters	Less than "A"		"A"[1]		More than "A"	
			Animals		Animals		Animals	
	no.	*no.*	*no.*	*%*	*no.*	*%*	*no.*	*%*
Mn-supplemented	22	8	0	0	6	27	16	73
Mn-deficient	39	15	22	56	11	28	6	15

[1] "A" refers to stage of development equal to that in figure 4.

Six of the 39 manganese-deficient young exhibited development of the osseous labyrinth comparable to the most advanced of the supplemented young. (These 6 animals came from two litters.) Thus only 15% of the deficient young, as compared with 73% of the normal young, showed a relatively advanced stage of development. The remaining 11 manganese-deficient animals (28%) showed a stage of development comparable to the least-developed of the manganese-supplemented young.

The number of samples available from control animals at one and two days of age was inadequate to permit a direct comparison with deficient young of these ages. However, examination of 13 one-day old, and 14 two-day old manganese-deficient young revealed that only 6 of these 27 animals exhibited a stage of development comparable to the most advanced of the newborn normal young. In the few two-day old normal animals examined, development had progressed to the formation of the first cochlear spiral (fig. 6).

Comparison of figures 1 to 3 (deficient young) and 4 to 6 (supplemented young) reveals an abnormal size and shape (shortening and doming) of the skull in the manganese-deficient young. Data concerning this observation will be presented in a later publication.

DISCUSSION

Ossification of the inner ear has not, to our knowledge, been previously described in the rat. Wada ('23) studied some aspects of development of the inner ear in this species, but presented little information on ossification. Walker and Wirtschafter ('57), in their report on osteogenesis of the rat skeleton, make only a fragmentary reference to the inner ear. Bast, in his classic studies on development of the ear in the human embryo, observed that ossification begins in the cochlea (Bast, '30; Bast and Anson, '49). However, the present study showed that in the rat ossification of the otic capsule begins in the vestibular region instead. Although there was a range in the degree of development in both the normal and manganese-deficient animals, the results clearly demonstrate an anomalous development of ossification of the osseous labyrinth following maternal manganese deficiency.

This finding is, to our knowledge, the first discovery of a structural defect referable to the congenital ataxia of manganese-deficient young. The results suggest that an abnormality of the inner ear may be a causative factor in this ataxia. Such a hypothesis is compatible with previous work. The delayed development of the righting reflexes observed in manganese-deficient offspring (Hurley and Everson, '59) could be due to abnormal labyrinthine function. The observation of anomalous ossification of the inner ear is also compatible with previous knowledge regarding the importance of manganese for normal skeletal development (Norris and Caskey, '39; Barnes et al., '41; Ellis et al., '47; Wolbach et al., '53; Frost et al., '59).

It remains to be determined whether the abnormal ossification of the inner ear reported here is a simple retardation of development, and if so, whether it persists throughout the life of the animal. It also remains to be determined whether or not there is an anomalous development of the membranous labyrinth as well. These questions are currently being investigated.

SUMMARY

Ossification of the inner ear was studied in young of manganese-deficient and normal rats in cleared specimens stained with alizarin red S for visualization of the skeleton. The pattern of early ossification of the otic capsule is described. Examination of 39 manganese-deficient newborn offspring revealed that more than half (56%) showed less development of the osseous labyrinth than was seen in any of 22 newborn normal young. Only 15% of the deficient young, as compared with 73% of the manganese-supplemented young, showed development beyond the minimum observed in normal newborns. The results demonstrate an anomalous development of ossification of the otic capsule following maternal manganese deficiency. The implications of this observation of a defect referable to the congenital ataxia of manganese-deficient offspring are discussed.

ADDENDUM

The authors wish to point out that in the preparation of this manuscript, an error was noted in the first paper of this ser-

ies (Hurley, L. S., G. J. Everson and J. F. Geiger 1958 Manganese deficiency in rats: Congenital nature of ataxia. J. Nutrition, *66:* 309). The amounts of copper, iron and iodine added to the milk were incorrectely stated and are now included in the correct amounts in the present paper.

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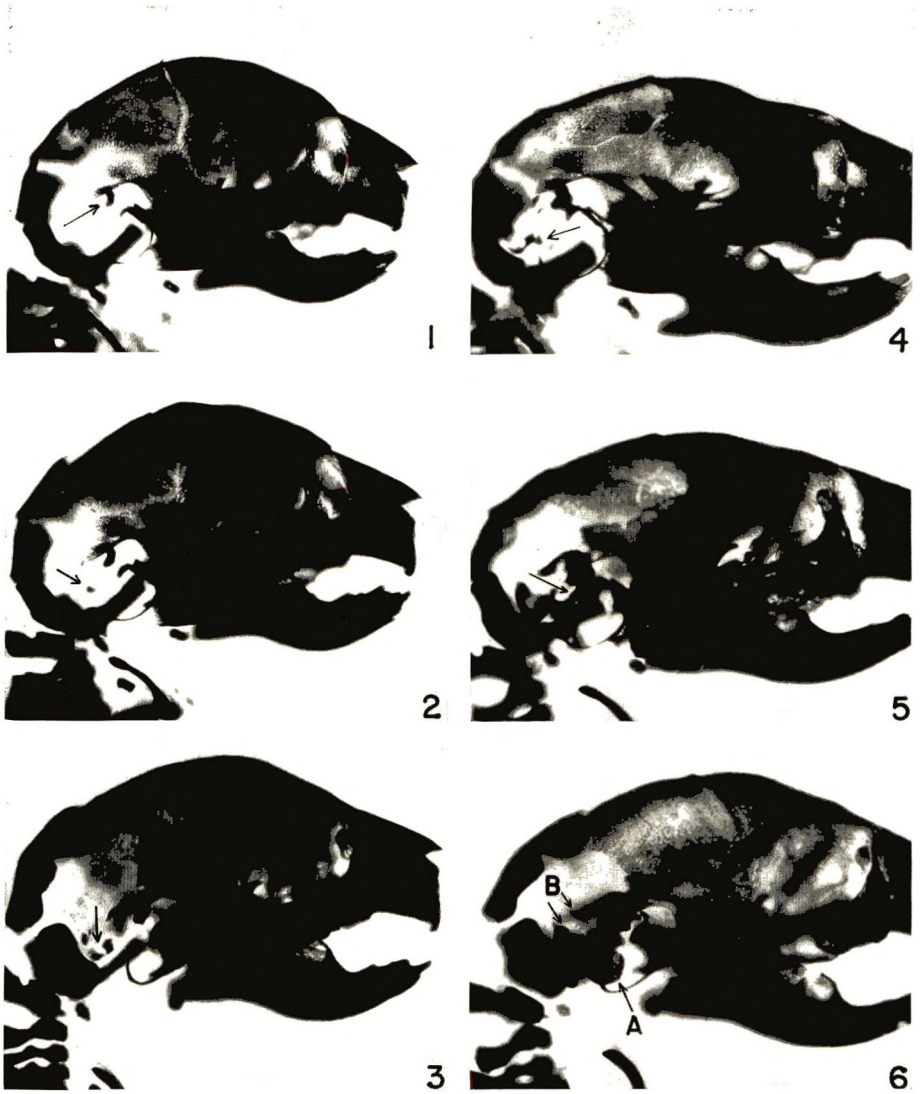

Figs. 1–6 Cleared, alizarin-stained skulls of young rats showing ossification in the otic capsule. × 4.

1 Newborn manganese-deficient rat. Beginning ossification of anterior arch.

2 Newborn manganese-deficient rat. First ossification center of posterior arch.

3 Newborn manganese-deficient rat. Curvature of posterior arch.

4 Newborn manganese-supplemented rat. Beginning ossification of cochlea.

5 Newborn manganese-supplemented rat. Ossification of lateral wall of cochlea and ossification around the fenestra rotunda.

6 Two-day-old manganese-supplemented rat. (A) First cochlear spiral and (B) beginning formation of third arch.

Measurement of the Adaptation Response to Urea-Nitrogen Utilization in the Ruminant[1,2]

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Urea is commonly used as a source of nitrogen for ruminants. However, its practical use is restricted because high levels are not compatible with optimum performance in terms of growth, lactation and wool development. A more precise understanding of factors influencing the utilization of urea or other non-protein nitrogen compounds (NPN) may permit the removal or revision of presently accepted limitations.

Indirect evidence from the review of Reid ('53), the work of Repp and associates ('55) and Ewan et al. ('58) suggests that the utilization of non-protein nitrogen compounds may be improved as the period during which they are fed is extended. Metabolism studies conducted at the West Virginia Station during the past three years have shown that non-protein nitrogen (NPN) utilization by the lamb is significantly improved by extending the period during which the compounds of this type are fed,[3] (Welch et al., '57; McLaren et al., '59). This improvement has been termed the "adaptation response" and will be referred to as such in this paper.

The purpose of the present study was to measure the effect of this adaptation response upon nitrogen utilization by the lamb fed high levels of urea.

EXPERIMENTAL

The effects of prolonged feeding of urea-containing rations was estimated through regression analyses using data obtained from 63 lambs in 19 digestion and metabolism trials. The ration fed contained approximately 1.70% of nitrogen, about two thirds of which was provided by urea. The composition of the ration was as follows: chopped wheat straw, 400 gm; blackstrap molasses (78% solids), 170 gm; and a concentrate mixture, 200 gm, which contained: soybean protein,[4] 13.2; urea, 18.4; cornstarch,76.0; glucose,[5] 17.6; corn oil, 29.4; fish oil,[6] 2.6; and mineral mixture (Thomas et al., '51), 27.2 gm, respectively. Differences in the composition of the wheat straw and molasses used in the nitrogen metabolism and digestibility trials conducted over the three-year period resulted in slight, but important variations in the ration.

Trials consisted of a 10-day preliminary feeding period followed by one or more consecutive 10-day collection periods. Grade wether lambs weighing from 30 to 34 kg were used. Procedures and analytical methods used have been described by Anderson et al. ('59) and Campbell et al. ('59).

A summary of the data studied is presented in table 1. Of the Y values listed, the first 6 and the 8th are means for two lambs, whereas the remaining values are means for 4 lambs. The use of means permitted an appreciable reduction in the labor of computing sums of squares and cross products while retaining adequate degrees

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[2] Based upon data taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree Doctor of Philosophy in Agricultural Biochemistry and Nutrition: Smith, G. S. 1959 Interrelationships of effects of roughage source energy, diethylstilbestrol, vitamin B_{12} and adaptation upon digestibility and urea-nitrogen utilization by lambs fed semipurified rations. West Virginia University, Morgantown.

[3] See footnote 2.

[4] Drackett Assay Protein C-1.

[5] Cerelose.

[6] Nopco XX.

TABLE 1

Summary of observations, means and sums of squares

Trial	Nitrogen utilization	Digestion coefficients		Ration N supplied by urea	Ration nitrogen content	Days from onset of urea feeding	Year in which trial was conducted	Ration crude fiber content
		Organic matter	Crude fiber					
	(Y_a)	(Y_b)	Y_c)	(X_1)	(X_2)	(X_3)	(X_{4a})	(X_4)
	%	%	%	%	%			%
BJ69–1	41.8	62.5	55.5	66.2	1.49	10	1	26.5
BJ69–2–1	41.7	61.1	50.2	66.2	1.49	10	1	25.0
BJ69–2–2	44.6	62.0	52.8	66.0	1.50	20	1	25.1
BJ69–6	44.1	61.0	48.5	66.1	1.56	10	1	24.3
BJ69–7	42.6	60.5	50.7	64.4	1.56	20	1	24.5
BJ69–8	35.7	59.2	43.5	67.2	1.78	10	2	23.2
BJ69–9	37.0	59.5	44.5	68.3	1.67	30	2	24.7
BJ69–10	46.4	59.0	41.3	65.7	1.80	50	2	23.7
BJ69–11–1	41.1	62.3	45.1	62.0	1.66	10	2	23.6
BJ69–11–2	45.1	63.3	45.5	59.3	1.73	20	2	22.8
BJ69–13–1	52.0	63.1	49.9	63.2	1.82	40	2	24.3
BJ69–13–2	52.2	61.8	46.7	62.7	1.84	50	2	23.8
BJ69–14	45.6	64.8	50.1	60.4	1.84	10	2	23.3
BJ69–15–1	46.9	63.0	49.3	61.5	1.68	10	3	22.6
BJ69–15–2	48.2	66.5	53.3	59.7	1.62	20	3	21.9
BJ69–15–3	50.3	63.6	43.1	58.6	1.68	30	3	19.7
BJ69–16–1	47.9	63.3	40.9	60.7	1.82	10	3	19.2
BJ69–16–2	55.5	60.7	46.3	54.1	2.03	20	3	22.4
BJ69–17	46.9	65.6	51.0	61.4	1.77	10	3	21.8
Means	45.6	62.3	47.8	62.8	1.70	20.5	2.05	23.3
Sums of squares	455.03	77.21	308.76	234.64	0.3708	3294.7	10.95	57.81

of freedom to detect any effects of practical importance. The regressions of nitrogen utilization[7] (Y_a), organic matter digestibility (Y_b), and crude fiber digestibility (Y_c) on the variables listed, $X_1 \ldots X_4$, were determined by the procedure for multiple linear regression as outlined by Snedecor ('56).

Differences in the percentage of urea nitrogen and the percentage of the total nitrogen in the ration fed over the three year period occurred as the result of variations in the nitrogen content of the wheat straw and the molasses. These variables, percentage of urea nitrogen and total percentage of nitrogen in the ration, are represented by the terms X_1 and X_2, respectively. The period of time during which the lambs had received non-protein nitrogen prior to any particular trial is represented as the variable X_3. The regressions of nitrogen utilization (Y_a), digestibility of organic matter (Y_b), and digestibility of crude fiber (Y_c) on the length of time urea was fed (X_3), while holding the other independent variables constant, were the measures of adaptation response in NPN utilization in the three analyses. The year in which a trial was conducted (X_{4a}) was included as an independent variable in deriving the regression equation for nitrogen utilization (Y_a), but for the regressions of digestibility of organic matter (Y_b) and crude fiber (Y_c) the year of a trial (X_{4a}) was excluded and the percentage content of crude fiber in the rations (X_4) was incorporated.

RESULTS

A summary of regression analyses and statistical evaluations is presented in table 2.

Nitrogen utilization. The equation $\hat{Y}_a = 111.93 - 1.093X_1 - 1.065X_2 + 0.201X_3 - 0.006X_{4a}$ represents the regression of the percentage of absorbed nitrogen upon the following variables:

X_1, percentage ration urea nitrogen;
X_2, percentage ration nitrogen;
X_3, length of time urea was fed;
X_{4a}, year of trial.

The following observations may be made from the summary of the regression analysis presented in table 2.

[7] The term nitrogen utilization is equivalent to the percentage of absorbed nitrogen retained (Anderson et al., '59).

TABLE 2
Summary of statistical evaluations

Item	Nitrogen utilization	Organic matter digestibility	Crude fiber digestibility
Determination coefficients, $R^2 = \Sigma\hat{y}^2/\Sigma y^2$:	0.827	0.309	0.605
Mean square due to regression, $\Sigma\hat{y}^2/4$:	94.14[1]	5.96	46.73[1]
Mean square due to deviations from regression, $\Sigma d^2/14$:	5.61	3.81	8.70
Standard partial regression coefficients: $b'_{y1.234}$	−0.717	−0.009	−0.655
$b'_{y2.134}$	−0.030	−0.898	−0.497
$b'_{y3.124}$	+1.455	+0.065	−0.163
$b'_{y4.123}$	−0.001	−0.294	+0.718
Standard error of regression coefficients ($d/f = 15$): $S_{by1.234}$	0.116; t = 9.42[1]	0.144; t = 0.03	0.218; t = 2.71[2]
$S_{by2.134}$	0.703; t = 1.51	4.319; t = 3.01[1]	6.527; t = 2.20[2]
$S_{by3.124}$	0.039; t = 5.21[3]	0.038; t = 0.23	0.057; t = 0.88
$S_{by4.123}$	0.008; t = 0.78	0.336; t = 1.01	0.507; t = 3.27[1]
Regression equations: $\hat{Y}_a = 111.9 - 1.093\,X_1 - 1.065\,X_2 + 0.201\,X_3 - 0.006\,X_{4a}$			
$\hat{Y}_b = 85.3 - 0.005\,X_1 - 13.02\,X_2 + 0.01\,X_3 - 0.34\,X_4$			
$\hat{Y}_c = 81.6 - 0.75\,X_1 - 14.35\,X_2 - 0.05\,X_3 + 1.66\,X_4$			

[1] $P < 0.01$. [2] $P < 0.05$. [3] $P < 0.001$.

(a) Of the total variance in nitrogen utilization, 82.7% is attributable to the 4 variables considered.

(b) The variance of per cent nitrogen utilization due to regression is highly significant, ($P < 0.01$).

(c) A highly significant ($P < 0.01$) effect upon nitrogen utilization was exerted by percentage ration urea nitrogen, (X_1) and length of time urea was fed, (X_3).

(d) The length of time urea was fed (X_3) had a very large effect on nitrogen utilization relative to the effects of the other variables included.

As can be seen in table 2, the partial regression of nitrogen utilization (Y_a) on year (X_{4a}) is $b_{y4.123} = -0.006$. The maximum change in Y_a resulting from this variable would be from -0.006 to -0.018 or a change of 0.012. The minor influence of this variable upon nitrogen utilization values is emphasized by the small standard partial regression coefficient $b'_{y4.123} = -0.001$. The "t" statistic leads one to conclude that nitrogen utilization (Y_a) was not influenced by the year of the trial (X_{4a}). Because of the small magnitude of the regression coefficient ($b_{y4.123} = -0.006$), X_{1a} can be deleted from the regression equation without jeopardizing accuracy. The revised equation would be:

$$\hat{Y}_a = 111.93 - 1.093X_1 - 1.065X_2 + 0.201X_3$$

By inserting the mean value for X_2 and the value 10 for X_3 into the above equation the adaptation response is adjusted to a standard 10-day previous feeding period and the equation reduces to $Y_a = 112.2 - 1.093X_1$. This equation expresses the effect of percentage ration urea nitrogen (X_1) upon the percentage of absorbed nitrogen retained (Y_a). This effect is presented in figure 1.

During the three years covered in this study the percentage of ration urea nitrogen varied from 54.1 to 68.3. As can be seen in figure 1, the retention of absorbed nitrogen is inversely proportional to the amount of urea nitrogen present in the ration. It is to be recognized that this relationship must become curvilinear as maximum retention values are obtained with rations low in urea nitrogen and when minimal values are obtained with rations high in urea nitrogen. Unpublished data from this laboratory show that only 30 to 35% of the absorbed nitrogen is retained

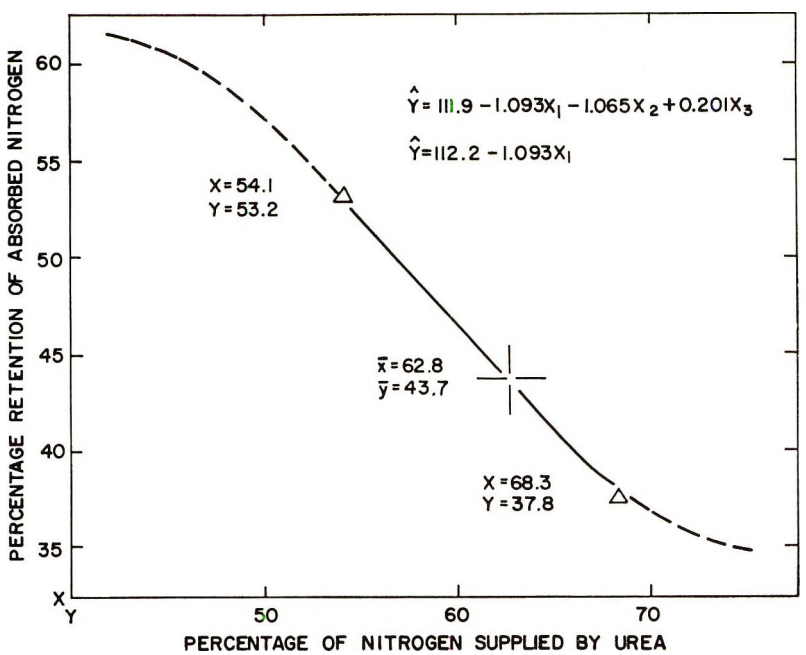

Fig. 1 Effect of percentage of ration urea nitrogen upon nitrogen utilization.

when approximately 90% of the ration nitrogen is supplied by urea.

The partial regression coefficient for time (X_3) $b_{y3.124} = +0.201$ reveals that retained nitrogen absorbed is increased by 0.201 percentage units with each day of urea feeding. This is equivalent to an adaptation response of 10 percentage units or approximately a 20% increase in the retention of absorbed nitrogen during a feeding period of 50 days (fig. 2). The equation $Y = 41.5 + 0.201X_3$, which is derived from the multiple regression equation by inserting the mean values for percentage ration urea nitrogen (X_1) and percentage ration nitrogen (X_2) describes the average adaptation response in the range studied, 10 to 50 days. Although this relationship is linear as presented in figure 2, it should be recognized that the regression must become curvilinear as maximum nitrogen utilization values are obtained.

Application of these equations to the original data is presented in figure 3. The lower curve represents the percentage retention of absorbed nitrogen predicted as the result of ration changes, whereas the upper curve expresses predicted values after considering ration changes and the adaptation response. It is worthy of note that the adaptation response was largely lost during the 50 days of hay feeding, but was re-established when feeding of the experimental ration was resumed.

Digestibility. The equations:

$$\hat{Y}_b = 85.3 - 0.005X_1 - 13.02X_2 + 0.01X_3 - 0.34X_4$$

$$\hat{Y}_c = 81.6 - 0.75X_1 - 14.35X_2 - 0.05X_3 + 1.66X_4$$

represent respectively the regression of organic matter digestibility and crude fiber digestibility upon the following variables:

X_1, percentage ration urea nitrogen;
X_2, percentage ration nitrogen;
X_3, length of time urea was fed;
X_4, percentage of crude fiber.

The determination coefficients for these regressions are given in table 2. The following observations in terms of organic matter digestibility may be made from these data.

(a) Of the total variance in the coefficients of organic matter digestibility 30.6% is attributable to the variables considered.

(b) The variance of organic matter digestibility due to regression is not significant ($P > 0.05$).

(c) A highly significant ($P < 0.01$) effect upon organic matter digestibility was exerted by the percentage ration nitrogen (X_2).

(d) Length of time urea was fed (X_3) did not affect organic matter digestibility.

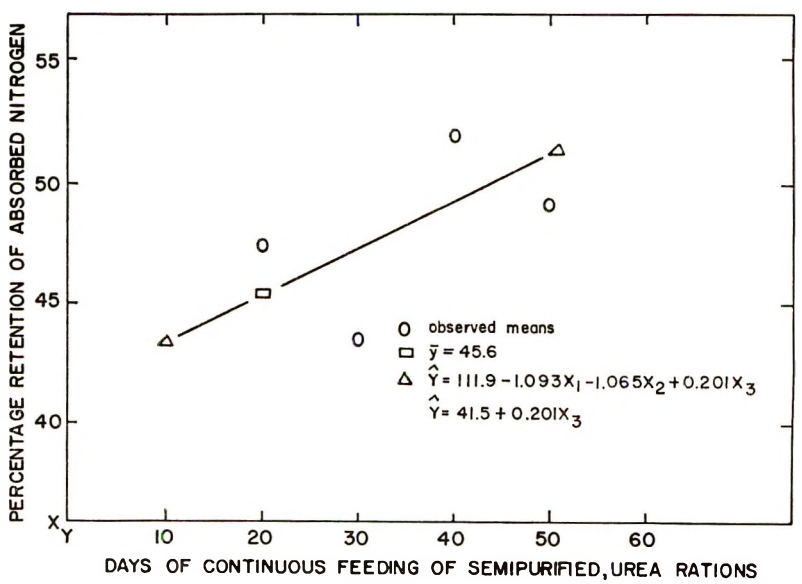

Fig. 2 Improvement in NPN utilization due to continuous feeding of urea.

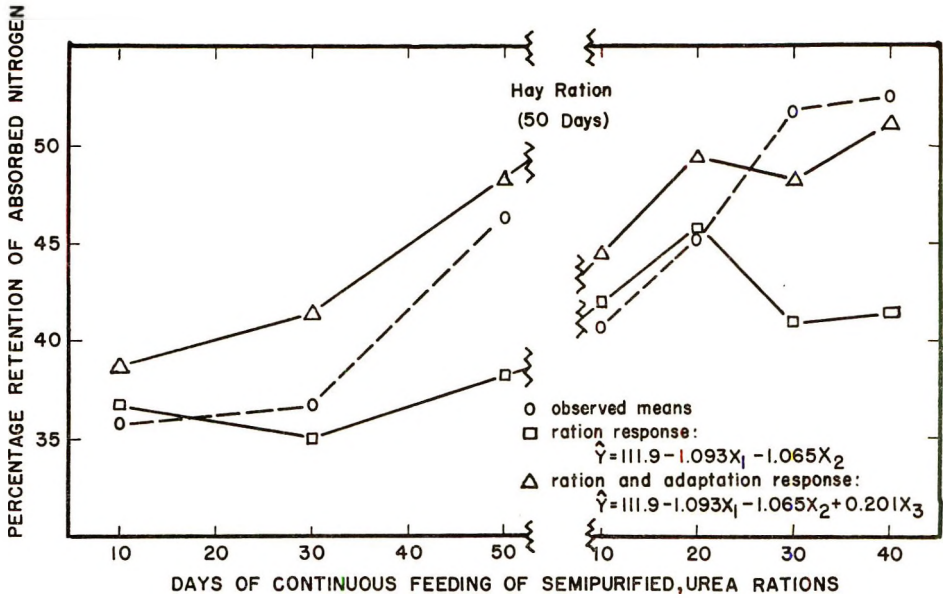

Fig. 3 Response in nitrogen utilization resulting from ration variables and adaptation.

The summary of the crude fiber digestibility regression analyses presented in table 2 indicates the following:

(a) The variables considered accounted for 60.5% of the variance in crude fiber digestibility.

(b) The variance of crude fiber digestibility due to regression is highly significant ($P < 0.01$).

(c) Percentage ration urea nitrogen (X_1) and percentage ration nitrogen (X_2) significantly ($P < 0.05$) influenced crude fiber digestibility, and the effect of percentage of crude fiber (X_4) was highly significant ($P < 0.01$).

(d) Length of time urea was fed (X_3) did not influence crude fiber digestibility.

DISCUSSION

The magnitude of the adaptation response in retention of absorbed nitrogen to prolonged non-protein nitrogen feeding in this study is in general agreement with previous reports from the West Virginia Station (Welch et al., '57; Campbell et al., '59; and McLaren et al., '59, '60). Differences in the magnitude of the adaptation responses reported by these authors result from the use of various non-protein nitrogen compounds as well as the fact that various treatments were superimposed upon the time studies.

The increase in percentage retention of absorbed nitrogen which follows prolonged feeding of urea as measured in this study is sufficient to explain the discrepancies which appear in many comparisons among results from relatively short-term metabolism studies with those from longer-term trials.

Application of the regression equations obtained in this study should be helpful in the interpretation of data obtained in digestion and metabolism studies in which similar variations in the composition of rations and lengths of feeding periods occur.

The mechanism of the adaptation response described is not known. However, two of several possible explanations might be considered. The first is that a specific rumen microbial population capable of efficiently using non-protein nitrogen may have to become established. A second consideration which might contribute to the adaptation response in NPN utilization is an adjustment in the tissues of the host animal as suggested by McLaren et al. ('60).

SUMMARY

Observations from 63 lambs receiving a high-urea, semipurified ration employed in 19 digestion and metabolism trials were subjected to multiple regression analysis in order to measure independently the effect of adaptation to urea feeding and the effects of changes in ration composition. The retention of absorbed nitrogen was significantly improved by approximately two percentage units with each consecutive 10-day feeding period up to 50 days, with no measurable change in the digestibility of organic matter or crude fiber. Increasing the percentage of total nitrogen supplied as urea from 54 to 68% significantly depressed the retention of absorbed nitrogen to the extent of approximately 12 percentage units; had no significant effect upon the digestibility of organic matter; and significantly depressed the digestibility of crude fiber by approximately 8 percentage units. Increasing the total nitrogen content of the ration from 1.5 to 2.0% had no significant effect upon the retention of absorbed nitrogen, but significantly decreased the digestibility of organic matter and crude fiber. Increasing the crude fiber content of the ration from 20 to 26% had no significant effect upon the digestibility of organic matter, but significantly improved the digestibility of crude fiber by approximately 10 percentage units.

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Serum Cholesterol Concentrations of Various Ethnic Groups in Hawaii[1,2]

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In recent years, attention has been focused on the theory that increased concentrations of serum cholesterol are characteristic of persons who are suffering from or are susceptible to atherosclerosis. Because of the difficulty of detecting the presence or severity of atherosclerosis in its early stages, much of the evidence available on this point was obtained by epidemiological methods. This evidence is for the most part consistent in indicating that populations which exhibit a relatively high incidence of coronary disease are also characterized by an increased average serum cholesterol concentration. Many workers believe, however, that the data do not justify the conclusion that there is a causal relationship between increased serum cholesterol concentration and atherosclerosis or even that the two are always coincidental (see reviews by Friedman et al., '55; Van Itallie, '57; Stamler, '58).

Still more uncertain are the answers to the questions of whether diet plays a role in the causation of coronary disease and of what the nature of that role, if any, might be. Some authors (see review by Stamler, '58) believe that the epidemiological studies have shown that increases in the incidence of coronary disease and/or of mean serum cholesterol concentrations are directly correlated with certain characteristics of the typical diet (usually with high fat or with high saturated-fat intakes). Others (Friedman et al., '55; Epstein et al., '56; Pihl, '52; Stare et al., '57; Yerushalmy and Hilleboe, '57) maintain that this correlation is not consistent and that the evidence is inadequate to show whether the observed variations in coronary disease, serum cholesterol and diet are directly related or are incidental to other more basic influences.

Much of the support for the theory that diet is instrumental in producing high serum cholesterol and atherosclerosis is based on comparisons of populations in the United States and in Asia (Snapper, '41; Benjamin, '46; Steiner, '46; Keys et al., '57; Gore, '59). When populations in two different countries are compared, many variables (including climate, diet, physical activity, psychological tensions, economic level and the hereditary characteristics of the population) are necessarily uncontrolled and may influence to an unknown extent the factors being specifically studied. Consequently, any attempt to explain the results in terms of only one variable, such as diet, has been suspect (see review by Friedman et al., '55). Because over one half of the present population of Hawaii are descendants of immigrants from Asia, Hawaii offers an unusual opportunity to supplement the studies which have compared Asian and United States populations. In Hawaii, it is possible to compare Americans of Asian or of European ancestry, but with most of the above differences (with the exception of heredity) eliminated. Keys et al. ('58) have already taken some advantage of this fact by comparing heart disease, serum cholesterol and diets of Japanese with those of Japanese-Americans living in Hawaii and in Los Angeles. For all three factors, the values from Hawaii were found to be intermediate between those of Japan and of Los Angeles.

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In the present study, serum cholesterol concentrations were measured in samples from Hawaii residents of Caucasian, Chinese, Filipino, Hawaiian, Japanese or mixed descent.

METHODS

Cholesterol. Classical procedures for the quantitative measurement of blood cholesterol are time consuming, involve several steps for separation of the cholesterol from most of the other blood constituents and require careful control of such factors as temperature, illumination and time of color development (Sobel and Mayer, '45; Sperry and Webb, '50a). Because of the relative inconvenience of such procedures, various simpler analytical methods have been devised which eliminate one or more of the isolation steps, increase the stability of the measured color, or are applicable on a micro scale (Brown et al., '53; Carpenter et al., '57; Galloway et al., '57).

For the present study a micromethod was required, since it was intended that the blood samples be obtained from finger punctures. The micromethod of Galloway et al., ('57) has been shown by extensive use in several laboratories to be reliable and convenient for the assay of free and of esterified cholesterol in finger-tip blood samples. However, if it is desired to measure only the total rather than both the nonesterified and total cholesterol content of serum, the steps involved in saponification and in isolation of cholesterol digitonide can be avoided. In the method published by Pearson et al., ('53), a modified Lieberman-Burchard reaction is described in which equal molecular amounts of free and esterified cholesterol yield reaction mixtures of the same color intensity. By use of this reaction it is possible to measure total cholesterol concentration without preliminary saponification of the esterified fraction. For a determination, in duplicate, of total cholesterol, the published method requires 0.3 ml of serum. Since this amount of serum cannot be obtained conveniently and routinely from a finger stab, a micromodification of the method was developed.

Two modifications of the Pearson procedure were introduced: (a) Protein precipitation, ommitted in the published method, was found to be necessary for the micromodification. Protein removal from 0.05 ml of serum was carried out as in the method of Galloway et al. ('57). Triplicate cholesterol and lipid phosphorus aliquots of the extract were measured into 10-ml pyrex test tubes immediately after the extract was decanted from the protein precipitate. These aliquots were dried in a vacuum oven or allowed to air-dry before the reagents were added.

(b) By use of microcuvets and microburets, it was possible to decrease the volumes of sample (extract equivalent to 0.005 to 0.01 ml of serum was used) and of all reagents to 1/10th those of the Pearson procedure. The microcuvets and adapter for the Beckman-DU are described by Bessey ('50).

With these two exceptions, the method used for cholesterol assay in the present study was the same as the Pearson method. Readings for triplicate aliquots of a given sample usually agreed within less than 5%. Readings which disagreed by more than 10% were rejected. The reported straight-line relationship between optical density and cholesterol concentration (Pearson et al., '53; Leppanen, '56) was confirmed.

The Pearson method was evaluated by Leppanen ('56) and found to be well suited for routine analysis. However, because of the modifications introduced here, the analytical results obtained in the present study were continuously monitored by the use of a control serum. The mixed reference serum was routinely analyzed with each group of unknown samples. The cholesterol concentration found for this serum remained constant during an entire year and provided a continuous check on the reproducibility of the method. The mean concentration found for 85 analyses of this serum was 198 mg/100 ml with a standard deviation of 13.

Lipid phosphorus. The lipid phosphorus method of Beveridge and Johnson ('49) was found to be readily adaptable to the microscale required, allowing phosphorus analyses in dried serum extracts equivalent to 0.005 ml of serum. The analysis was carried out as published except that the volumes of all reagents used

were reduced to 1/25th of those of the published method. Digestion and color development were carried out in 10-ml pyrex test tubes calibrated at 2 ml, which was the final volume of the colored solution. Optical density was measured in a Beckman Model C colorimeter.

The reference serum used for the cholesterol assay was also used to maintain a continuous check on the lipid phosphorus analytical results.

The methods described have been used for the analysis of approximately 1000 serum samples in this laboratory. One of the advantages which they offer is their relative rapidity. For the analysis of 5 serum samples, an actual working time of about one-and-a-half hours is needed for the cholesterol assay and an additional hour for the lipid phosphorus determination. These time intervals do not include the periods required for color development or phosphorus digestion, during which the attention of the technician is not required.

Subjects and samples. Almost all of the Chinese and Japanese immigration to Hawaii took place before 1900, whereas the first Filipinos did not arrive until 1906 and Filipino immigration continued until 1946. Therefore, while the greater part of the subjects in the present study who were of Chinese and Japanese ancestry were second or third generation Americans, many of the "Filipino" subjects were born in the Philippine Islands. However, all serum samples (with the exception of the Thai group) were from subjects who had lived in Hawaii for at least 10 years, the great majority of them having been born there.

Cholesterol and lipid phosphorus concentrations were measured in 5 groups of sera:

(1) 800 venous blood samples which were collected by personnel of the Territorial Board of Health from Honolulu residents of various (chiefly manual and clerical) occupations.

(2) 100 venous blood samples drawn from plantation workers on the island of Lanai by Dr. Edwin Willet.

(3) 100 samples from University of Hawaii students.

(4) Samples from approximately 100 Honolulu adults.

(5) Approximately 200 samples collected in Thailand in December, 1958.

The samples of groups 3, 4, and 5 were drawn into heparinized capillary tubes from a finger puncture.

Information on body weight, physical activity, and caloric intake was unavailable for groups 1, 2, and 5. It has therefore been impossible to relate these factors to the results.

RESULTS AND DISCUSSION

Dietary patterns. In general, the foods eaten by people of Asian ancestry in Hawaii are more nearly typical of the continental United States than of Asia. The economic standard of living which plays such a major role in controlling the diets in much of Asia is, in Hawaii, largely independent of ethnic group and is equivalent to that of the continental United States. Ice cream, milk, cheese, mayonnaise, eggs and meats, all of which hold a minor place in the diets of Japan and China, are consumed liberally by all groups in Hawaii. Foods ordinarily eaten in the United States are, however, supplemented by others, some of which (such as papaya) are typical of Hawaii, whereas others (such as rice and soy sauce) are representative of the diets of the Far East.

In the present study, to supplement the above general knowledge concerning local diets, detailed dietary information was obtained from 109 University students, most of whom were living at home. They were asked to complete, in class, detailed questionnaires concerning their diets. It is realized that due to inadequacies of the questionnaire method of obtaining dietary data, the figures obtained merely represent approximations of the actual diets of the students. However, it is believed that since the same questionnaire was used by all of the students, the comparisons of different ancestry groups should be meaningful. The results of the analysis of the questionnaires are summarized in table 1 and compared with figures published by Keys et al. ('58). It can be seen that the amount of fat and the proportion of animal fat in the diets of the University students of Japanese and Chinese ancestry appear to be normal for Americans. This is what would be expected from cursory observation of local food habits.

TABLE 1
Percentage of calories derived from fat

City	Subjects and ancestry	No.	Mean age	Calories from fat		
				Animal	Vegetable	Total
				%	%	%
	Present study					
Honolulu	Univ. students: Caucasian	5	21	28	13	41
Honolulu	Univ. students: Japanese	86	20	27	13	40
Honolulu	Univ. students: Chinese	18	19	29	11	40
	Keys et al. ('58)					
Los Angeles	Adult men: Caucasian	(?)	40–49	28	14	42
Los Angeles	Adult men: Japanese	45	40–49	29	10	39
Honolulu	Adult men: Japanese	35	40–49	22	10	32
Shime, Japan	Adult men: Japanese	55	40–49	9	3	12

Serum cholesterol: Honolulu men. The results of the serum cholesterol analyses for Honolulu men are presented by ethnic and age group in table 2. The most obvious ethnic group differences are between the values for the Filipinos (who will be discussed at greater length later) and the other ancestry groups. In table 3, the significance of the differences in table 2 are analyzed. The Filipino value is significantly lower than the Japanese or Caucasian values for age 20 to 39; in the 40 to 49 age group the Filipino value is significantly lower than the Chinese value; in the 50 to 59 age group the Filipino mean is significantly lower than the Hawaiian mean. For age 20 to 39 the Chinese mean value is significantly higher than the Japanese. Other differences in group means for a given age in table 2 are less significant.

The Japanese and Chinese values are similar to that for the Caucasians in each age group, although the Chinese tend to be higher. It is apparent that the low concentrations reported for serum cholesterol in Japan (Keys et al., '58) are not maintained in Hawaii. This indicates that for the population studied here, the influence of race on serum cholesterol concentration is negligible, whereas some shared environmental influence has effectively equalized the cholesterol levels.

The cholesterol concentrations reported here tend to be about 10% higher than those generally reported for normal American men by other authors. Of particular interest are the data obtained in Hawaii by Keys et al. ('58). For the men of Japanese ancestry, age 40 to 49, they reported 223 mg/100 ml, whereas the corresponding value from the present study is 269. Most of the normal serum cholesterol values reported by different laboratories for 40 to 49-year-old American men fall between the values of 232 mg/100 ml published by Barker ('39) and 261 mg/100 ml given by Ackerman et al. ('59). Interlaboratory variation in the results of cholesterol analyses is often considerable even if the different laboratories are all asked to analyze the same sample (King, '53). It is therefore quite possible that the apparent difference between our sample of Honolulu Japanese and that of Keys is not a real one. This possibility is heightened by the fact that the analytical method used in the present study is less specific (Holmgard, '57) than is the procedure (used by Keys' group) which involves digitonin precipitation. Therefore, although the data from the present study are internally consistent (due to the inclusion of a sample of a pooled reference serum with each group of unknowns analyzed), they may not be comparable with data from other laboratories.

Serum cholesterol: Honolulu women. Relatively few serum samples were obtained from Honolulu women. The cholesterol concentrations for Japanese and Caucasian women of two age groups are compared in table 4. Although the numbers of subjects in three of the 4 groups are so small as to be of doubtful significance, the general pattern is the same as that observed for the men: the serum cholesterol concentration rises from about 200 mg/100 ml at age 20 to approximately 250 mg/100 ml at 50, with the

TABLE 2

Serum cholesterol concentrations in Honolulu men

Ancestry	20–39 years			40–49 years			50–59 years			All ages		
	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol
			mg/100 ml			*mg/100 ml*			*mg/100 ml*			*mg/100 ml*
Caucasian	23	27	226 ± 42[1]	39	44	266 ± 50[1]	19	55	269 ± 44[1]	81	41	256 ± 48[1]
Chinese	18	28	247 ± 45	32	43	278 ± 39	25	57	282 ± 51	75	44	272 ± 47
Filipino	16	28	196 ± 31	124	45	251 ± 41	57	54	263 ± 40	197	46	250 ± 44
Hawaiian (part and full)	75	27	240 ± 46	44	44	252 ± 49	22	55	289 ± 55	141	37	251 ± 51
Japanese[2]	57	23	230 ± 34	89	43	269 ± 39	29	54	276 ± 44	175	38	257 ± 43
Various others	18	24	219 ± 32	25	43	263 ± 47	12	56	275 ± 44	55	40	251 ± 48

[1] Mean ± standard deviation.
[2] Data were also obtained for Japanese men aged 60–89 years: N = 19; mean age = 77; mean cholesterol = 225.

TABLE 3

Significance of observed differences in serum cholesterol of various ethnic groups

Groups compared (adult men)	Age range	Difference in group means	Degrees freedom	t	Probability of larger t value[1]
		mg/100 ml			*less than*
Honolulu: Japanese vs. Filipino	20–39	34	71	3.45	0.1%
Caucasian vs. Filipino	20–39	30	37	2.34	2.5
Chinese vs. Japanese	20–39	17	73	2.44	2.5
Chinese vs. Filipino	40–49	27	154	3.39	0.1
Hawaiian vs. Filipino	50–59	26	77	2.26	5.0
Chinese vs. Caucasian	20–59	16	163	2.18	5.0
Honolulu Filipino vs. Lanai Filipino	50–59	37	91	4.20	0.1
Honolulu Filipino vs. Lanai Filipino	40–49	30	139	2.82	1.0
Honolulu Caucasian vs. Lanai Filipino	20–39	26	38	2.12	5.0

[1] The probabilities that the difference among other groups of a given age range occurred by chance are calculated to be greater than 5%. Probabilities were taken from Snedecor ('56).

TABLE 4

Serum cholesterol in Honolulu women

Ancestry	20–39 years			40–49 years		
	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol
			mg/100 ml			*mg/100 ml*
Japanese	51	21	214 ± 32[1]	18	44	248 ± 48[1]
Caucasian	6	23	200 ± 18	21	44	252 ± 43

[1] Mean ± standard deviation.

Caucasian and Japanese values being essentially the same.

Serum cholesterol: Filipinos. The Honolulu data given above and exclusive of the Filipino data indicate that groups with different ethnic background living in the Hawaiian environment have similar serum cholesterol concentrations. What is to be expected with two groups of the same ethnic background living in different environments? The studies of Scrimshaw et al. ('57) and of Keys et al. ('58) have indicated that subjects of a given race have blood cholesterol concentrations characteristic of their environment rather than of their race. However, the environmental differences between Los Angeles and Japan or rural Guatamala and North America are many and extreme. Thus, there is the possibility that " . . . strong environmental influences can modify and even nullify the genetic predisposition" as suggested by Adlersberg and Schaefer ('59). The report of Gopalan and Ramanathan ('57), in which different economic groups living in India were compared, differs from most studies in that serum cholesterol was compared among groups with large dietary differences in a constant geographical environment.

As pointed out above, the Filipinos in Hawaii are the most recent group of immigrants from the Orient. Serum cholesterol concentrations of two groups of Hawaii Filipinos (Honolulu and Lanai) are compared in table 5. The environmental differences between Honolulu and Lanai are essentially those between an urban and a rural locale. Lanai is a small island 60 miles from Honolulu. The economy of the island is based on the growing of pineapples and the serum samples were from pineapple plantation workers. Although the men live in a small plantation town, they raise most of their own food, in contrast to the Honolulu Filipinos who do not. In addition to the environmental difference for the two groups, there also may be a difference in the physical activity: although the Honolulu sample was chiefly composed of longshoremen and gardeners, detailed occupational records were not available.

It can be seen that the average serum cholesterol concentration for Filipinos of less than 40 years is the same for Lanai and Honolulu and is lower than for Honolulu Caucasians. In the 40 to 49 and 40 to 59 age groups, however, the Honolulu Filipino values increase (equalling the level of Honolulu Caucasians), whereas the corresponding increase with age in the Lanai concentrations is less than one-half as great. As a result there are significant differences (table 3) between Honolulu and Lanai Filipinos of these age groups.

A similar contrast is observed between rural and urban Thai samples with the difference again increasing with age (table 5). The Chiengmai samples were from farmers in northern Thailand where the average diet (adult man) is reported to furnish 1851 calories (with only 11% of these calories coming from fat) and 46 gm of protein. The diet of the average Bangkok adult man has been reported to contain 1807 calories (27% from fat) and 47 gm of protein (Chandrapanond, '57).

Here, too, the evidence indicates that environment (including diet and occupation) rather than race controls cholesterol concentrations, even when the contrasted environments are identical in their geographical characteristics.

TABLE 5

Serum cholesterol of adult men in rural and urban environments

Subjects	20–39 years			40–49 years			50–59 years			All ages		
	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol	No.	Mean age	Cholesterol
			mg/100 ml			*mg/100 ml*			*mg/100 ml*			*mg/100 ml*
Lanai Filipino	17	29	200 ± 30[1]	17	46	221 ± 39[1]	36	55	226 ± 41[1]	70	46	218 ± 40[1]
Honolulu Filipino	16	28	196 ± 31	124	45	251 ± 41	57	54	263 ± 40	197	46	250 ± 44
Honolulu Caucasian	23	27	226 ± 42	39	44	266 ± 50	19	55	269 ± 44	81	41	256 ± 48
Rural Thai (Chiengmai)	19	30	193 ± 44	6	44	155 ± 25	5	53	184 ± 33	30	37	184 ± 42
Urban Thai (Bangkok)	41	30	218 ± 46	19	43	241 ± 52	5	53	232 ± 24	65	36	226 ± 48

[1] Mean ± standard deviation.

TABLE 6

Lipid phosphorus and cholesterol/lipid phosphorus ratios

Subjects (men)	20–39 years		40–49 years		50–59 years		All ages	
	Lipid P	Chol./P	Lipid P	Chol./P	Lipid P	Chol./P	Lipid P	Chol./P
	mg/100 ml		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*	
Honolulu Caucasian[1]	9.95	23.01	10.70	25.04	10.44	25.94	10.43	24.68
Honolulu Chinese	10.22	24.39	11.04	25.29	11.02	22.89	10.83	24.27
Honolulu Filipino	9.35	21.52	10.45	20.20	10.93	24.32	10.50	24.95
Honolulu Hawaiian (part and full)	10.40	23.13	10.79	23.54	11.81	24.54	10.74	23.48
Honolulu Japanese	10.02	23.32	10.63	25.54	11.28	24.53	10.54	24.65
Honolulu—various others	10.51	20.91	10.55	24.93	11.14	24.67	10.66	23.56
Lanai Filipino	8.56	23.47	9.53	23.14	9.22	24.54	9.14	23.95
Bangkok Thai	7.00	31.27	7.90	30.75	7.77	30.01	7.35	30.97
Chiengmai Thai	6.48	30.65	5.32	29.03	5.79	31.74	6.14	30.51

[1] Numbers and ages for the various groups are the same as in tables 3 and 4.

Serum cholesterol: change with age. There seems to be no doubt that in the United States, serum cholesterol concentrations are higher in subjects of increasing age up to approximately age 50, after which they tend to decrease (Barker, '39; Jones et al., '51; Keys et al., '52; Swanson et al., '55; Ackerman et al., '59). It has been pointed out that "this does not necessarily mean . . . that the cholesterol level actually declines in old age. The oldest persons . . . are survivors . . . who may not have been typical, at younger ages, of the general population. It is entirely possible that persons . . . characterized by having relatively low cholesterol concentrations tend to survive longer than their fellows . . ." (Keys et al., '50). Whether or not this is actually the case can only be determined by long-term studies of individuals. No such studies have been reported. In three studies in which measurements on a few individuals were repeated once after various lengths of time (3 to 48 years), results are inconsistent (Sperry and Webb, '50b; Keys, '52; Man and Peters, '53).

The frequent observation of constant cholesterol after age 30 in low cholesterol populations (Keys et al., '52; Gopalan and Ramanathan, '57; Scrimshaw et al., '57; Abraham and Miller, '59) renders questionable the assumption that the age-cholesterol trend observed in the United States would be observed in a truly normal population.

In the Honolulu data reported here, the usual age-cholesterol pattern for the United States is seen: cholesterol concentrations in the 40 to 49 age range are appreciably higher than for age 20 to 39 for all groups. The increase is most marked in the Filipino group. However, the corresponding increases for the Lanai Filipinos and for the Thai were much less pronounced. These data therefore lend support to the hypothesis that the increase of cholesterol with age is a consequence of environmental influences rather than of age *per se*.

Serum lipid phosphorus. The lipid phosphorus concentrations and the cholesterol/lipid phosphorus ratios of the various groups are shown in table 6. In general the phosphorus concentrations repeat the cholesterol relationships. Correlation coefficients of cholesterol and phosphorus were more than 0.7 for most groups. However, the cholesterol increases with age somewhat more than does the phosphorus, with the result that the cholesterol/lipid phosphorus ratio shows an increase with age in most groups.

In view of the theory that the cholesterol/phospholipid ratio is increased in cases of atherosclerosis (Gertler et al., '50), it is interesting to note that this ratio is appreciably higher in the Thai samples than in the Hawaii ones. This high ratio is chiefly due to the fact that the lipid phosphorus of the Thai samples was much lower than in the Hawaii sera. The Lanai samples, with cholesterol concentrations nearly as low as those of the Thai but with higher phosphorus levels, had cholesterol/lipid phosphorus ratios equivalent to the Honolulu samples.

SUMMARY

Fats in the diets of the various ethnic groups among University of Hawaii students (Japanese, Chinese, Caucasian) provide approximately 40% of the total caloric intake by each group.

The serum cholesterol and lipid phosphorus concentrations for various ancestry groups in adult Honolulu men (Caucasian, Chinese, Filipino, Japanese, Hawaiian) were essentially the same in all groups with some exceptions: in the 20 to 39 age group, Honolulu Filipinos had lower serum cholesterol and lipid phosphorus than the other groups, and the Chinese had higher serum cholesterol than the Japanese. The Chinese and Japanese values for all ages were as high as or higher than the corresponding values for Caucasians.

Filipino plantation workers over 40 years old living in a rural environment on the island of Lanai had significantly lower serum cholesterol and lipid phosphorus concentrations than did Honolulu Filipinos. The increase in the Lanai Filipino cholesterol concentrations after age 30 was appreciably less than that observed for all of the Honolulu groups.

Serum cholesterol concentrations of Thai residents of Bangkok were approximately equivalent to those of the Lanai Filipinos. Values for farmers in Chieng-

mai (northern Thailand) were lower than for the Bangkok Thai. The relative constancy of cholesterol concentration with age was also observed in the Thai samples from both locations.

The data support the viewpoint that the frequently observed increase of serum cholesterol with age is not obligatory but is attributable to environmental hypercholesterolemic influences.

There was good correlation between serum lipid phosphorus and serum cholesterol concentrations in all groups. The cholesterol/lipid phosphorus ratio was higher in the Thai serum samples than in the Hawaii sera.

These results are consistent with the view that race is of negligible influence in determining serum cholesterol concentrations, whereas undetermined environmental factors are of great influence.

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A Study on Pigment Deposition by Intravenous Fat Emulsions

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Meyer and associates ('57) reported on the observation of a brown pigment within the reticuloendothelial system of human beings and dogs receiving multiple intravenous infusions of a sterile aqueous fat emulsion. This emulsion consisted of 15 gm of cottonseed oil, 4 gm of glucose, 1.2 gm of soybean phosphatide and 0.3 gm of a polyoxyethylene-propylene polymer (Pluronic F-68) per 100 ml of the mixture. Thompson et al. ('58) observed that the pigment reported by Meyer et al. was encapsulated by lipoid substances. They designated the pigment-lipoid complex "intravenous (i.v.) fat pigment" and described its histochemical characteristics. Thompson and associates ('60a) demonstrated that the i.v. fat pigment is a specific entity and not an artifact incident to the fixation and processing of the tissues. Thompson et al. ('60b) reported that the i.v. fat pigment persists within reticuloendothelial cells of rabbits up to and including 18 months postinfusion.

To date, the literature of i.v. fat pigment has dealt solely with fat emulsions identical in composition to that reported by Meyer et al. ('57). The present report deals with the testing for pigment deposition of emulsions containing fats other than cottonseed oil and emulsifiers other than soybean phosphatide and Pluronic F-68.

MATERIALS AND METHODS

Splenic tissue was obtained at necropsy of rabbits and rats receiving multiple intravenous infusions of 11 sterile aqueous fat emulsions or suitable control mixtures containing no fat. The composition of the fat emulsions and the control mixtures and the data relative to their administration are presented in table 1. All animals were killed and necropsied one to three days after receiving the final infusion. At necropsy, blocks of splenic tissue were placed in 10% formalin buffered to pH 7.0 and in absolute ethyl alcohol. Using fresh reagents and chemically clean glassware, the fixed tissues were processed and infiltrated on the Autotechnicon. Replicate tissue sections were cut 6 to 8 μ in thickness from paraffin-embedded blocks. Formalin and alcohol-fixed tissue sections from each animal were stained by Harris' hematoxylin and aqueous eosin (Gridley, '57) and by Gomori's stain for iron (Mallory, '42).

Alcohol-fixed tissue sections from animals receiving fat emulsions were subjected to several additional stains and histochemical procedures. These included immersion in Oil red O stain in propylene glycol for 24 hours (Gridley, '57), exposure to osmium tetroxide for 24 hours (Mallory, '42), exposure to anhydrous pyridine for 24 hours in the Soxhlet apparatus followed by immersion in Oil red O stain in propylene glycol for 24 hours, exposure to equal parts of methanol and chloroform for 24 hours in the Soxhlet apparatus followed by immersion in Oil red O stain for 24 hours, and examination by fluorescence microscopy of unstained glycerine-mounted tissue sections. For this procedure a Philips CS 150 high pressure mercury vapor lamp was used with two filter systems: (1) a 6 mm UG1 transmission filter and a 2.5 mm Euphos absorption filter and (2) a 4 mm BG12 transmission filter and a 2.5 mm OG1 absorption filter. The first system produces

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TABLE 1

Composition of infusions and the responses induced by intravenous administration to rabbits or rats

Group	Fat/100 ml	Emulsifier system/100 ml	Number of animals	Number of infusions	Dosage	Production i.v. fat pigment
					ml/kg	
1	15 gm Coconut oil	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68[2]	5 rabbits[1] 5 rabbits[3]	29 29	15 15	+ —
2	15 gm Cottonseed oil	4 gm glucose 0.3 gm Pluronic F-68	5 rabbits[1] 5 rabbits[4]	29 29	15 15	+ —
3	15 gm Cottonseed oil	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68	5 rats[1] 5 rats[3]	20 20	20 20	+ —
4	15 gm Cottonseed oil[5]	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68	5 rats[1] 5 rats[6]	20 20	20 20	+ —
5	15 gm Olive oil	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68	5 rats[1] 5 rats[6]	20 20	20 20	+ —
6	15 gm Corn oil	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68	5 rats[1] 5 rats[6]	20 20	20 20	+ —
7	15 gm Peanut oil	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68	3 rats[1] 3 rats[6]	20 20	20 20	+ —
8	15 gm Butyro olein[7]	4 gm glucose 1.2 gm soybean phosphatide 0.3 gm Pluronic F-68	3 rats[1] 3 rats[6]	20 20	20 20	+ —
9	10 gm Safflower oil	5 gm glucose 8 gm glycerine USP 0.83 gm Gliddophil SM[8]	5 rabbits[1] 5 rabbits[3]	20 20	22.5 22.5	+ —
10	15 gm Synthetic fat[9]	5 gm glucose 0.1 gm Na cholate 1 gm Aldo 28[10] 0.5 gm TEM-4T[11]	2 rabbits[1] 1 rat[1] 1 rat[3]	20 13[12] 12[12]	15 15 15	+ + —
11	15 gm Coconut oil	5 gm glucose 1 gm Pluronic F-68	5 rabbits[1] 5 rabbits[3]	20 20	15 15	— —

[1] Animals receiving fat emulsion.
[2] Polyoxyethylene-propylene polymer, Wyandotte Chemicals Corporation, Wyandotte, Michigan.
[3] Animals receiving emulsifier system without fat.
[4] Animals receiving 5% aqueous glucose solution.
[5] Midfraction of molecularly distilled cottonseed oil.
[6] Same control rats as in group 3.
[7] A mixed butyro olein prepared by interesterification of methyl oleate and tributyrin.
[8] Sixty per cent soybean phosphatides dispersed in a partially hydrogenated vegetable oil carrier.
[9] Oleic acid, 60%; palmitic acid, 30%; stearic acid, 7.4%; linoleic acid, 2.6%; iodine number, 51 to 55; hydroxyl value, 2.19; peroxide value, 0; free fatty acids as oleic acid, 0.02 to 0.05%.
[10] Glyceryl monostearate, Glyco Products Company, Inc.
[11] Reaction product of glycerol monostearate and acetylated tartaric acid.
[12] Animals sacrificed due to gangrene of tail.

an exciting light of 360 mμ wave length. The second system produces an exciting light of 400 mμ wave length. The unstained tissue sections in addition to the hematoxylin and eosin stained sections also were examined by the polarizing microscope.

RESULTS

A brown, granular birefringent and iron-negative pigment was observed within the spleens of all animals receiving intravenous infusions of fat emulsions with the exception of that group of rabbits receiving the coconut oil-nonphosphatide emulsion (figs. 1–3). The pigment was not observed within the spleens of animals receiving intravenous infusions of control mixtures containing no fat (fig. 4). All examined sections exhibited a pigment interpreted as hemosiderin, but no section contained pigment that was interpreted as acid formalin hematin.

In stained and unstained tissue sections, the pigment appeared as light-brown to dark-brown granules within the cytoplasm of the reticuloendothelial cells. In unstained tissue sections, a faint-yellow capsule surrounding the granules was often seen. Under ultraviolet light the capsule appeared as a halo emitting a yellowish-green fluorescence, except in the animals receiving olive oil or corn oil emulsions. In these two groups of tissues the fluorescence of the capsule was orange. The Oil red O stain showed that the capsule was Oil red O positive. Although the quantity of capsular material varied between groups and between animals within a group, all examined pigment granules were encapsulated by Oil red O-positive material. Neither the capsule nor pigment granule reduced osmium tetroxide. Treatment with anhydrous pyridine consistently removed the pigment granules from the tissue sections. Treatment with methanol-chloroform removed the pigment granules except for a small number remaining in the tissues of the cottonseed oil-phosphatide emulsion group. Oil red O staining of the tissue sections after exposure to anhydrous pyridine or methanol-chloroform demonstrated that Oil red O-positive material remained in quantities that varied little from that present in the untreated tissue sections.

DISCUSSION

The pigment associated with multiple intravenous infusions of a fat emulsion is evidently related to the presence of fat in the emulsion since the pigment was not observed in animals receiving the emulsifier system free of fat. The fact that one fat emulsion, coconut oil-nonphosphatide, produced no pigment under the conditions of this experiment indicates that the coconut oil is free from the pigment-producing agent. However, when coconut oil is combined with a phosphatide emulsifying system, which by itself does not produce pigment, small but detectable quantities of pigment are found in the injected animals. All fat emulsions containing soybean phosphatide produced pigment, but two of three fat emulsions without soybean phosphatide also produced pigment. Whatever the source of the pigment may be, this study shows that the pigment associated with one fat emulsion is not significantly different, histologically, from the pigment associated with any other of 9 fat emulsions. In a previous study (Thompson et al., '58) the pigment associated with a cottonseed oil-phosphatide emulsion was described as a pigment-lipoid complex. Since the pigment granules observed in this study were routinely encapsulated by fluorescent Oil red O-positive material that did not reduce osmium tetroxide and that was not readily removed by lipid solvents, the term pigment-lipoid complex may also be used to describe these pigments. Likewise, the title of i.v. fat pigment should include the pigment-lipoid complexes described here in addition to the pigment-lipoid complex originally associated with the cottonseed oil emulsion.

SUMMARY

Eleven fat emulsions containing various synthetic or vegetable oils were administered intravenously to rabbits or rats. In addition the emulsifying systems without fat were similarly tested. A pigment-lipoid complex, i.v. fat pigment, was found within the cytoplasm of the splenic reticuloendothelial cells of animals receiving coconut oil-phosphatide, cottonseed oil-nonphosphatide, cottonseed oil-phosphatide, molecularly distilled cottonseed oil-phosphatide, olive oil-phosphatide, corn oil-

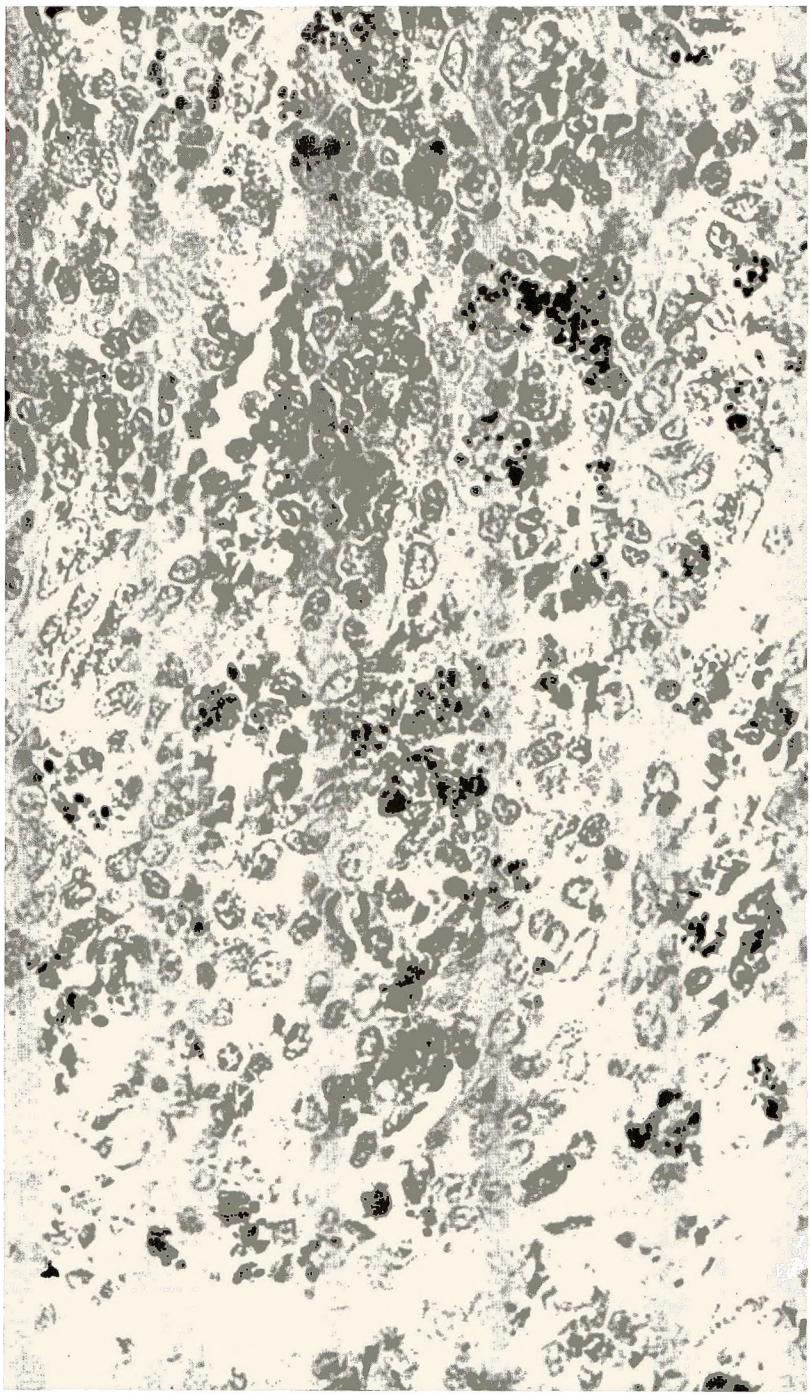

Fig. 1 Spleen of a rabbit receiving the coconut oil-phosphatide emulsion. Intravenous fat pigment is present in the reticuloendothelial cells. Alcohol fixation and Gomori's stain for iron with nuclear fast red counterstain. (× 825.)

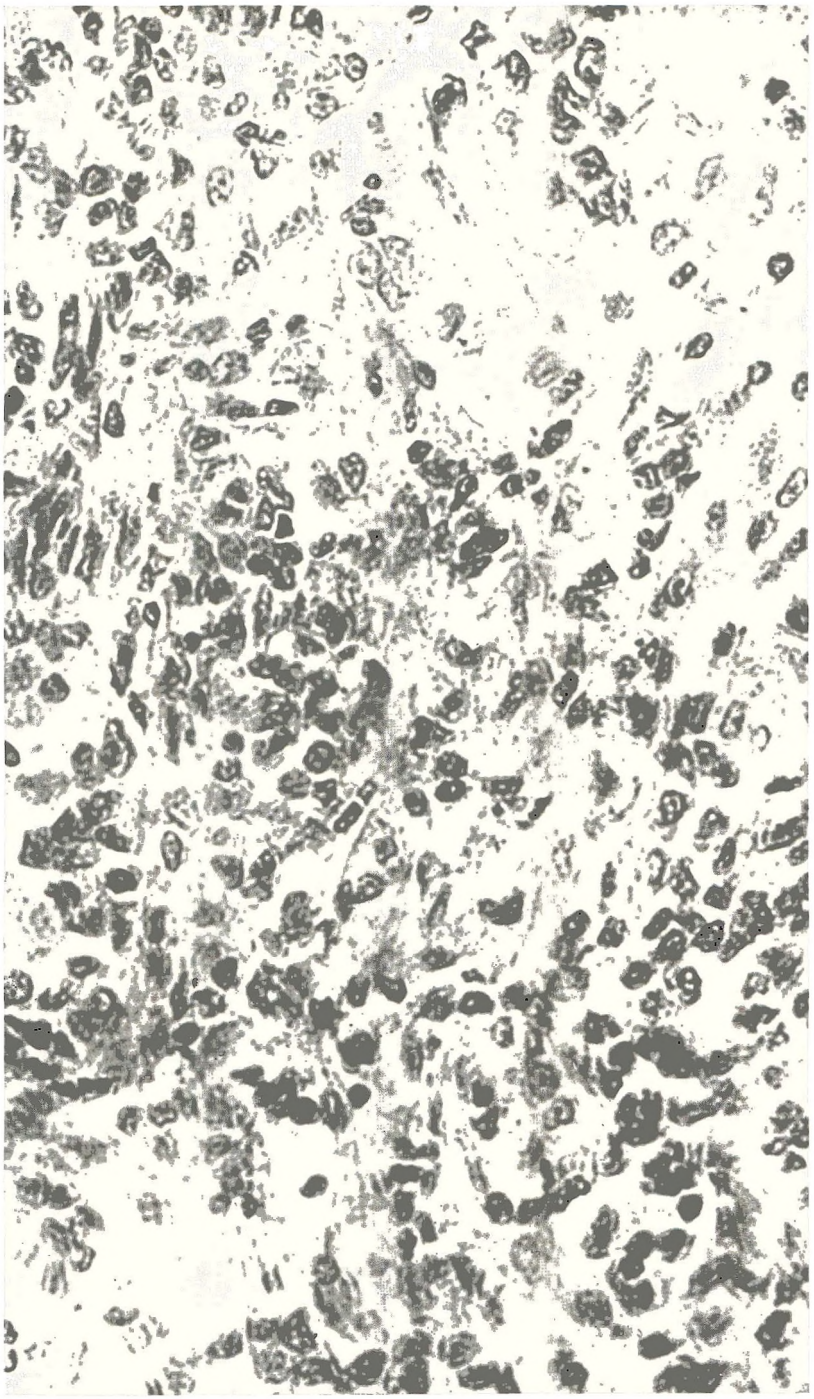

Fig. 2 Spleen of a rabbit receiving the coconut oil-nonphosphatide emulsion. Intravenous fat pigment is absent from the reticuloendothelial cells. Alcohol fixation and Gomori's stain for iron with nuclear fast red counterstain. ($\times$ 825.)

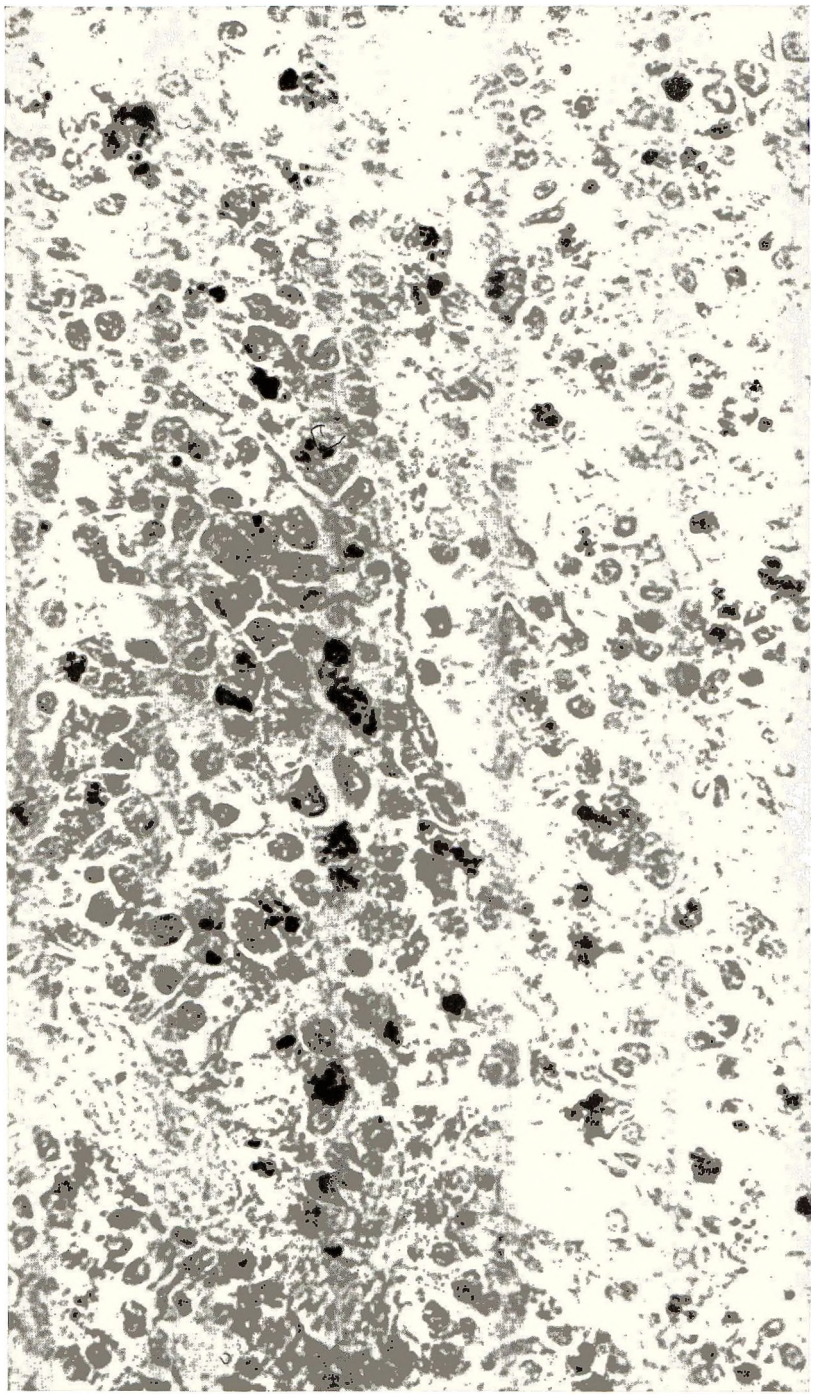

Fig. 3 Spleen of a rat receiving the corn oil-phosphatide emulsion. The i.v. fat pigment in the reticuloendothelial cells is representative of all i.v. fat pigment. Alcohol fixation and Gomori's stain for iron with nuclear fast red counterstain. (× 825.)

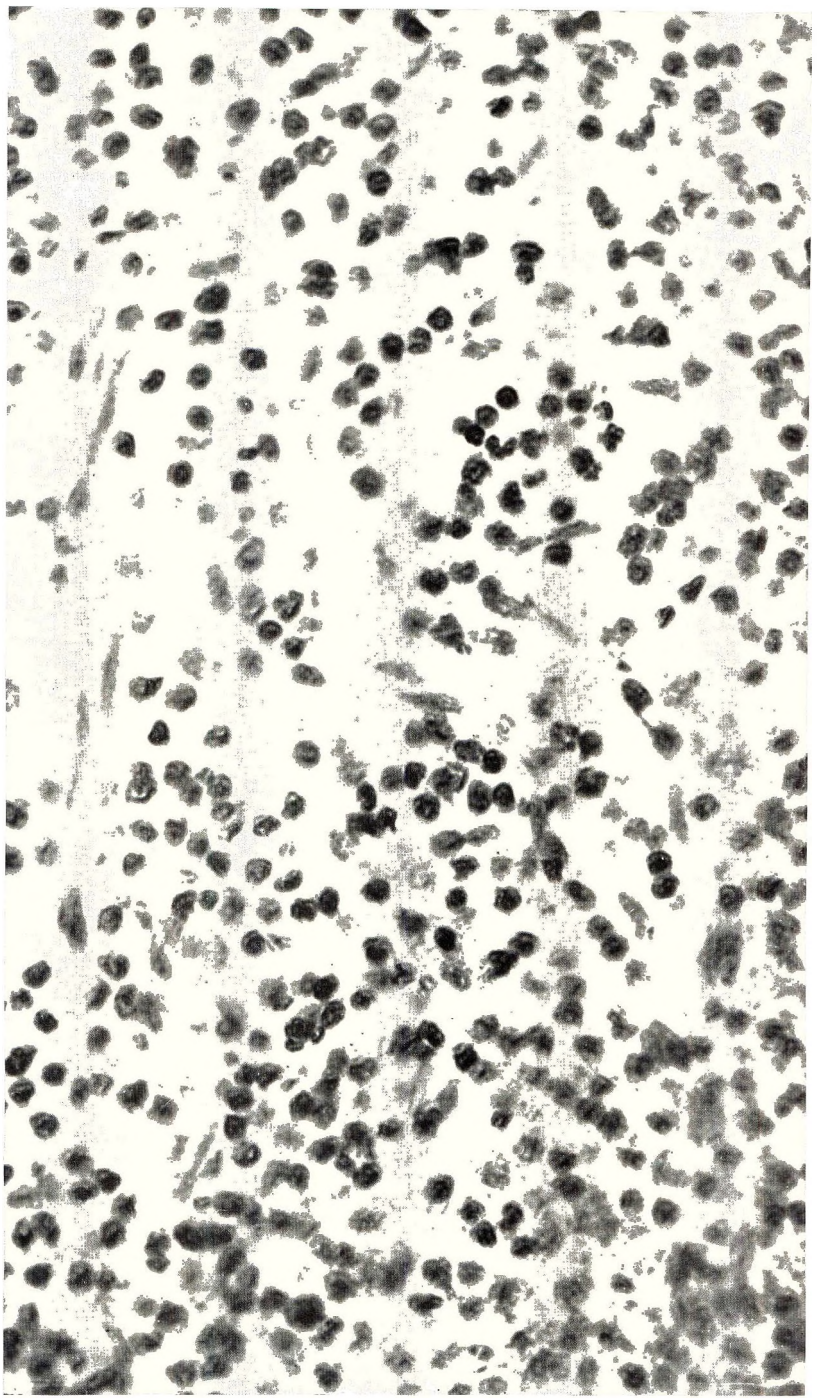

Fig. 4 Spleen of a rat receiving an emulsifier system without fat. The reticuloendothelial cells are free of pigment. Alcohol fixation and Gomori's stain for iron with nuclear fast red counterstain. (× 825.)

phosphatide, peanut oil-phosphatide, butyro olein-phosphatide, safflower oil-phosphatide or synthetic oil-nonphosphatide emulsions. No i.v. fat pigment was observed in animals receiving coconut oil-nonphosphatide emulsion or in the animals receiving the emulsifying mixture without fat.

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Effects of Diet on Fish Oil Toxicity in the Rat

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It is well established that the prolonged administration of fish oils will induce or accentuate symptoms of vitamin E deficiency in a number of animal species (Agduhr, '26a, b, '28; Madsen et al., '35; Dam, '43; Singsen et al., '55a). This effect has been ascribed to one or more of the following mechanisms: (1) destruction or inactivation of vitamin E in the diet, the gastrointestinal tract or the tissues by the autoxidation of the polyunsaturated fatty acids of fish oil or (2) an increased requirement for (or an impaired utilization of) tocopherol induced by (a) toxic substances in, or toxic metabolites of, fish oil and/or (b) toxic substances produced by the interaction of fish oil with other substances in the diet.

Symptoms of vitamin E deficiency induced by fish oil administration such as muscular dystrophy in herbivora or encephalomalacia in chicks can readily be counteracted by the concurrent feeding of α-tocopherol (Dam et al., '38; Mackenzie and McCollum, '40). They can also be counteracted, however, by various non-tocopherol supplements. Thus, Ni ('36, '37, '38, '40) found that a water-soluble material derived from Chinese gelatin effectively prevented the occurrence of muscular dystrophy and encephalomalacia using diets containing fish liver oils. Jukes and Babcock ('38) described a paralytic disorder in chicks which could be prevented either by a fat-soluble fraction prepared from soybean oil (presumably vitamin E) or by a water-soluble fraction prepared from fat-extracted alfalfa meal.

More recently Dam et al. ('48, '51) observed that a number of apparently unrelated compounds including methylene blue, ascorbic acid and nordihydroguaiaretic acid had significant activity in preventing chick encephalomalacia using diets containing cod liver oil. Similar findings were obtained by Singsen et al. ('55b) with N, N′ - diphenyl - *p* - phenylenediamine (DPPD) and by Bunnell et al. ('55) with other antioxidants including 2, 6-ditertiary butyl-4-methoxyphenol (BHT), dibutyl and diamyl hydroquinones; di-sec-butyl- *p*-phenylenediamine; and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquin). Methylene blue, ascorbic acid and tetraethylthiuram disulfide (antabuse) were also active in protecting against the development of such symptoms of vitamin E deficiency as fat coloration and peroxidation, incisor depigmentation, hepatic damage and sterility in rats fed diets containing cod liver oil (Dam et al., '49). Methylene blue was also active in preventing dystrophy in calves fed a diet containing cod-liver oil (Blaxter et al., '53). Since all the materials indicated above have antioxidant activity, it would appear that their activity was due either to their sparing effect on the destruction of vitamin E in the diet, gastrointestinal tract or tissues or to their ability to replace vitamin E, at least in part, in the tissues.

There is evidence, however, that the toxic effects of fish oil administration are not confined to the induction or accentuation of a vitamin E-deficiency state. Thus, Scott ('51a) observed that the addition of fish liver oil to the diet of poults resulted in a high incidence of enlarged hock disorder. Vitamin E deficiency does not appear to be the cause of this condition since high levels of α-tocopheryl acetate (50 mg/pound of diet) failed to prevent the disorder (Scott, '51b). Intramuscular injections of α-tocopheryl phosphate (10 mg per poult weekly) were also ineffective in this regard (Scott, '51b). The high incidence of enlarged-hock disorder in poults fed diets containing fish liver oil could be prevented completely however, by the concurrent feeding of dried brewers' yeast (Scott, '51b). In the present communication further data are presented indicating

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that toxic effects may occur following fish oil administration due to factors other than the induction of a vitamin E-deficiency state.

PROCEDURE AND RESULTS

A series of experiments was designed to study the effects of various dietary supplements on the growth of immature rats fed a purified low-fat ration supplemented with 10% of fish oil. The basal low-fat ration used in these experiments consisted of sucrose, 71%; casein,[1] 24%; and salt mixture,[2] 5%. To each kilogram of the above diet were added the following synthetic vitamins: (in milligrams) thiamine·HCl, 20; riboflavin, 20; pyridoxine·HCl, 20; Ca pantothenate, 60; nicotinic acid, 100; ascorbic acid, 200; biotin, 4; folic acid, 10; *p*-aminobenzoic acid, 400; inositol, 800; 2-methyl-1,4-naphthoquinone, 5 mg; and vitamin B_{12}, 150 μg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units;[3] vitamin D_2, 500 U.S.P. units;[3] and α-tocopheryl acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Fish oils and the various test supplements were incorporated in the above diet in the amounts listed in tables 1–4, replacing equal amounts of sucrose.

[1] Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Crystalets 500 A—50 D, crystalline vitamin A acetate and crystalline vitamin D_2 stabilized with gelatin and sugar, 500,000 U.S.P. units vitamin A and 50,000 U.S.P. units vitamin D_2 per gm, Chas. Pfizer and Co., Inc., New York.

TABLE 1

Effects of fish oil administration on the weight increase of immature male rats fed a purified low-fat diet and a similar ration supplemented with cottonseed oil (6 animals per group)[1,2]

Dietary group	Average gain in body weight after following days of feeding			
	7th	14th	21st	28th[3]
	gm	*gm*	*gm*	*gm*
Basal low-fat diet	27	64	93	124 ± 6.1
Basal low-fat diet plus following supplements:				
10% Crude tuna oil	2	14	29	38 ± 3.4
10% Refined tuna oil	1	9	21	37 ± 2.9
10% Crude sardine oil	−3	3	13	28 ± 2.1
10% Menhaden oil	−1	4	15	21 ± 4.2
10% Cod liver oil	6	34	45	61 ± 3.1
Basal low-fat diet plus 10% cottonseed oil	33	80	118	153 ± 5.8
Basal low-fat diet plus 10% cottonseed oil plus following supplements:				
10% Crude tuna oil	29	68	97	138 ± 4.4
10% Refined tuna oil	26	59	86	119 ± 3.9
10% Crude sardine oil	29	56	84	120 ± 4.6
10% Menhaden oil	30	58	88	129 ± 4.1
10% Cod liver oil	32	66	98	147 ± 2.9

[1] The average initial body weight of rats in the various groups ranged between 46.4 and 48.0 gm.

[2] The crude and refined tuna oil and the crude sardine oil were kindly provided by the late Dr. E. Geiger and Mr. H. J. Dunn of the Van Camp Sea Food Co., Inc., Terminal Island, California. The menhaden oil was obtained from the Pacific Vegetable Oil Corp., Los Angeles; the cod liver oil from E. R. Squibb and Sons, New York. The cod liver oil had a vitamin A content of 1700 U.S.P. units/gm and a vitamin D_3 content of 170 U.S.P. units/gm. When fed at a 10% level in the diet it supplied 170,000 U.S.P. units of vitamin A and 17,000 U.S.P. units of vitamin D_3/kg of ration. The tuna oils contained approximately 200 U.S.P. units of vitamin A and 50 U.S.P. units of vitamin D_3/gm, and the sardine oil approximately 500 U.S.P. units of vitamin A and 100 U.S.P. units of vitamin D_3/gm. No data are available on the vitamin A and D content of the menhaden oil.

[3] 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.

TABLE 2

Comparative effects of cottonseed oil, methyl linoleate and other sources of dietary fat on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil (6 animals per group)[1,2]

Dietary group	Average gain in body weight after 14 days of feeding
	gm
Basal low-fat diet	63
Basal low-fat diet + 10% cottonseed oil	81
Basal low-fat diet + 10% crude tuna oil	17
Basal low-fat diet + 10% crude tuna oil plus following supplements:	
10% Cottonseed oil	54
5% Cottonseed oil	26
2% Cottonseed oil	14
10% Soybean oil	67
5% Soybean oil	51
2% Soybean oil	22
10% Sesame oil	68
5% Sesame oil	54
2% Sesame oil	21
10% Wheat germ oil	72
10% Corn oil	62
10% Olive oil	28
10% Coconut oil	18
10% Lard	15
10% Butter fat	14
10% Hydrogenated cottonseed oil	24
10% Methyl linoleate	−12
5% Methyl linoleate	−9
1% Methyl linoleate	−2
Dextrin (substituted for sucrose in diet)	−4
Corn starch (substituted for sucrose in diet)	2

[1] The average initial body weight of rats in the various groups ranged between 45.2 and 46.4 gm.

[2] The fats were obtained from the following sources: cottonseed oil, Wesson Oil and Snowdrift Sales Co., New Orleans, La.; wheat germ oil, VioBin Corporation, Monticello, Ill.; corn oil, Corn Products Refining Co., Argo. Ill.; stripped lard, Distillation Products Industries, Rochester, (the tocopherol content of this material according to the manufacturers was less than 5 μg/gm); hydrogenated cottonseed oil, Best Foods, Inc., New York; and butter, Challenge Creamery Co., Los Angeles. The butter fat was prepared by melting the butter and removing the oil layer. The soybean oil, sesame oil, olive oil and cocount oil were obtained from the Pacific Vegetable Oil Corp., Los Angeles. The methyl linoleate was kindly provided by Dr. Roslyn B. Alfin-Slater, Department of Home Economics, University of California, Los Angeles. The purity of methyl linoleate as determined by iodine value assuming the contaminant to be methyl oleate was 92%.

Male rats of the Holtzman strain were selected, 21 to 24 days old, having a body weight between 40 and 54 gm for the present series of experiments. The rats were housed in metal cages with raised screen bottoms (two animals per cage) and were provided with food and water ad libitum. Diets were made up biweekly and stored under refrigeration when not in use. The animals were fed daily (6 rats per group) and all food not consumed 24 hours after feeding was discarded. Feeding was continued for either 14 or 28 days as indicated in tables 1–4. Animals were weighed once weekly during the course of the experiment.

Experiment 1

Deleterious effect of fish oil on the growth of immature rats fed a purified low-fat diet. Considerable data are available indicating that animals fed low-fat diets are particularly sensitive to a number of stressor agents including X-irradiation (Decker et al., '50; Cheng et al., '52), thyroid administration (Greenberg and Deuel, '50; Greenberg, '52), mineral oil administration (Bacon et al., '52; Ershoff and Greenberg, '54) and administration of succinylsulfathiazole, atabrine and other drugs (Bosshardt et al., '50; Bosshardt and Huff, '53). In each of the experiments indicated above a protective effect was obtained by increasing the fat content[4] of the diet.

Present findings indicate that immature rats fed a purified low-fat diet supplemented with 10% of fish oil exhibited a highly significant retardation in growth which was evident during the very first week of feeding. During this period rats fed the above diets exhibited an almost complete cessation of growth or actual loss in body weight accompanied by diarrhea. Subsequent to this period a small increase in body weight occurred, particularly during the third and 4th week of feeding. With the exception of their smaller size, however, rats fed the low-fat diet supplemented with fish oils appeared grossly normal in all respects. Similar findings were obtained with both crude and refined

[4] The fats used in these experiments were either vegetable oils with a high content of unsaturated fatty acids or methyl linoleate *per se*.

tuna oil, crude sardine oil and menhaden oil. Growth also was retarded using diets containing 10% of cod liver oil but to a lesser extent than with the other fish oils tested. In contrast to the above, the growth-retarding effect of the various fish oils as well as the occurrence of diarrhea was largely if not completely counteracted by the concurrent administration of cottonseed oil at a 10% level in the diet.

Experiment 2

Comparative effects of cottonseed oil, methyl linoleate, and other sources of dietary fat on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. In agreement with experiment 1 the incorporation of crude tuna oil at a 10% level in the basal purified low-fat ration resulted in a significant retardation in growth accompanied by diarrhea which was largely counteracted by the concurrent administration of 10% of cottonseed oil. Soybean oil, sesame oil, corn oil and wheat germ oil when fed at a 10% level in the diet were active also in this regard but olive oil, coconut oil, hydrogenated cottonseed oil, butter fat and lard at a 10% level of feeding had little if any growth-promoting effect. The above findings indicate that the fats most active in counteracting fish oil toxicity under conditions of the present experiment were vegetable oils with a high content of unsaturated fatty acids, whereas the more saturated fats (such as hydrogenated cottonseed oil, vegetable oils of low unsaturated fatty acid content or animal fats) had little if any activity in this regard.

Methyl linoleate, however, when incorporated at levels of 1, 5 or 10% in the diet was without protective effect. On the contrary the rats fed these supplements lost weight during the course of the experiment in contrast to the small weight increase of rats fed the basal low-fat ration with 10% of crude tuna oil. Studies conducted with cottonseed oil, soybean oil and sesame oil at levels of 2, 5 and 10% of the diet indicate that the effects obtained were proportional to the level fed. At the 2% level of feeding, findings were comparable to that obtained using the low-fat plus 10% of crude tuna oil ration. A significant increase in body weight over that using the latter diet occurred in rats fed the 5% soybean oil or sesame oil supplements and to a lesser extent in rats fed the diet containing 5% of cottonseed oil. A still further increase in body weight resulted when these supplements were fed at a 10% level. Replacing the sucrose in the purified low-fat diet with dextrin or corn starch did not improve the growth of rats fed the crude tuna oil supplement. On the contrary the weight increase of rats fed dextrin or corn starch as the source of carbohydrate in a low-fat diet supplemented with 10% of crude tuna oil was significantly less than that of rats fed a similar ration containing sucrose as the dietary carbohydrate.

Experiment 3

Comparative effects of α-tocopherol, α-tocopheryl acetate, DPPD, Santoquin and sesamol on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. Findings indicate that α-tocopherol, when fed at levels of 0.025, 0.05 or 0.1% in the diet, partially counteracted the growth retardation (and completely prevented the diarrhea) of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. Its growth-promoting effect was more marked at the 0.05% than the 0.025% level; no further increase in weight occurred, however, when fed at a higher (i.e., 0.1%) level. In contrast to the effects obtained with α-tocopherol, α-tocopheryl acetate, when fed in comparable amounts, was without protective effect. Intraperitoneal injections of α-tocopheryl phosphate (10 mg/rat/day, 6 times/week) were also without growth-promoting effect. DPPD and Santoquin, when fed at a 0.05% level in the diet and sesamol (the methylene ether of oxyhydroquinone, a constituent of sesame oil with antioxidant properties) when administered at a 0.05 or 0.017% level in the ration, counteracted completely the growth retardation caused by crude tuna oil when incorporated at a 10% level in the basal purified low-fat diet. Results are summarized in table 3.

TABLE 3

Comparative effects of α-tocopherol, α-tocopheryl acetate, DPPD, Santoquin and sesamol on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil (6 animals per group)[1,2]

Dietary group	Average gain in body weight after 14 days of feeding
	gm
Basal low-fat diet	61
Basal low-fat diet + 10% cottonseed oil	76
Basal low-fat diet + 10% crude tuna oil	14
Basal low-fat diet + 10% crude tuna oil + following supplements:	
0.025% α-tocopherol	32
0.05% α-tocopherol	49
0.1% α-tocopherol	49
0.025% α-tocopheryl acetate	14
0.05% α-tocopheryl acetate	15
0.1% α-tocopheryl acetate	12
Tocopheryl phosphate (10 mg/rat/day 6 times weekly I.P.)	4
0.05% DPPD	65
0.05% Santoquin	70
0.017% Sesamol	72
0.05% Sesamol	74

[1] The average initial body weight of rats in the various groups ranged between 45.4 and 46.8 gm.

[2] Test supplements were obtained from the following sources: DL-α-tocopherol and DL-α-tocopherol phosphoric acid disodium salt from Nutritional Biochemicals Corp., Cleveland; DL-α-tocopheryl acetate (Dry Vitamin E Acetate Powder 25%), Hoffman-La Roche Inc., Nutley, N. J.; DPPD, B. F. Goodrich Chemical Co., Cleveland; and Santoquin, Monsanto Chemical Co., St. Louis, Mo. The sesamol used in the present experiment was a twice recrystallized product prepared by the Trubek Laboratories, Inc., East Rutherford, N. J., and supplied by the Western Utilization Research and Development Division Agricultural Research Service, U.S. Department of Agriculture, Albany, Calif.

Experiment 4

Comparative effects of known nutrients and other materials of plant and animal origin on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. Findings indicate that a number of natural source materials other than unsaturated vegetable oils or substances present therein were also effective in promoting the growth (and preventing diarrhea) of immature rats fed a purified low-fat diet supplemented with 10% of crude tuna oil. Alfalfa meal at a 20% level in the diet and desiccated liver N.F. and Torula yeast at a 10% level of feeding were particularly effective in this regard. Both liver residue at a 10% level and liver concentrate powder at a 2.5% level of supplementation had growth-promoting and anti-diarrheal activity. Increasing the casein content of the diet by an additional 10% of the ration or supplementing the basal ration with defatted fish flour or a mixture of crystalline amino acids to the extent of 10% of the diet also resulted in a significant increase in body weight, although less than that obtained with the 20% alfalfa meal or 10% liver and yeast supplements. DL-Methionine when fed at a 0.6% level in the diet was similar to the casein, fish meal and amino acid supplements in activity. Doubling the vitamin or salt-mixture content of the basal ration or adding 0.5 or 1% of ascorbic acid, 2% of mixed flavonoids, 10% of cellulose or 1 gm of Aureomycin·HCl per kilogram of diet had little if any protective effect. A combined supplement of 10% of casein, 5% of salt mixture, 2 gm of choline chloride per kilogram of diet plus the various vitamins in an amount equal to the level at which they were incorporated in the basal low-fat ration counteracted completely, however, the growth retardation and diarrhea of immature rats fed the basal low-fat ration supplemented with 10% of crude tuna oil. Results are summarized in table 4.

DISCUSSION

Present findings indicate that immature rats fed a purified low-fat diet supplemented with 10% of fish oil (i.e., crude or refined tuna oil, crude sardine oil, menhaden oil or cod liver oil) showed a highly significant retardation in growth accompanied by diarrhea which was evident during the first week of feeding. These effects were counteracted largely by the concurrent administration of cottonseed oil at a 10% level in the diet. Soybean oil, sesame oil, corn oil and wheat germ oil at a 10% level of feeding were also active in counteracting the growth retardation and diarrhea of immature rats fed the basal low-fat ration supplemented with 10% of crude tuna oil; but olive oil, coconut oil, hydrogenated cottonseed oil, butter

fat and lard, when fed at a 10% level in the diet, had little if any protective effect. These observations indicate that the fats most active in counteracting fish oil toxicity under conditions of the present experiment were vegetable oils with a high content of unsaturated fatty acids, whereas the more saturated fats (such as hydrogenated cottonseed oil, vegetable oils of low unsaturated fatty acid content or animal fats) had little if any activity in this regard.

TABLE 4

Comparative effects of known nutrients and other materials of plant and animal origin on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil (6 animals per group)[1]

Dietary group	Average gain in body weight after following days of feeding	
	14th	28th
	gm	*gm*
Basal low-fat diet	64	132
Basal low-fat diet + 10% cottonseed oil	80	154
Basal low-fat diet + 10% crude tuna oil	12	45
Basal low-fat diet + 10% crude tuna oil + following supplements:		
Vitamins A, D and E[2]	12	41
Vitamins B, C and K[3]	16	40
5% Salt mixture[4]	24	61
10% Casein[5]	38	93
Combined supplements[6]	88	155
0.5% Ascorbic acid	23	54
1% Ascorbic acid	20	53
2% Mixed flavonoids[7]	15	44
Aureomycin·HCl (1 gm/kg of diet)	22	54
10% Cellulose[8]	20	65
10% defatted fish flour[9]	50	116
10% Amino acid mixture[10]	44	101
0.6% DL-methionine	55	94
20% Alfalfa meal	83	158
10% Alfalfa meal	51	89
Desiccated liver N.F.	87	160
10% Liver residue[11]	57	129
2.5% Liver concentrate powder N.F.	51	99
10% Torula yeast[12]	67	131

[1] The average initial body weight of rats in the various groups ranged between 48.8 and 49.6 gm.

[2] The following vitamins were added per kilogram of diet: 5000 U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D_2 and 100 mg of α-tocopheryl acetate.

[3] The following were added per kilogram of diet: thiamine·HCl, 20 mg; riboflavin, 20 mg; pyridoxine·HCl, 20 mg; Ca pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-1,4 naphthoquinone, 5 mg; and choline chloride, 2 gm.

[4] Wesson Modification of Osborne-Mendel. See footnote 2 in text.

[5] Vitamin-free Test Casein. See footnote 1 in text.

[6] These include the supplements indicated in footnotes 2, 3, 4 and 5.

[7] The following flavonoids were each fed at a 0.4% level in the diet: calcium flavonate glycoside, naringen (naringenin-5-rhamnosidoglucoside), hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex.

[8] Solka Floc BW 200, Brown Co., Boston.

[9] VioBin Corp., Monticello, Illinois.

[10] Schultze's amino acid mixture (Schultze, '57) consisting of the following: L-arginine monohydrochloride, 7.65; L-histidine monohydrochloride, 5.21; DL-isoleucine, 19.94; L-leucine, 18.97; L-lysine hydrochloride (95%), 11.06; DL-methionine, 5.40; DL-phenylalanine, 8.00; DL-threonine, 12.00; DL-tryptophan, 2.78; DL-valine, 21.60; DL-aspartic acid, 9.72; DL-alanine, 8.66; L-cystine, 5.01; L-glutamic acid, 36.74; L-tyrosine, 9.90; and glycine, 0.76.

[11] Extracted liver residue, Wilson Laboratories, Chicago. This consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

[12] Dried Torula Yeast U.S.P., Lake States Yeast Corp., Rhinelander, Wisconsin.

Methyl linoleate, however, when fed at levels of 1, 5 or 10% of the diet was without protective effect, suggesting thereby that the beneficial effects of the more unsaturated vegetable oils were caused by some factor or factors other than their essential fatty acid content. Since tocopherol is present in considerable concentration in a number of unsaturated vegetable oils and in view of the extensive literature on the destruction or inactivation of vitamin E by fish oils, experiments were conducted to determine the effects of graded amounts of vitamin E (in addition to that present in the basal ration) on the response of immature rats fed the basal low-fat diet supplemented with 10% of crude tuna oil. Findings indicate that α-tocopherol, when fed at levels of 0.025, 0.05 or 0.1%, partially counteracted the growth retardation (and completely prevented diarrhea) on the above diet. Its growth-promoting effect was more marked at the 0.05% than the 0.025% level; no further increase in weight occurred, however, when fed at a higher (i.e., 0.1%) level.

In contrast to the effects obtained with α-tocopherol, however, α-tocopheryl acetate, when fed in comparable amounts, was without protective effect. Intraperitoneal injections of α-tocopheryl phosphate (10 mg/rat/day, 6 times/week) were also without growth-promoting or antidiarrheal effect. These findings suggest that the beneficial effects of α-tocopherol (which has marked antioxidant activity in contrast with that exhibited by α-tocopheryl acetate) were due not to its vitamin E activity *per se* but rather to its antioxidant properties, either in the diet itself or in the intestinal tract. Further evidence that the beneficial effect of α-tocopherol was due to its antioxidant activity was the observation that DPPD and Santoquin (both potent antioxidants) were also active in counteracting the growth retardation and diarrhea of immature rats fed the basal low-fat diet supplemented with 10% of crude tuna oil.

Available data indicate, however, that the beneficial effect of the unsaturated vegetable oils in counteracting the toxic effects of tuna oil administration were due, at least in part, to some factor other than its tocopherol content. This is indicated by (1) findings that cottonseed oil, soybean oil, sesame oil, corn oil and wheat germ oil, when fed at a 10% level in the diet, were more effective than the α-tocopherol supplements in counteracting the growth retardation caused by crude tuna oil under conditions of the present experiment, despite the fact that they contained less tocopherol; and (2) the demonstration that sesamol, a constituent of sesame oil with antioxidant properties, was completely effective when fed as the sole supplement, even at 1/6th the dosage of α-tocopherol used, in counteracting all symptoms of fish oil toxicity on the purified low-fat diet. These findings suggest that the unsaturated vegetable oils indicated above contained, besides tocopherol, one or more additional materials with significant antioxidant activity equal to or exceeding that of tocopherol itself.

In addition to the protective effect of unsaturated vegetable oils and substances present therein, a number of natural source materials of both plant and animal origin, as well as some of the known nonlipid nutrients, were also active in preventing growth retardation and diarrhea in immature rats fed the purified low-fat ration supplemented with 10% of crude tuna oil. Alfalfa meal at a 20% level in the diet and desiccated liver N.F. and Torula yeast at a 10% level of feeding were particularly effective in this regard. A supplement of 10% of casein, 10% of fish meal or a mixture of crystalline amino acids at a 10% level in the diet, as well as DL-methionine alone at a 0.6% level of feeding, also resulted in a significant increase in body weight, although less than that obtained with the 20% of alfalfa meal, 10% of liver or 10% of Torula yeast supplements. A combined supplement of 10% of casein, 5% of salt mixture, 2 gm of choline chloride per kilogram of diet plus the various vitamins in an amount equal to the level at which they were incorporated in the basal low-fat ration were also active in counteracting the growth retardation (and diarrhea) of immature rats fed the basal low-fat diet supplemented with 10% of crude tuna oil. No data are available as to the cause of the toxic effects produced by fish oils

when incorporated in the purified low-fat diet nor the mechanism (or mechanisms) whereby α-tocopherol, sesamol, DPPD, Santoquin, DL-methionine and the other supplements indicated above exerted their protective effect.

SUMMARY

Immature rats fed a purified low-fat diet supplemented with 10% of fish oil (i.e., crude or refined tuna oil, crude sardine oil, menhaden oil or cod liver oil) showed a highly significant retardation in growth accompanied by diarrhea. These effects were largely counteracted by the concurrent administration of cottonseed oil at a 10% level in the diet. Soybean oil, sesame oil, corn oil and wheat germ oil at a 10% level of feeding were also active in this regard but olive oil, coconut oil, hydrogenated cottonseed oil, butter fat and lard when fed at a 10% level in the diet had little if any protective effect. Methyl linoleate when fed at levels of 1, 5 or 10% of the diet was without protective effect. α-Tocopheryl acetate when incorporated at levels of 0.025, 0.05 or 0.1% in the diet was also inactive. Intraperitoneal injections of α-tocopheryl phosphate (10 mg/rat/day, 6 times/week) were likewise ineffective. α-Tocopherol, however, when administered at levels of 0.025, 0.05 or 0.1% in the diet had a significant protective effect although less than that obtained with DPPD (N,N′-diphenyl-*p*-phenylenediamine) and Santoquin (6-ethoxy-2,2,4-trimethyl-1, 2-dihydroquinoline) when fed at a 0.05% level in the diet or sesamol (the methylene ether of oxyhydroquinone, a constituent of sesame oil with antioxidant properties) when fed at 0.05 or 0.017% levels in the diet. In addition to the materials indicated above, alfalfa meal at a 20% level in the diet, desiccated liver N.F. and Torula yeast at a 10% level of feeding and supplements of casein, fish meal or a mixture of crystalline amino acids at a 10% level in the diet or DL-methionine at a 0.6% level of feeding also had significant activity in preventing growth retardation and diarrhea in immature rats fed a purified low-fat diet supplemented with 10% of crude tuna oil.

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Effect of Variations of Rations on the Incidence of Teratogeny in Vitamin E-Deficient Rats[1]

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The experimental production of congenital malformations by various metabolic means has been recently reviewed by Kalter and Warkany ('59). Previous studies (Thomas and Cheng, '52; Cheng and Thomas, '53) have shown that congenital malformations may be produced in the vitamin E-deficient rats if a single dose of one or more milligrams of *dl*-α-tocopheryl acetate is given during the second week of gestation from the 9th to the 12th day inclusive. If the therapeutic dose is given before the 8th day of gestation, only normal young are obtained. On the other hand, if it is given after the 12th day of gestation, complete resorption ensues. When the supplementation is given on the 9th day of gestation, hydrocephalus is the most prevalent abnormality, and may be accompanied by club feet, exencephalus, harelip, receding maxillae and other malformations. If it is given on the 11th day of gestation, exencephalus and gastroschisis are the predominant malformations with receding maxillae, club feet, hydrocephalus, edema, ectocardia, kinked tail and others occurring in an order of decreasing incidence (Cheng and Thomas, '53).

The development of the gross malformations has been followed. Arrested development characterizes the 11-day old embryos from the experimental group which has received a single dose of vitamin E on the 10th day of gestation. By the 13th day of gestation, exencephalus when present in the abnormal embryos is clearly apparent (Cheng et al., '57). Table 1 illustrates the type of abnormalities observed in 112 abnormal fetuses of various gestational age. The administration of progesterone or estrone at certain levels, in one or several doses, either reduces or eliminates the incidence of teratogeny in vitamin E-deficient rats given 2 mg of the vitamin on the 10th day of gestation (Cheng, '59). Preliminary observations on the histological changes in the tissues of the term fetuses (Cheng and Thomas, '55) and on glycogen content of developing embryos have also been reported (Cheng and Scranton, '59).

The purpose of the experiments was to breed the females in a borderline state of vitamin E-deficiency which would alter the normal development of the young without causing complete intrauterine death of the embryos. Up to the present time congenital abnormalities were produced by using vitamin E-deficient rations (semisynthetic rations 1 and 2, SSR1, SSR2) in which yeast was used as the source of most of the B vitamins and cod liver oil as the source of vitamins A and D. It was deemed advisable, therefore, to evaluate the effect of substituting pure crystalline sources of the vitamins (with the exception of vitamin E) for the natural food materials used in the original rations. Accordingly, after due consideration of the diets used by Pan et al. ('49), Everson et al. ('54) and Nelson and Evans ('56) such a vitamin E-deficient ration (pure synthetic ration 1, PSR1) was formulated and used in one series of experiments. In addition, another group of rats was maintained on a vitamin E-deficient ration containing 2% of cod liver oil in addition to the crystalline vitamins. This 4th vitamin E-deficient ration was designated as PSR2, pure synthetic ration 2.

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TABLE 1

Frequency of malformations observed in embryos of different gestational age from vitamin E-deficient rats given 2 mg of dl-α-tocopheryl acetate on the 10th day of gestation as a supplement to ration SSR 2

Type of abnormalities	Gestational age, day								Total (112)
	14 (6)[1]	15 (19)	16 (11)	17 (24)	18 (11)	19 (12)	20 (13)	21 (16)	
Viscera:									
Umbilical hernia	—	—	—	9	9	8	12	16	54
Ectocardia	4	0	0	0	0	0	1	0	5
Skeletal system:									
Scoliosis	0	2	7	8	2	4	5	5	33
Club feet	0	1	7	3	2	0	7	7	27
Webbed front feet	0	4	3	0	0	0	0	0	7
Syndactylism	0	0	0	1	1	0	2	1	5
Kinked tail	0	1	1	0	0	1	1	0	4
Central nervous system:									
Exencephalus	2	4	1	5	3	1	3	3	22
Hydrocephalus	0	2	3	3	0	0	0	0	8
Anencephalus	1	0	0	0	1	0	1	2	5
Microphthalmia	0	1	0	1	0	0	0	0	2
Circulatory system:									
Hemorrhagic spots	0	8	3	8	0	0	3	0	22
Edema	0	0	0	1	1	0	3	3	8
Mouth:									
Harelip	0	3	1	2	0	0	2	1	9
Receding maxillae	0	0	2	2	0	0	1	2	7
Receding mandibles	0	0	0	0	1	0	0	1	2
Cleft mandibles	1	1	0	0	0	0	0	0	2
Agnathus	0	0	0	0	0	1	0	0	1
Cleft palate	0	0	0	1	0	0	0	0	1
Total	8	27	28	44	20	15	41	41	224

[1] Figures in parentheses indicate number of abnormal embryos observed.

In the present communication the effect of these 4 vitamin E-deficient rations on the gestational performance, including the incidence of teratogeny, is reported and compared.

EXPERIMENTAL

The detailed procedure for producing congenital malformations in vitamin E-deficient rats was reported earlier (Cheng and Thomas, '53). For this study weanling female rats of the Holtzman strain, weighing approximately 40 gm, were fed the different vitamin E-deficient rations, the compositions of which are presented in table 2.[2] The rats received these rations ad libitum throughout the period of growth and pregnancy. Distilled water was available at all times. The temperature of the animal room was kept constant at 72 to 76°F. When the rats weighed approximately 175 gm, they were mated with male rats previously fed a pellet diet.[3] Mating was ascertained by the presence of sperm in the vaginal smears. The day on which sperm were found was designated zero day of gestation. On the 10th day of gestation, 2 or 4 mg of *dl-α*-tocopheryl acetate in 1 ml of corn oil[4] was given by gavage to the rats in the experimental groups. Positive control rats received 2 mg of tocopheryl acetate each day from the first to the 5th day of gestation inclusive, whereas negative control rats received no tocopherol supplementation. All rats were laparotomized on the 21st day of gestation, and the conceptuses were examined for gross congenital abnormalities. Furthermore, blood, muscle and liver were collected

[2] We are indebted to Dr. P. W. Wilcox of Merck, Sharp and Dohme, West Point, Pa., for crystalline vitamins, and Dr. J. P. Kass of Pabst Laboratories, Milwaukee, for brewers' yeast.

[3] Purina Pellets, Ralston Purina Co., St. Louis.

[4] Mazola, Corn Products Refining Co., New York.

TABLE 2

Composition of vitamin E-deficient rations

Ingredients	Pure synthetic		Semisynthetic	
	PSR 1	PSR 2	SSR 1	SSR 2
	%	%	%	%
Dextrose[1]	62	60	—	—
Dextrin	—	—	49	49
Casein (vitamin-free)[2]	24	24	—	18
Casein (crude)	—	—	18	—
Lard	10	10	22	22
Salt mixture (U.S.P. XIV)	4	4	4	4
Dried brewers' yeast	—	—	5	5
Cod liver oil	—	2	2	2
	mg/kg ration			
β-Carotene	3	3		
Pyridoxine	5	5		
Riboflavin	10	10		
p-Aminobenzoic acid	10	10		
Thiamine·HCl	15	15		
Niacin	20	20		
2-methyl-1, 4-naphthoquinone	25	25		
Ca pantothenate	50	50		
Pteroylglutamic acid	50.5	50.5		
Inositol	400	400		
Choline	1000	1000		
	μg/kg ration			
Vitamin D_2[3]	10	10		
Vitamin B_{12}	50	50		
d-Biotin	300	300		

[1] Cerelose.
[2] Vitamin-Free Casein, Nutritional Biochemicals Corp., Cleveland.
[3] Calciferol.

from both the mother rats and the normal, as well as abnormal, fetuses for chemical assay of their vitamin E content. These results will form a later report.

The percentage of abnormal young was calculated in two ways. First, it was calculated in terms of the total number of implantation sites, the same as the other categories in table 3. Then, in order to facilitate comparison with comparable data from other maternal deficiencies in the literature, it was also calculated in terms of total number of live young.

For statistical analysis of the significance, at the one or 5% level, of the incidence of normal and abnormal young and dead and resorbed fetuses the chi-square test was used. The significance of the difference between groups with regard to the average number of implantation sites per rat was assessed by means of the "Student" t test.

RESULTS AND DISCUSSION

The gestational performance of the rats receiving the 4 vitamin E-deficient rations is presented in table 3. The vitamin E-deficient rats (negative controls) on all rations showed 100% resorption. The positive control rats fed PSR1 yielded 100% normal young, whereas those receiving SSR1 and SSR2 showed 9.7 and 1% resorption, respectively. The 9.7% resorption was slightly higher than the 6.5% normally found in stock colony rats (Cheng and Thomas, '53). The results from the positive and negative control rats indicated that all these rations were vitamin E-deficient.

Differences between rats fed PSR1 and those receiving PSR2. Compared with rats fed PSR1, the rats receiving PSR2 showed greater weight gain during gestation due to a significantly higher percentage of normal young ($P < 0.05$) with concurrent significant decrease in the percentage of resorption ($P < 0.01$). Although the incidence of teratogeny as calculated in terms of the total number of implantation sites, appeared reduced, the difference between the two groups was not significant statis-

TABLE 3

Gestational performance of rats fed various vitamin E-deficient rations and receiving various treatments

Ration	Dose of *dl*-α-tocopheryl acetate/day	Given on day(s) of gestation	No. rats bred	Weight gain during gestation	Live fetuses			Dead fetuses		Implantation sites[1]
					Normal	Abnormal		Dead	Resorbed	Av. no. ± S.D.
	mg			*gm*	%[2]	%[2]	%[3]	%[2]	%[2]	
PSR 1	2	1–5	11	56	100	—	0	—	—	7.9 ± 2.9
	2	10	15	25	6.1	4.1	40.0	1.0	88.8	6.5 ± 3.8
	2	10	(26)[4]	(53)	(19.1)	(2.6)	(12.0)	(1.7)	(76.1)	(8.8 ± 3.3)
	4	10	18	44	11	6.5	37.0	0	82.5	8.6 ± 2.8
	—	—	12	25	0	0	0	0	100	8.3 ± 2.9
SSR 1[5]	2	1–5	19	73	90.3	0	0	0	9.7	8.6 ± 1.4
	2	10	20	47	8.5	15.0	63.8	2	74.5	10.0 ± 1.1
	4	10	20	60	17.7	12.8	42.0	0.4	69.1	11.3 ± 5.2
	—	—	21	26	0	0	0	0	100	9.9 ± 3.9
SSR 2	2	1–5	46	73	98.0	0	0	1.0	1.0	9.0 ± 2.1
	2	10	38	45	11.0	5.1	31.2	0.3	83.7	7.9 ± 2.9
	4	10	79	44[6]	15.3	2.9	15.8	0.5	81.3	8.0 ± 3.3
	—	—	40	26	0	0	0	0	100	8.5 ± 2.7

[1] Including those of live, resorbed and non-resorbed dead fetuses.

[2] In terms of total number of implantation sites.

[3] In terms of total number of viable young.

[4] Rats fed ration PSR 2.

[5] Unpublished data: Cheng, D. W. 1954 A study of the occurrence of teratogeny in vitamin E-deficient rats and associated abnormalities in blood and tissues. Ph.D. thesis, Iowa State College, Ames.

[6] Average of 13 rats.

tically. When the frequency of abnormality was determined as a function of viable young, however, a significant ($P < 0.01$) reduction of abnormal young was observed in rats fed PSR2. The average number of implantation sites was raised slightly but not significantly. All these differences were due probably to the additional incorporation of cod liver oil in PSR2. The additional vitamins A and D in the cod liver oil plus fat *per se* might explain the beneficial effects of PSR2 on the gestational performance. Certainly our results are contrary to those of Singsen et al. ('55) who observed that, other things being equal, the addition of 2% of cod liver oil to the vitamin E-deficient diet of chicks reduced the body weight gain, and augmented the incidence of mortality and encephalomalacia.

Differences between rats fed PSR1 and those receiving SSR1. Compared with corresponding rats fed PSR1, the rats receiving SSR1 with 2 mg of vitamin E supplementation gave a better gestational performance, namely, more weight gain during gestation, significantly lower percentage of resorption ($P < 0.01$) and higher average number of implantation sites ($P < 0.05$). However, there was a significantly higher incidence of abnormal young ($P < 0.01$), determined either as a function of total number of implantation sites or as a total number of live young. This probably indicates that SSR1 might not be as vitamin E-deficient as PSR1. When the corresponding groups that had received 4 mg of vitamin E were compared, the same trend was accentuated by the fact that the percentage of normal young was also significantly higher in SSR1 group ($P < 0.01$) although difference in abnormality as a function of viable young was not significant.

Difference between rats fed PSR1 and those receiving SSR2. When observations of rats fed PSR1 and those that had received 2 mg of vitamin E with SSR2 were compared, we found that rats fed SSR2 showed better gestational performance in that in addition to greater weight gain during gestation, there was significant enhancement of the percentage of normal young ($P < 0.01$) with a significant decrease in the incidence of resorption ($P < 0.01$). No significant difference was observed in the incidence of abnormal young determined either in terms of total number of implantation sites or live young. The difference between the average numbers of implantation sites was not statistically significant.

When the corresponding groups receiving 4 mg of vitamin E were compared, the results showed that in the group fed SSR2 there was a significantly greater percentage of normal young ($P < 0.01$) with concomitant significant decrease in the incidence of abnormal young calculated both ways ($P < 0.01$). No significant difference was noted between the results from any of the other categories.

When the obseravtions of rats fed PSR1, SSR1 and SSR2 are examined together, it seems that PSR1 was just as vitamin E-deficient as SSR2, if not more so, but both were more deficient than SSR1.

Differences between rats fed PSR2 and SSR1. Compared with rats receiving SSR1, the rats fed PSR2 showed a significantly higher percentage of normal young ($P < 0.01$) with a concurrent significant decrease in the percentage of abnormal young, determined either way ($P < 0.01$). The differences between the results from the other categories were not significant statistically.

Differences between rats fed PSR2 and SSR2. When the observations of rats receiving PSR2 were compared with those of rats fed SSR2, it was seen that the rats fed PSR2 showed a significantly higher percentage of normal young ($P < 0.05$) and lower percentages of both the abnormal young, determined both ways, ($P < 0.01$) and resorption ($P < 0.01$).

When the comparisons between rats fed PSR2 and SSR1, and those between rats receiving PSR2 and SSR2 were viewed together, we found that the difference between the two sets of data was the significantly higher incidence of resorption in rats fed SSR2 in which the casein used was vitamin-free. In other words, other things being equal, the removal of these trace vitamins from the crude casein appears to have caused the increase in the incidence of resorption under the conditions of the experiments.

Differences between rats fed SSR1 and those receiving SSR2. Compared with rats fed SSR1 that had received 2 mg of vitamin E supplementation, those fed SSR2 had a significantly higher incidence of normal young ($P < 0.01$) and resorption ($P < 0.01$) with a significantly lower incidence of abnormal young, calculated both ways, ($P < 0.01$), and average number of implantation sites ($P < 0.05$). The weight gain during gestation was approximately the same for both groups. When the groups receiving 4 mg of vitamin E were compared, that fed SSR2 showed a significantly lower percentage of abnormal young, determined either way, ($P < 0.01$) and average number of implantation sites ($P < 0.01$). There was also a significantly higher percentage of resorption ($P < 0.01$). It appears that the rats fed SSR1 derived a good deal of benefit from the 4 mg of vitamin E supplementation, resulting in a significant rise in the percentage of normal young ($P < 0.01$) and a coincident significant reduction in the percentage of resorption ($P < 0.01$). Otherwise, the trend of significantly lower incidence of abnormal young and average number of implantation sites, together with a higher incidence of resorption, in rats fed SSR2, prevailed in both the group with two and that with 4 mg of vitamin E supplementation. These differences were probably due to the use of vitamin-free casein instead of crude casein in SSR2. Apparently the trace vitamins in the crude casein minimized the incidence of resorption as well as augmented the incidence of abnormal young in the rats fed SSR1.

Effect of increase in the level of vitamin E supplementation. If we viewed the experiments as a whole, disregarding the compositions of the rations, we noted that compared with rats that had received 2 mg of vitamin E supplementation, those fed 4 mg of vitamin E showed uniformly a significant increase in the percentage of normal young ($P < 0.01$) resulting usually in an augmentation of the weight gain during gestation. The percentage of abnormal young were usually slightly reduced, but, except in the rats fed SSR2 which showed a significant decrease ($P < 0.01$), the differences between the 2 and 4 mg groups fed PSR1 and SSR1 were not statistically significant. There was little increase in the average number of implantation sites.

From the foregoing it can be seen that congenitally malformed young can be produced when the rats are fed any one of these 4 vitamin E-deficient rations. However, the percentage of abnormal young varies greatly depending upon the composition of the ration. Under the conditions of the experiments it appears that ration SSR1 gives the highest incidence of congenitally abnormal young and consequently is the best from the standpoint of experimental teratology.

SUMMARY

Congenital malformations were produced in the offspring of rats reared and bred on 4 vitamin E-deficient rations. Variations in the composition of the rations influenced the yield of the abnormal as well as the normal young, the incidence of resorption and the average number of implantation sites. Increasing the level of vitamin E supplementation from 2 to 4 mg on the 10th day of gestation resulted in a uniformly significant increase in the percentage of normal young, but the percentage of abnormal young might be slightly or significantly reduced, or even slightly increased depending upon the ration used.

The trace vitamins in crude casein appeared to have caused a decrease in the incidence of resorption as well as an increase in the incidence of abnormal young and the average number of implantation sites. The addition of cod liver oil to a pure synthetic ration resulted in a significant increase in the yield of normal young together with a concomitant significant decrease in the percentage of resorption.

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The Response of Man to Dietary Cholesterol[1,2]

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Recent work in our laboratory has demonstrated unequivocally that the cholesterol present in butter accounts, at least in part, for its hypercholesterolemic activity (Beveridge et al., '59). The experiment described herein was performed to obtain further information on the relationship between dietary and serum cholesterol in man.

EXPERIMENTAL

Ninety-three university students (75 men and 18 women) consumed a homogenized formula fat-free diet (table 1)[3] for a period of 8 days at the end of which time they were divided into 8 groups of comparable serum cholesterol levels as indicated by values obtained on day 4, and continued for another 8 days on a diet modified by the substitution of a butter oil fraction,[4] low in cholesterol, for 30% of calories at the expense of an equicaloric amount of carbohydrate.

Supplements of highly purified cholesterol[5] were added to the rations given to the 8 groups in the following amounts in milligrams per 950 calories: zero, 25, 50, 100, 200, 400, 800 and 1600. The sterol was dissolved in the fat component by warming the latter, and then the cholesterol-enriched butter-fat fraction was homogenized along with the rest of the

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[2] Presented in abbreviated form at the meeting of the Council on Arteriosclerosis of the American Heart Association on November 8, 1959.

[3] The authors are indebted to the following firms for generous supplies of certain of the dietary ingredients and the preparation of fat fractions: Distillation Products Industries, Rochester, New York; Mead Johnson of Canada, Limited; and Mil-ko Products Limited, Hamilton, Ontario, Canada.

[4] The butter-fat fraction was prepared through the courtesy of the Distillation Products Industries of Rochester, New York. It was comprised of the residue remaining after removal of almost all of the cholesterol and some of the more volatile triglycerides by distillation in high vacuum. It represented about 60% of the original butter-fat and contained 0.02% of cholesterol.

[5] A sample of high purity prepared from sheep-wool fat and obtained from the Amerocholesterol Company, New York.

TABLE 1

Composition of basal diet.[1] Amounts required to make a 950-Cal. sample

Ingredient	Amount	Protein	Fat	CHO
	gm	*gm*	*gm*	*gm*
Skim milk powder[2]	28.1	10	0.1	14.6
Calcium caseinate[3]	28.4	25	0.57	—
Sucrose	20.0	—	—	20.0
Maltose and dextrins[4]	169.8	—	—	166.4
Total		35.0	0.67	201.0
Calories	950.03	140.0	6.03	804.0
Calories, %		14.74	0.63	84.63

[1] Two grams of iodized salt were added per 950-Cal. batch. A mixture of vitamins was also added to supply the following amounts of these substances (in milligrams) per 950 Cal.: thiamine, 0.6; riboflavin, 0.6; niacin, 5.0; pyridoxine, 5.0; Ca pantothenate, 5.0; ascorbic acid, 25; and 1700 I.U. of vitamin A was added per 950-Cal. portion.

[2] Mil-ko.

[3] Casec.

[4] Dextrimaltose.

dietary components. The only other items permitted were water, clear tea and clear coffee. The volunteers were instructed to consume a sufficient amount of the diet to maintain their body weight as nearly constant as possible. With a few exceptions, the subjects were relatively successful on this score. The average change in weight was −1.1 pound. Sixty-seven subjects (53 men and 14 women) completed the feeding period.

Total serum cholesterol was determined by the method of Abell et al. ('58) on blood obtained from the subjects in the fasting state at zero, 4, 8, 12 and 16 days.

RESULTS AND DISCUSSION

The behavior of the serum cholesterol expressed in terms of average percentage change at day 16 from the values found at day 8, is shown in figure 1 and the statistical data on the absolute changes are presented in table 2.

As expected, during the first 8 days when the fat-free diet was being consumed, the serum cholesterol levels dropped rapidly, there being as usual no significant difference between the values found at 4 and 8 days. The averages in mg/100 ml of serum for day zero, 4, and 8 were 201.0, 146.8 and 145.5 respectively. The rapidity with which such subjects reach a low plateau on the homogenized fat-free diet has been established in previous publications (Beveridge et al., '59). An examination of figure 1 reveals a remarkably good correlation between the dietary increments of cholesterol up to 800 mg/950 Cal. and its concentration in the serum. However, at the 1600-mg level there is no greater response than with the 200- or 400-mg supplements. These relationships are illus-

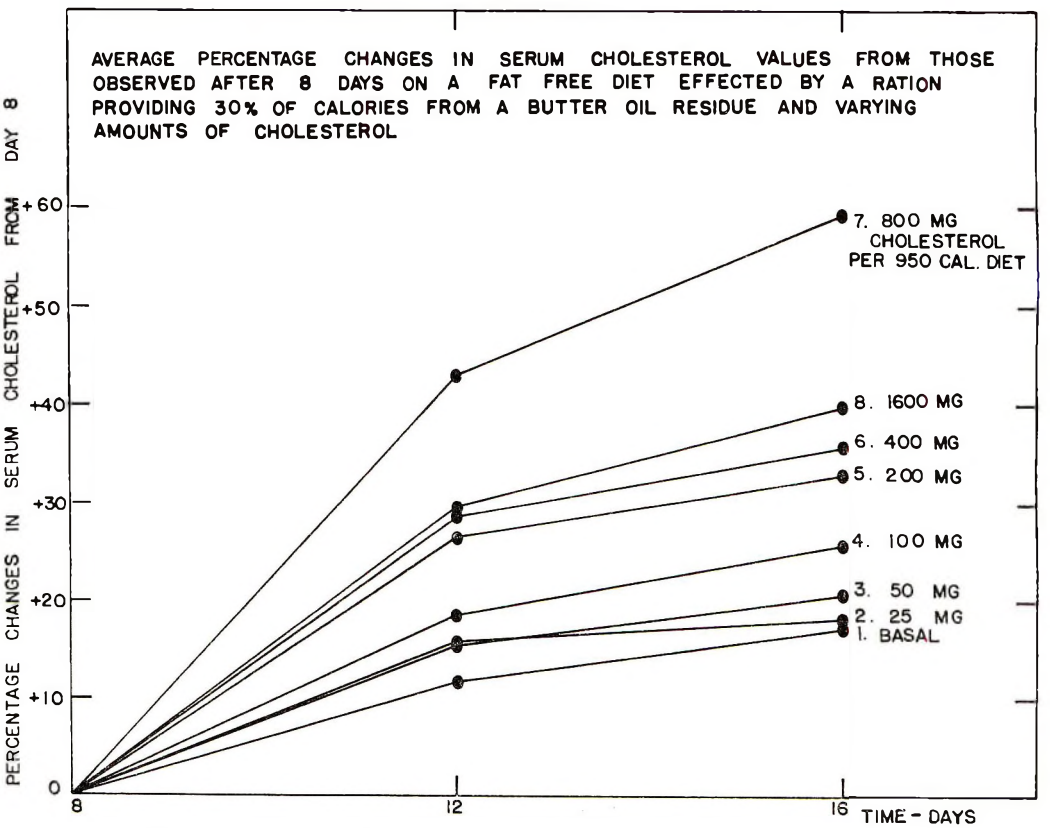

Fig. 1 The effect of supplementing a butter-fat fraction stripped of cholesterol by distillation in high vacuum with varying amounts of cholesterol. The number of subjects in each group was as follows: (1) 9; (2) 6; (3) 9; (4) 9; (5) 9; (6) 6; (7) 10; (8) 9 subjects.

TABLE 2

The effects on serum cholesterol levels of substituting a butterfat fraction[1] equicalorically for carbohydrate in a fat-free diet[2] at a level of 30% calories and supplementing with cholesterol

Group no.	Supplement of cholesterol	Estimated daily intake of cholesterol	No. of subjects[3]	Mean difference between day 8 and 16	Standard error of difference	t	P
	mg/950 calories	*mg*		*mg cholesterol/ 100 ml*			
1	0	13.3	9	+25.3	5.06	5.0	0.005–0.001
2	25	94.7	6	+27.6	7.33	3.77	<0.02–0.01
3	50	153	9	+29.1	6.51	4.47	0.005–0.001
4	100	293	9	+31.7	6.53	4.85	0.005–0.001
5	200	634	9	+41.9	6.74	6.22	< 0.001
6	400	1295	6	+48.0	5.77	8.32	< 0.001
7	800	2494	10	+71.7	7.96	9.00	< 0.001
8	1600	4493	9	+58.8	5.40	10.89	< 0.001

[1] See footnote 4 on page 61 for a description of this fraction.
[2] All subjects ate the fat-free basal ration for a period of 8 days.
[3] There were no females in group 6, one in group 2, two in groups 1, 3, 5, 7, and 8 and three in group 4.

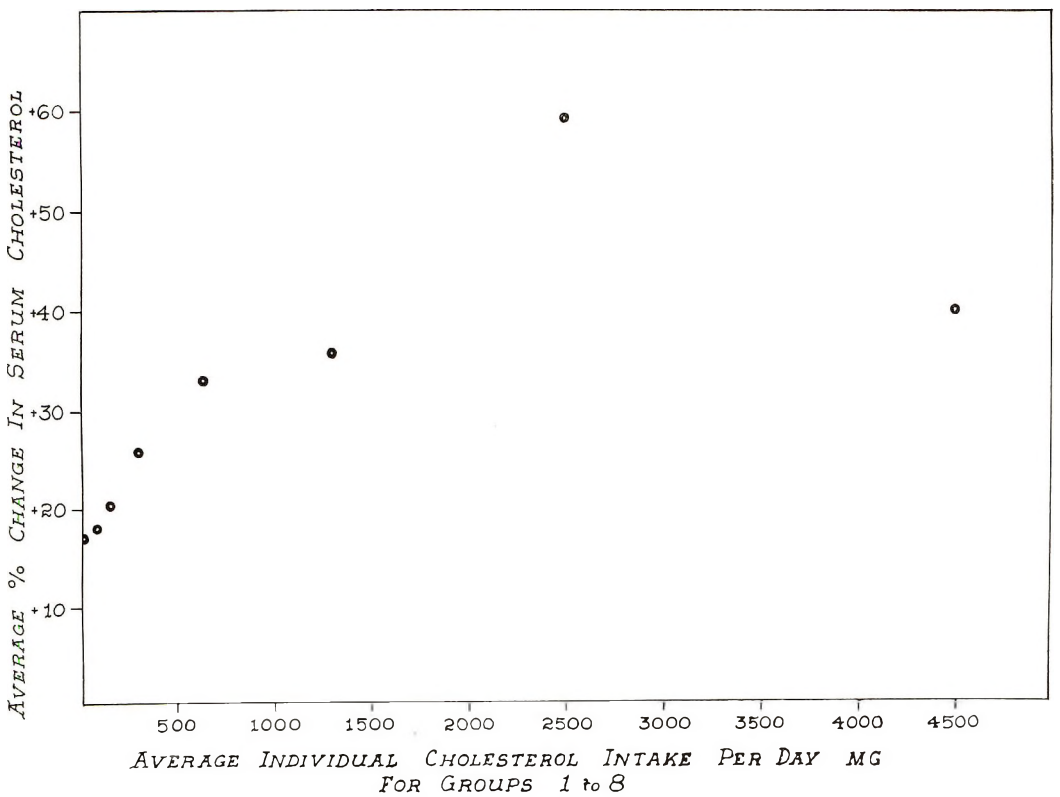

Fig. 2 The relationship between the estimated daily cholesterol intake and percentage change in serum cholesterol in groups of 6 to 10 subjects given a ration providing 30% of calories from a butter-fat fraction stripped of cholesterol by distillation in high vacuum and supplementation with varying amounts of cholesterol.

trated more clearly in figure 2 in which the average daily intakes of cholesterol, based upon the estimates of dietary consumption submitted by the volunteers, are plotted against the increase in serum cholesterol.

Between intakes of about 13 mg per day to 634 mg per day, the serum cholesterol increases sharply but there is no further significant increase obtained by daily intakes of 1300 to 4500 mg and the dose-response curve is relatively flat. The difference between the increase of 25.3 mg/100 ml for group 1 and 48.0 mg/100 ml for group 6 is highly significant ($P < 0.01$) as determined by an analysis of variance. At the present time we are at a loss to interpret the peak obtained at the daily intake of 2450 mg. This may very well represent a reproducible response and it is proposed to obtain further information on the effect of intakes of this order of magnitude and above.

It is concluded that the serum cholesterol concentration in man is affected by dietary levels of this sterol but that there is a very effective control in normal young subjects that prevents the development of hypercholesterolemia even when relatively large amounts of cholesterol are eaten. Presumably this mechanism has broken down in hypercholesterolemic subjects and is impaired in diabetics (Beveridge et al., '59).

After Anitschkow ('33) reported that the addition of cholesterol to the rations of rabbits led to an increase in serum cholesterol and the production of vascular lesions simulating those seen in human atherosclerosis, it was logical to assume that this sterol might be responsible for a similar sequence of events in man. Although many experiments have been performed over the past 50 years to test this point, equivocal results have been obtained. Some of the possible reasons for the lack of any clear-cut answer from these investigations were discussed in a previous publication (Beveridge et al., '59) and will not be repeated here. It is believed that an examination of the data reported in this publication provides the most cogent explanation in this regard. The response of serum levels to dietary cholesterol appears to plateau above a concentration of 200 mg/950 Cal. (fig. 1) corresponding to a daily intake of about 600 mg of the sterol (fig. 2). This amount approximates the intake of most people who are ingesting the usual variety of non-vegetarian foodstuffs.

Since most attempts to investigate the effect of dietary cholesterol have involved its addition—often in relatively large amounts, a point that may also be of some importance — to a non-vegetarian diet which already provided a daily intake of several hundred milligrams of cholesterol, it is not surprising that dramatic increases have not been observed. A consideration of the curve in figure 2 supplies ample reason for this relative lack of effect. It would seem redundant to point out that if one wishes to study the response of an animal or of man to a dietary component, it is mandatory that the basal ration be free from or at least essentially free from the substance to be tested.

Although such factors as plant sterols (or something associated with them) and degree of unsaturation of dietary fat presumably contribute to the well-known difference in serum cholesterol levels between vegetarians and non-vegetarians (Hardinge and Stare, '54), the presence of cholesterol in the diet of the latter group undoubtedly also accounts for at least part of this difference.

SUMMARY

Ninety-three university students were placed upon a homogenized fat-free diet for a period of 8 days at which time they were divided into 8 groups and given a ration modified by the substitution of a butter-fat fraction, low in cholesterol, for 30% of calories at the expense of carbohydrate. Cholesterol supplements varying from nil up to 1600 mg/950 Cal. were added. Sixty-seven subjects successfully completed the experiment which was terminated after a total of 16 days. Between intakes of 13 and 634 mg of cholesterol per day, the serum cholesterol increased sharply but no further significant increases were obtained at daily intakes of 1300 to 4500 mg.

ACKNOWLEDGMENTS

Our thanks go to the student volunteers who made posible the performance of this large-scale experiment. The technical assistance of J. Donaldson, J. Ludwig and

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Essential Fatty Acid Properties of Tuna, Herring and Menhaden Oils[1]

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The limited evidence that is presently available indicates that the structures of the major polyunsaturated fatty acids of marine oils are not of the linoleic acid type (Klenk, '57; Farquhar et al., '59; Stoffel and Ahrens, '58; Whitcutt, '57; Silk and Hahn, '54; Montag et al., '57; and Whitcutt and Sutton, '56). Nutritional studies (Privett et al., '59) demonstrated that the dermal symptoms of essential fatty acid (EFA) deficiency in rats were not relieved by the polyunsaturated fatty acids of tuna oil given at a rate of 100 mg per animal per day. Nevertheless marine oils do not appear to be entirely devoid of essential fatty acid activity. Menhaden, herring and whale oils were estimated to contain 4.4, 7.9 and 6.4% of essential fatty acids, respectively, by a rat bioassay method based on growth under conditions of restricted water intake (Thomasson, '53a, b).

In the present study, observations were made of the effects of tuna, menhaden and herring oils on the dermal syndrome of EFA deficiency in the rat as a prelude to the isolation and characterization of the fatty acids responsible for the EFA activity of these oils.

EXPERIMENTAL

Two nutritional experiments were performed. In the first, 50 male weanling rats of the Sprague-Dawley strain were maintained ad libitum on a fat-free diet (Privett et al., '59) for 16 weeks. At the end of this period the growth rate of the animals had reached a plateau and all animals exhibited distinct dermal symptoms of EFA deficiency. The rats were then divided into 5 equal groups. Group 1 was continued on the basal (fat-free) diet and served as a control group. Groups 2, 3, 4 and 5 received the basal diet in which 10% by weight of menhaden, tuna, herring and corn oils, respectively, were substituted for sucrose. The corn oil group served as the positive control for the experiment. The rats were weighed and scored for dermal symptoms of EFA deficiency each week for an additional 12 weeks, and the experiment was then terminated.

The second experiment was conducted in two parts. In the first part 50 male weanling rats were divided into 5 equal groups, and fed ad libitum the same fat-free diet used previously. Groups 1, 2, 3 and 4 were administered orally 200 mg per day of menhaden, tuna and herring oil ethyl esters and ethyl linoleate, respectively. Group 5 received no supplement and served as the negative control for this experiment. The animals were scored for dermal symptoms of EFA deficiency and weighed each week of the experiment for 14 weeks. At this point the administration of 200-mg quantities of esters was discontinued.

In the second part of the experiment, which followed immediately, the same groups of rats were fed the basal diet in which had been substituted for sucrose, 10% by weight of the oil of the same esters that had been administered orally; the ethyl linoleate group was given corn oil. The feeding was continued for an additional 14 weeks during which all of the animals were scored for dermal symptoms and weighed each week as before. The purpose of the second phase of this experiment was to confirm that the dermal symptoms of EFA deficiency could be cured, and the growth affected by feeding a larger amount of oil in the diet.

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RESULTS AND DISCUSSION

The results of the first experiment are summarized in table 1. It is evident from the dermal scores at the end of the 16th and 28th weeks that all of the fish oils cured the dermal symptoms of EFA deficiency and stimulated growth equally as well as corn oil. Figure 1 shows the results on the growth rate of the EFA-deficient animals of feeding 10% of fish oil.

In the first part of the second experiment in which 200 mg of the fish-oil esters were administered daily, the dermal symptoms of fatty acid deficiency developed equally as fast and as severely in animals receiving the fish-oil esters as in the animals fed the fat-free diet (table 2).

In contrast, the growth rates of rats fed the fish-oil esters compared favorably

TABLE 1

Average growth and gross symptoms of animals in experiment 1

Group no.	Supplement	Average weight		Average score[1]	
		Initial 16th week	Final 28th week	Initial 16th week	Final 28th week
		gm	*gm*		
1	Fat-free	232 ± 4.8[2]	252 ± 8.2[2]	5.2 ± 0.3[2]	5.6 ± 0.1[2]
2	10% Menhaden oil	237 ± 7.6	354 ± 15.4	6.0 ± 0.2	1.0 ± 0.4
3	10% Tuna oil	219 ± 15.2	329 ± 16.8	5.6 ± 0.2	0.7 ± 0.2
4	10% Herring oil	235 ± 11.9	354 ± 15.1	5.2 ± 0.2	1.4 ± 0.2
5	10% Corn oil	223 ± 4.0	340 ± 8.1	5.6 ± 0.3	0.1 ± 0.1

[1] Animals scored on basis of dermal symptoms of tail and legs, 0 to 7.
[2] Standard error of mean.

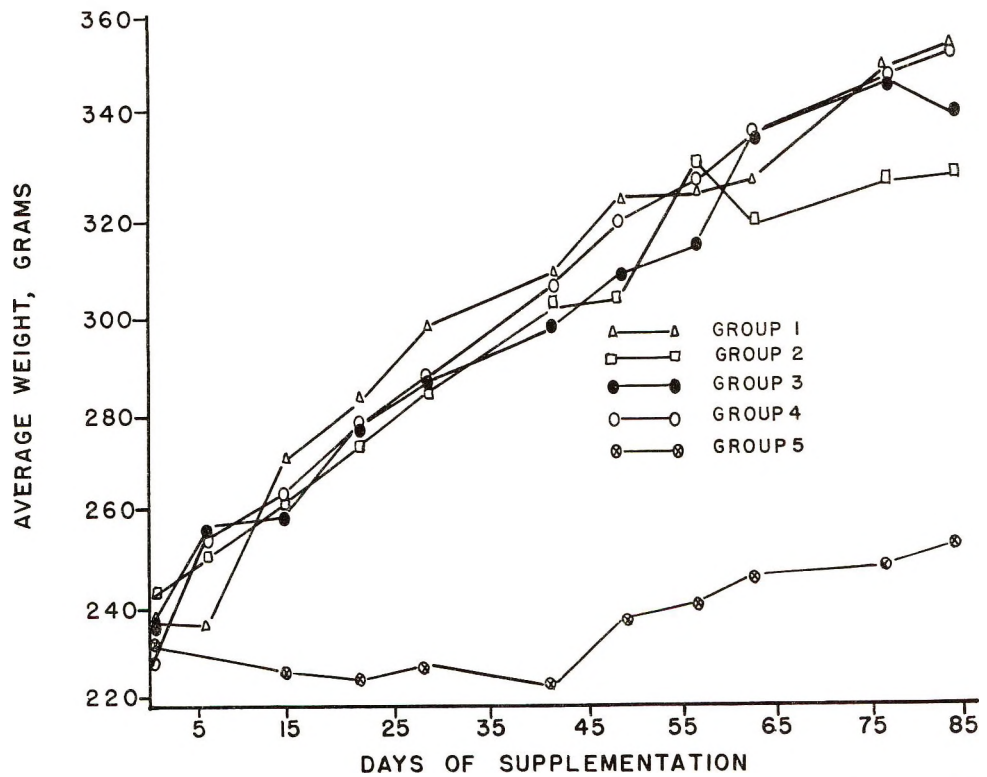

Fig. 1 Average growth rate of rats in experiment 1. Group 1 received the fat-free basal diet; groups 2, 3, 4 and 5 received the fat-free basal diet plus 10% of menhaden, tuna, herring and corn oil, respectively.

with that of the group receiving the ethyl linoleate, and were significantly greater than for the animals fed the fat-free diet as shown in table 2.

When the amounts of these oils were increased to 10% in the diet, in the second phase of this experiment, the dermal symptoms of EFA deficiency were cured. The cure occurred earlier in the animals receiving the corn oil, followed in order of decreasing effectiveness by tuna, menhaden and herring oils (fig. 2).

TABLE 2

Average weights and dermal scores[1] of animals in experiment 2

Group no.	Part 1			Part 2		
	Supplement 200 mg/ animal	14th week		Supplement 10%	28th week	
		Av. weight	Av. score[2]		Av. weight	Av. score[2]
		gm			*gm*	
1	Menhaden oil ethyl esters	290 ± 10.4[1]	5.0 ± 0.4[1]	menhaden oil	351 ± 11.8[1]	0.8 ± 0.2[1]
2	Tuna oil ethyl esters	308 ± 9.8	4.8 ± 0.3	tuna oil	357 ± 15.0	0.6 ± 0.1
3	Herring oil ethyl esters	290 ± 10.4	4.8 ± 0.3	herring oil	352 ± 13.1	1.6 ± 0.2
4	Ethyl linoleate	314 ± 9.0	0.2	corn oil	361 ± 12.5	0
5	Fat-free	245 ± 3.0	5.2 ± 0.2	fat-free	258 ± 5.7	5.5 ± 0.2

[1] Standard error of mean.
[2] Animals scored on basis of dermal symptoms of tail and legs, 0 to 7.

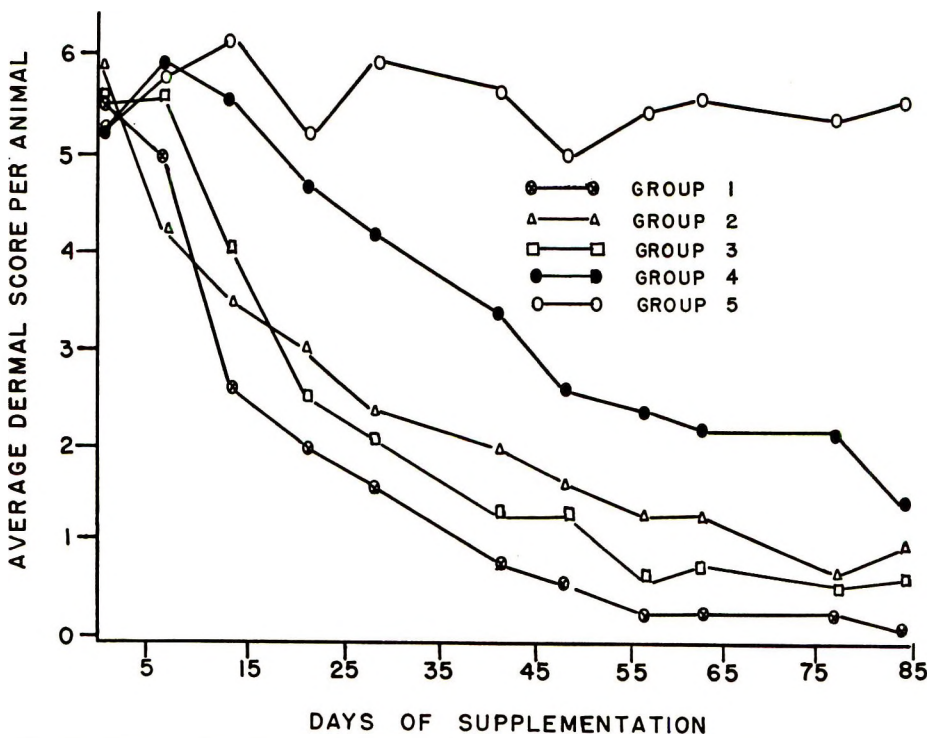

Fig. 2 Average dermal scores of rats in experiment 2. Group 1 received only basal fat-free diet; groups 2, 3, 4 and 5 received basal diet plus 10% of menhaden, tuna, herring and corn oils, respectively.

Since the oral administration of 200 mg per rat per day of tuna, herring or menhaden oils did not prevent the development of the dermal symptoms of EFA deficiency, if the prophylactic dose of linoleic acid is assumed to be about 20 mg per day (Holman, '56), and if "sparking" of polyunsaturated acids by small amounts of linoleate is assumed to be inoperative, these oils may be judged to contain less than 10% of EFA of the linoleate family. If the "sparking" effect on the cure of dermal symptoms (Privett et al., '55) operates with all polyunsaturated acids and one half of the oils are assumed to consist of polyunsaturated acids, the content of linoleate-type acids in these oils may be estimated to be less than 2%.

Inclusion of 10% of fish oils in the diet of rats having pronounced dermal symptoms completely relieved the symptoms. Thus the approximately one gram of oil consumed per rat per day may be estimated to contain at least 20 mg of linoleate-type acids equal to 2% of the oil.

The results presented here indicate that tuna, herring and menhaden oils contain small amounts of linoleate-type acids. In the case of menhaden oil, the estimate of about 2% of acids of the linoleate family agrees in magnitude with an analysis by gas chromatography of this oil by Farquhar et al. ('59). Thomasson's values are somewhat higher than ours which were estimated from dermal symptoms. His data were obtained by a method of assay which uses water restriction, with growth as a criterion, and which therefore may be sensitive to the activity of the acids of the linolenate family. These fish oils contain large amounts of acids of the linolenate family which undoubtedly bestow the growth promoting activity to the oil; and these acids probably account for the ability of the oils to lower serum cholesterol levels in humans (Ahrens et al., '59) and in rats.[2]

SUMMARY

Tuna, menhaden and herring oils cured the dermal symptoms of EFA deficiency and stimulated growth of EFA-deficient rats when fed at a level of 10% in the diet.

It was shown that although severe dermal symptoms of EFA deficiency developed with feeding 200 mg of the above oils in the form of their ethyl esters, the growth rate was not affected. The content of acids of the linoleate family is estimated to be approximately 2% of these oils, using dermal symptoms of EFA deficiency as the criterion.

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Placental Transfer of Fluorine to the Fetus in Rats and Rabbits[1]

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The literature concerning the placental transfer of fluorine is not extensive. In many species of domestic or laboratory animals, few or no reports on the amount of fluorine transferred to the fetus *in utero* could be found.

Smith and Smith ('35) observed that female rats which received sufficient fluorine to cause mottled teeth produced fetuses with normal teeth. As little as 7 to 14 ppm of fluorine in the diet of rats, however, will cause striations which are visible under a hand lens (Mitchell and Edman, '51) and with low levels of fluorine, mottled enamel is the only detectable lesion (Am. A. Adv. Sci., '54). Murray ('36) found that the rat fetuses from dams fed either a control ration or 500 ppm of added fluorine analyzed 1.1 and 5.1 ppm fluorine, respectively. Murray also stated that fluorine was transferred to the offspring through the dam's milk.

Evans and Phillips ('39) fed female rats a basal milk diet supplemented with minerals and added fluorine at various levels. Placental transfer occurred on the lowest level ingested (1.6 ppm of fluorine on a dry-weight basis). Above 10 ppm of fluorine in the diet, a definite increase was observed in fluorine passing the placenta. Mammary secretion was not affected by as much as 20 ppm of fluorine in the diet.

Lawrenz (cited by Mitchell and Edman, '51) fed rats diets containing 0.47 and 2.5 ppm of fluorine. The fetuses contained at birth 0.8 ppm of fluorine compared with a fluorine content of 5 to 7 ppm in young produced on a "stock diet of natural feeds."

Roholm ('37) cited the findings of Jodlbauer ('03) who found almost as much fluorine in the bone ash of new-born rabbits and guinea pigs as was present in their dams.

Phillips ('32) found the plasma phosphatase value to be an excellent index of the degree of fluorosis in cattle. The rise in plasma phosphatase was interpreted as an indication of a marked disturbance in the metabolism of bone and teeth caused by fluorosis. Plasma phosphatase concentrations were not considered to be a sensitive criterion of fluorosis in rats and man (Mitchell and Edman, '51).

MATERIALS AND METHODS

Rats. Twenty-one day old weanling rats of the Sprague-Dawley strain formed the basic breeding stock. They were fed the basal diet after weaning. In experiment 3 the Wistar strain of rats was used as a means of comparison with the Sprague-Dawley rats used in experiments 1 and 2.

Diets. The basal diet was that described by Jackson et al. ('50) with slight modifications and consisted of: ground wheat, 62; casein, 14; skim milk (powdered), 10; wheat germ meal, 6; butter (unsalted), 8; cod liver oil (vitamin A 1500 I.U. and vitamin D 400 I.U. per gm), 2; NaCl, 1; and $CaCO_3$, 1. Sodium fluoride U.S.P. grade was added to the basal diet to produce the desired levels of fluorine. The basal diet contained 3 ppm of fluorine. The rats received the experimental diets and drinking water ad libitum.

Analyses. Twenty-one days from the time that the males were placed with the

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females, the pregnant females were removed and anesthetized with ether. Samples of blood for phosphatase analyses were drawn by heart puncture and an overdose of ether was administered to complete euthanasia. Samples of liver, kidney and duodenal mucosa were taken for phosphatase determinations. The intact uterus and the entire gastrointestinal tract were removed from the dam's carcass. The carcass was skinned, weighed and placed in a can. The fetuses were removed from the uterus, washed three times with water, dried with a paper towel and placed in a can. In some trials one half of the litter was removed at birth and the remainder of the litter was carried through until 21 days of age.

The samples were processed using the technique of Jackson et al. ('50). The carcasses or fetuses were nearly covered with 3% of acetic acid; the cans were heated in flowing steam for 30 minutes to exhaust gases and capped while hot; heating was continued for two to three hours at 15 pounds steam pressure. The cans were then stored at 5°C until analyzed.

Rabbits. Weanling female rabbits of mixed breeding were kept on wood shavings in pens divided by wire screens. A commercial rabbit chow[5] was fed as the basal diet. The rabbit chow contained 46 ppm of fluorine. Sodium fluoride was added to the basal diet to produce the desired treatment levels. The diets and the drinking water were fed ad libitum.

At 4½ months of age, the females were mated to fertile males. In the groups fed the basal diet supplemented with 200 and 300 ppm of fluorine, because of errors in sexing, a male rabbit was included in each group. When this error was noticed some females were already pregnant; these were closely watched and both dams and their offspring were sacrificed during or immediately after kindling.

Thirty days after mating, the females were sacrificed and processed in a similar manner to the rats, with the exceptions that the long bones of the left hind leg were subjected to roentgenologic study and the tibia of the left leg and both femurs were canned for fluorine analyses, as opposed to whole-carcass determinations in rats. The canned samples of bone were autoclaved at 15 pounds pressure for 10 to 12 hours, whereas the entire fetuses were handled in a manner similar to those of the rat.

Fluorine determinations. The analytic method used was the Willard and Winter ('33) distillation technique as modified by Jackson et al. ('50).

Alkaline phosphatase determinations. The methods outlined by Motzok and Wynne ('50), Motzok ('50) and Motzok and Branion ('59) were used. The liver homogenates were found to contain appreciable amounts of water-soluble inorganic phosphorus. After centrifugation the supernatant liquid was discarded and the residue suspended again in distilled water. Although about half of the phosphatase activity was lost in the supernatant, the residue contained very little inorganic phosphorus and an accurate measure of enzyme activity could be obtained.

Phosphatase activity was defined in terms of milligrams of inorganic phosphate, expressed as phosphorus, liberated from sodium-glycerophosphate by the enzyme present in 1 gm of kidney, and bone and the residue of 1 gm of liver, on a wet-weight basis, in 1 ml of plasma, or in 0.01 gm of intestinal mucosa on a dry-weight basis.

Roentgenologic studies. Lateral and anterior-posterior views of the left hind leg of rabbits were made. The exposure was 40 K.V., 25 m.a.s. on non-screen film at 36 inches with 0.5 mm of aluminum filter.

EXPERIMENTAL AND RESULTS

Rats. In experiment 1, 38 weanling female rats of the Sprague-Dawley strain were randomized and placed into 4 groups containing 6, 6, 13 and 13 rats which were fed the basal diet supplemented with zero, 50, 100 and 200 ppm of fluorine, respectively.

The females were sacrificed on the 21st day of pregnancy with the exception of two females fed the basal diet plus 200 ppm of fluorine. These two dams were handled similarly to, and are thus included with, the rats in experiment 2.

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[5] Purina Rabbit Chow, Ralston Purina Co., Woodstock, Ontario.

TABLE 1

Fluorine content of the fetuses of rats (Sprague-Dawley strain) fed varying levels of fluorine before and during pregnancy (experiment 1)

Amount of added fluorine[1]	No. of dams	Duration of treatment	Accumulation of fluorine	
			Dams (skinned and eviscerated)	Fetus (whole carcass)
ppm		*days*	*ppm*[2]	*ppm*[2]
0	5[3]	208[4]	9 ± 2.6[5]	0.0
50	5[3]	207	106 ± 23.6	1.0 ± 1.2[5]
100	11[3,6]	207	183 ± 48.2	1.8 ± 0.8
200	11	196	438 ± 122.2	4.1 ± 1.3

[1] Added as NaF to the basal diet containing 3 ppm F.
[2] Wet basis.
[3] Barren female in this group; results not included.
[4] Mean.
[5] Mean ± standard deviation.
[6] One female ate her young as soon as they were born.

The experimental findings of experiment 1 are summarized in table 1. The data show that the dams fed the basal diet (containing 3 ppm of fluorine) stored a mean of only 9 ppm of fluorine in their carcasses; their fetuses contained no detectable amounts of fluorine. The amount of fluorine in the dams and fetuses increased with the higher levels of fluorine. The ratio of fluorine in the carcasses of the dams to that in the fetuses was approximately 100:1 at all levels of added fluorine.

Plasma intestinal mucosa, kidney and liver residue contained respectively, 0.21, 2.4, 14 and 0.22 units of alkaline phosphatase activity, on the average. The added dietary fluorine did not significantly modify these levels.

Experiment 2 was a further check on the placental transfer of fluorine and the transfer of fluorine by way of the dam's milk was also investigated. Thirty-nine weanling female Sprague-Dawley rats were randomized into 5 groups and fed the basal diet supplemented with zero, 50, 100, 200 and 300 ppm of fluorine. At whelping, one half of the litter from each dam was removed for fluorine analysis. The remainder of each litter were left with their dams until they were 21 days old and then sacrificed. Attempts were made to carry the dams through subsequent gestation periods.

The experimental findings are summarized in tables 2 and 3. Whereas the analyses obtained on second and third whelpings were quite limited, nevertheless it appeared that the amount of fluorine transferred to the offspring did not increase with succeeding gestations. The carcasses of the dams showed increased storage of fluorine with each increase in dietary level of fluorine. No dams on the highest level of fluorine (300 ppm) survived for a third whelping.

Experiment 3 was conducted with 15 21-day-old rats of the Wistar strain. This was an attempt to determine whether there was a strain difference in the placental transfer of fluorine. The findings are summarized in table 4. The findings between the two strains are in good agreement considering the lengths of time the rats were on test.

Rabbits. In experiment 4, 27 weanling female rabbits were randomized into 5 groups and fed the basal diet supplemented with zero, 50, 100, 200 and 300 ppm of fluorine. Since the basal diet was found to contain 46 ppm of fluorine, the total amount of fluorine in the rabbit diets was raised accordingly.

The observations from experiment 4 are summarized in table 5. The bone of the dams fed the experimental diets supplemented with zero, 50, 100, 200 and 300 ppm of fluorine contained 311, 920, 1176, 2107 and 2768 ppm of fluorine whereas the fetuses contained 3.1, 5.8, 9.3, 15.8 and 21.5 ppm of fluorine, respectively.

Plasma, intestinal mucosa, kidney, bone and liver residue contained respectively, 0.20, 0.17, 8, 1.1 and 0.40 units of alkaline phosphatase activity on the average. The

TABLE 2

Fluorine content of the offspring of rats (Sprague-Dawley strain) fed varying levels of fluorine before and during pregnancy and lactation (experiment 2)

Amount of added fluorine[1]	No. of dams	Duration of treatment	Accumulation of fluorine in offspring	
			At birth (whole carcass)	At 21 days of age (skinned and eviscerated)
ppm		*days*	*ppm*[2]	*ppm*[2]
		First whelping		
0	5	172[3]	0.3 ± 0.1[4]	0.0[3]
50	6	153[3]	1.4 ± 1.9	2.1 ± 1.1[4]
100	5	159[3]	1.7 ± 1.6	4.7 ± 2.4
200	10[5]	172[3]	2.8 ± 2.2	10.1 ± 5.9
300	7	170[3]	5.6 ± 2.8	9.9 ± 2.3
		Second whelping		
0	4	224[3]	1.0 ± 1.0[4]	0.0[3]
50	2	214[3]	0.0[3]	—[6]
100	3	215[3]	2.0 ± 1.7	4.7 ± 2.9[4]
200	1	236	1.8[3]	2.5
300	1	214	3.5	15.4
		Third whelping		
0	2	338[3]	0.2[3]	
50	1	337	0.0	
100	1	333	0.3	
200	1	333	6.9	

[1] Added as NaF to basal diet containing 3 ppm F.
[2] Wet weight.
[3] Mean.
[4] Mean ± standard deviation.
[5] Two dams from experiment 1 included.
[6] Dams ate remainder of litters.

TABLE 3

Fluorine content of the carcasses of dams (Sprague-Dawley strain of rats) fed varying levels of fluorine (experiment 2)

Amount of added fluorine[1]	No. of dams	Duration of treatment	Accumulation of F in dams (eviscerated and skinned)
ppm		*days*	*ppm*[2]
0	4[3,4]	289[5]	9.2 ± 2.9[6]
50	6[4]	274	77 ± 0.1
100	7[4]	266	208 ± 26
200	8[4,7,8]	232	343 ± 112
300	7[4]	226	539 ± 245

[1] Added as NaF to the basal diet containing 3 ppm F.
[2] Wet weight.
[3] Two barren females and one female that ate her young as soon as she whelped are not included.
[4] One dam from each of these groups was removed for bacteriological and pathological examination and no F determinations were made.
[5] Mean.
[6] Mean ± standard deviation.
[7] Two carcasses unfit for test—partially eaten by other rats.
[8] Includes two dams from experiment 1.

TABLE 4

Fluorine content of dams and their offspring (Wistar strain of rats) fed varying levels of fluorine before and during pregnancy (experiment 3)

Amount of added fluorine[1]	No. of dams	Duration of treatment	Accumulation of F	
			Dams (skinned and eviscerated)	Offspring (whole carcass)
ppm		*days*	*ppm*[2]	*ppm*[2]
0	5	161[3]	11 ± 1.7[4]	0.0[3]
50	3[5]	159	66 ± 27	0.6 ± 0.1[4]
300	5	154	357 ± 122	3.5 ± 3.5

[1] Added as NaF to the basal diet containing 3 ppm F.
[2] Wet weight.
[3] Mean.
[4] Mean ± standard deviation.
[5] Figures do not include two barren females.

TABLE 5

Fluorine content of the fetuses and the bone of dams from rabbits fed varying levels of fluorine before and during pregnancy (experiment 4)

Amount of added fluorine[1]	No. of dams	Duration of treatment	Accumulation of F	
			Dams (bone)	Fetus (whole carcass)
ppm		*days*	*ppm*[2]	*ppm*[2]
0	4[3]	157[4]	312 ± 192[5]	3.1 ± 0.2[5]
50	4[3]	128	920 ± 243	5.8 ± 3.4
100	4	133	1176 ± 149	0.3 ± 3.6
200	6[6]	133	2090 ± 510	14.8 ± 7.1
300	5[6]	133	2768 ± 683	21.5 ± 4.1

[1] Added as NaF to the basal diet containing 46 ppm F.
[2] Wet weight.
[3] Barren dam in group; data not included.
[4] Mean.
[5] Mean ± standard deviation.
[6] One male rabbit in group; data not included.

added dietary fluorine did not significantly modify these levels.

Roentgenologic studies of the left hind leg showed no bone abnormalities although excess growths of tissue were visible grossly on the mandibles and long bones of some of the rabbits fed the higher levels of fluorine.

DISCUSSION

The data presented show that the placenta of the rat was a definite and efficient barrier to the passage of fluorine to the fetus. Whereas increased fluorine in the diet caused increases of fluorine in the fetuses and dams, the increases in the dams were marked while those in the fetus were slight. None of the fetuses from the groups fed the basal diet contained measurable amounts of fluorine while the carcasses of the dams fed this diet showed a mean of 9 ppm of fluorine.

The observations in this study are in agreement with those reported by Murray ('36) and Lawrenz (cited by Mitchell and Edman, '51) but appear to conflict with those of Evans and Phillips ('39). Whereas the dams fed the basal diet in the experiments of Evans and Phillips and in the present study contained approximately the same amount of fluorine, this was not true for the fetuses. The fetuses from dams fed the basal diet alone contained practically no detectable fluorine, whereas the fetuses from dams fed the basal diet in the experiments of Evans and Phillips contained more fluorine than was found here in fetuses from dams receiving diets containing 300 ppm of fluorine. Both groups of rats were on experiment approximately

the same length of time. The rat dams fed Lawrenz' experimental diets (0.47 to 2.5 ppm of fluorine) stored fluorine at the same rate as the dams fed the basal diet (3 ppm of fluorine) in the experiments reported herein.

The rats of Lawrenz which were born from the dams on low fluorine rations compared closely with those born from dams fed the basal diet in these experiments. Lawrenz, however, also found that young produced from dams fed a "stock diet of natural foods" contained 5 to 7 ppm of fluorine. This compares closely with a mean of 5.6 ppm of fluorine for fetuses from dams fed the basal diet supplemented with 300 ppm of fluorine.

When the higher levels of fluorine were fed for extended periods the rats became saturated with fluorine; offspring born of these dams did not show increasingly higher levels of fluorine which demonstrated that the placenta maintained its function as an effective barrier even under extreme conditions.

Wide variations were observed in the fluorine levels obtained in both dams and offspring at all levels of fluorine supplementation. Differences were not seen between the offspring of the first, second and third whelpings in experiment 2. Offspring contained more fluorine at 21 days of age than at birth. Whereas only milk curd was seen in the digestive tracts, which were removed from the carcasses before analysis, it is possible that the offspring ingested some feed which would raise their fluorine level.

While rats of the Wistar strain were not on test as long as those of the Sprague-Dawley strain, the storage level of fluorine and the ratio of fluorine in the dam and offspring fed the various supplementation levels were similar (ratio approximately 100:1).

In experiment 4 there was an increasing amount of fluorine in the bone of the rabbit dams, and in the fetuses, with increasing feed intake. Since the basal diet contained more fluorine than had been anticipated, all levels of supplementation were raised accordingly and no really low-fluorine diet was fed.

The levels of fluorine obtained indicate a greater placental passage of fluorine in rabbits than in rats. This is difficult to explain on the basis of morphological structure of the placenta, for if the rabbits are classed with the higher rodents they would be in the hemo-endothelial group which also includes the rat. If, on the other hand, they are included with the lower rodents they would be in the hemo-chorial placenta group which has a more primitive placenta structure and, on the basis of morphology, would be expected to be less permeable than the hemo-endothelial type. It has been pointed out, however, that many factors are concerned in placental transfer and that the morphology of the placenta is only one factor (Cohen, '50).

From this work it appeared that little or no change occurred in alkaline phosphatase activity in rats or rabbits fed the experimental diets. This agrees with the conclusions of Mitchell and Edman ('51). Thus on the basis of these experiments, alkaline phosphatase activity could not be considered as a suitable criterion for the detection or diagnosis of fluorosis in rats and rabbits.

All of the roentgenograms were normal, even in those animals in which gross observation showed excess tissue growth on the long bones or mandibles. These findings are in agreement with those of Largent et al. ('43).

SUMMARY

1. Various levels of fluorine were fed to rats (Sprague-Dawley and Wistar strains), and to rabbits, for varying periods of time in attempts to determine the permeability of the placenta to fluorine added as sodium fluoride to the basal diet. Passage of fluorine to the fetus by way of the dam's milk was also studied in rats. In these experiments the placentae of the rats and rabbits were an extremely efficient barrier to the passage of fluorine. This effect may be related to the permeability of the placenta, to the molecular size of any compounds in which fluorine had become fixed, or it is possible that the transfer of fluorine may have been conditioned by other factors which affect placental transfer.

2. Alkaline phosphatase activity of various tissues of rats and rabbits as affected by the diets containing various levels of

fluorine was also studied. In these studies alkaline phosphatase activity of plasma, kidney, liver residue, intestinal mucosa and bone were not suitable criteria of the degree of fluorosis in rats and rabbits.

3. Roentgenologic studies of the hind legs of the experimental rabbits were made. The roentgenograms did not reveal any abnormalities although excess growths of tissue were observed on the long bones of some rabbits fed high levels of fluorine.

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Blood Plasma Tocopherol and Phosphorus Levels in a Herd of Beef Cattle[1]

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Nutritional muscular dystrophy ("white muscle disease") is one of the major diseases affecting young calves in beef herds in Ontario. During investigational work on clinical cases of muscular dystrophy it became apparent that suitable information on the blood plasma tocopherol of Ontario beef cattle was not available. Since Schofield and Bain ('39) and Schofield ('53) reported benefit in the alleviation of signs of muscular dystrophy in lambs and calves, from the inclusion of a phosphorus supplement, the plasma inorganic phosphorus levels were also determined. In the present study the beef herd of the Ontario Agricultural College was selected as a well-fed herd. This herd has never experienced any cases of muscular dystrophy.

METHODS AND MATERIALS

Twenty-seven cows (13 Shorthorns, 8 Herefords and 6 Angus), were selected from the beef herd of the Ontario Agricultural College and divided at random into three groups of 9. A 50-ml sample of blood was drawn from the jugular veins of the cows into a bottle containing 120 mg of dried sodium oxalate. Each cow was sampled at three-week intervals (one group of 9 per week) except during the summer months. Because of the difficulty of "rounding up" cows from pasture, during the summer all of the cattle were bled at monthly intervals. When calves were born, sampling of them was usually begun on the day that the dam was normally sampled. Thus no precolostrum samples were collected from the calves and comparisons between plasma levels of the dams and their calves during this period were not possible.

Determinations of total tocopherols of the plasma were made according to the macromethod of Quaife and Harris ('44), Quaife and Biehler ('45) and Latschar et al. ('49). Analyses for acid-soluble phosphorus were made as described by Hawk et al. ('54).

During the first winter the cows were fed mixed hay of variable composition which was not of top quality. Corn silage of good quality was also fed. A grain mixture of rolled oats with a small amount of barley was fed at the rate of 1 to 1.5 pounds per day to the dry cows and 5 to 6 pounds per day to the milking cows. A standard mineral supplement was available ad libitum in the exercise yard until the 60th day of the experiment (February 26) at which time a mixture of equal parts of disodium phosphate (Na_2HPO_4) and bone meal was substituted. The dry heifers were fed better-quality mixed hay, grass silage of fair quality and 4 to 5 pounds daily of the following mixture: 60% of rolled oats and 40% of a mixture of: oats, 700; corn, 420; barley, 250; wheat, 250; linseed oil cake, 100; bran, 200; iodized salt, 20; and mineral, 40. Mineral supplement was fed ad libitum to the cows and heifers. During the summer, cows were placed on pasture and mineral was available ad libitum. Because of a period of prolonged drought, the pasture was inadequate for the cattle and hay was fed during August. The onset of fall rains

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caused rapid regrowth of pasture and no further supplementation was necessary for the remainder of the pasture feeding period. When the cows were stabled again, the feeding practices were similar to those of the previous winter except that some concentrate feeds were fed during the period from January to March in order to fit the herd for a livestock show. At approximately the 475th day of the test (April 7) the ration was changed gradually so that the milking cows received 15 pounds of grass silage daily and all the mixed hay they would eat. There was insufficient silage to provide more per day and since the hay quality was only fair, 2 to 3 pounds of oats and bran (equal parts) were fed per head per day. Heifers and dry cows were changed at this time to 25 pounds of grass silage, all the mixed hay they would eat and 2 to 3 pounds of oats and bran (equal parts) per head per day.

RESULTS AND DISCUSSION

The experimental findings are depicted in figures 1 to 5. In figure 1 the mean values of total tocopherols in the plasma of the three breeds throughout the 520 days of the experiment are shown. The curve for the mean has been smoothed by calculating the mean at each sampling time from (a) the individual values at each sampling time, and (b) the interpolated individual values of cows sampled at the preceding and following sampling dates. There is a steady decline from the start of the experiment to the 135th day (May 10) when the cows were turned out on lush pasture. At that time plasma tocopherol values rose rapidly. Plasma tocopherol levels dropped sharply during a period of prolonged summer drought at which time the pasture had to be supplemented with hay. With the onset of fall rains and a consequent regrowth of pasture, tocopherol levels again rose markedly. When the cows were stabled for the winter feeding period a steady decline in plasma tocopherol levels began. This decline was reversed at approximately 375 days (January of the second winter) when the entire herd was fed concentrate feeds to

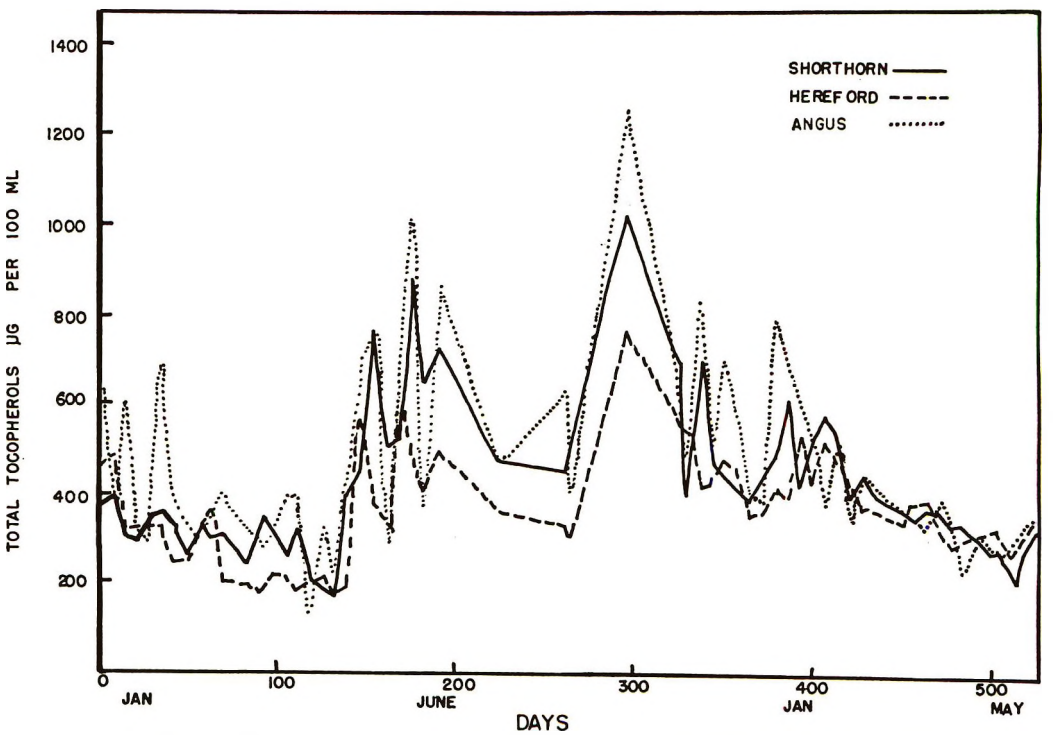

Fig. 1 Plasma total tocopherol concentration of three beef breeds.

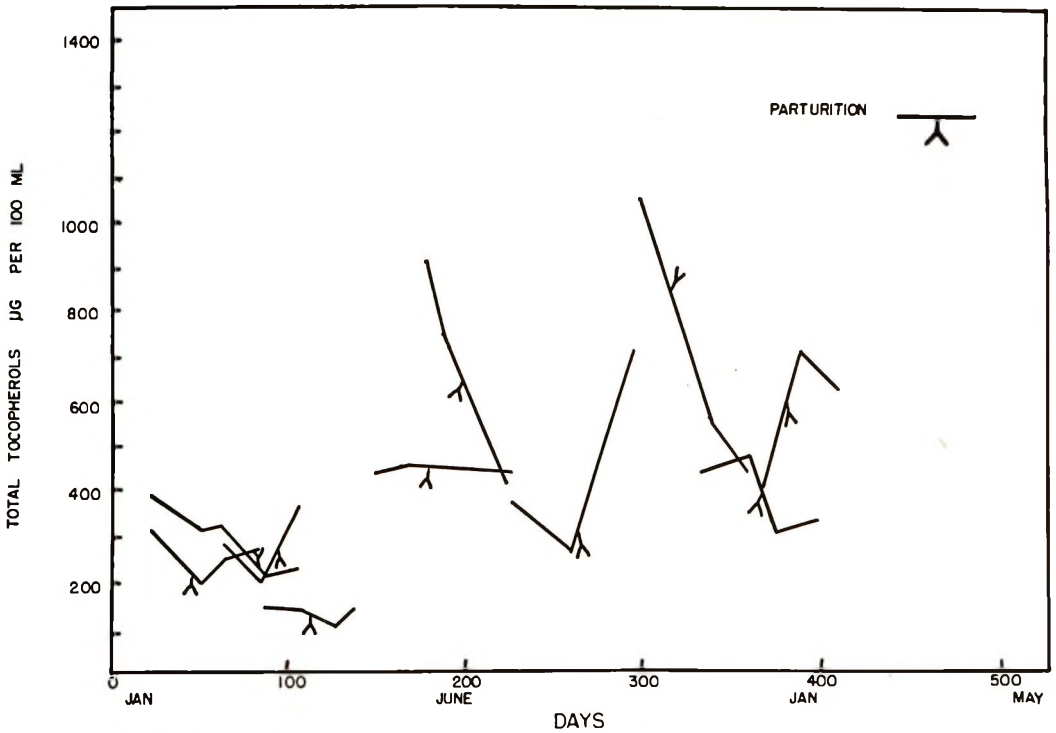

Fig. 2 Plasma total tocopherol concentration of Shorthorn cows at parturition.

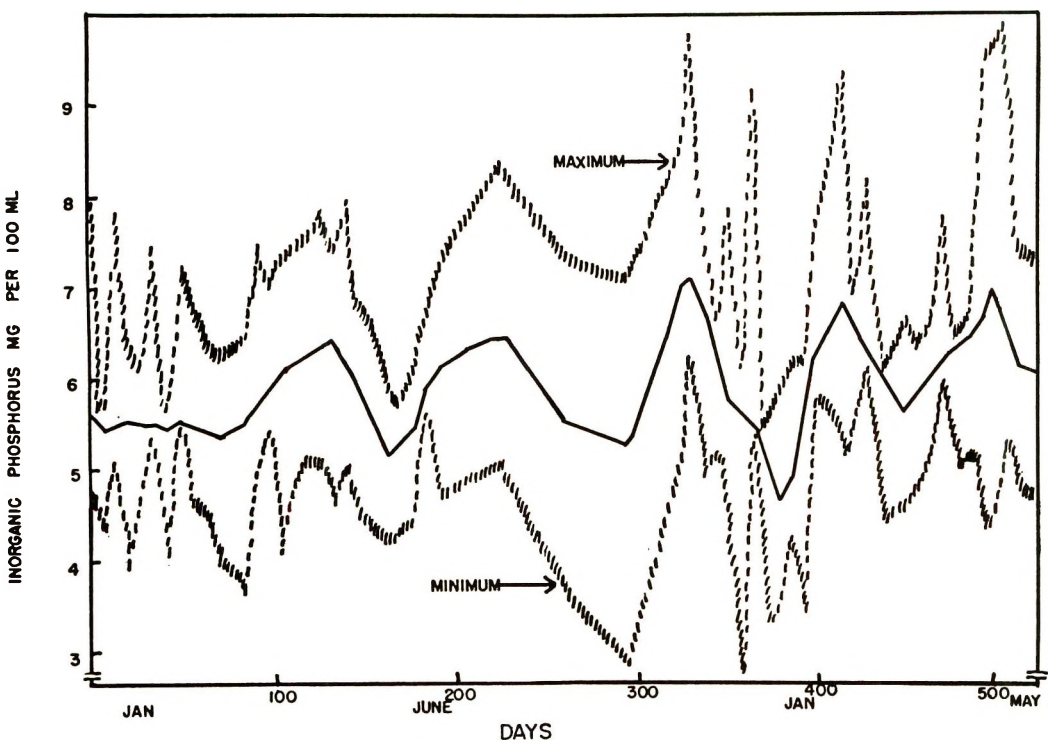

Fig. 3 Plasma acid-soluble phosphorus of beef cows.

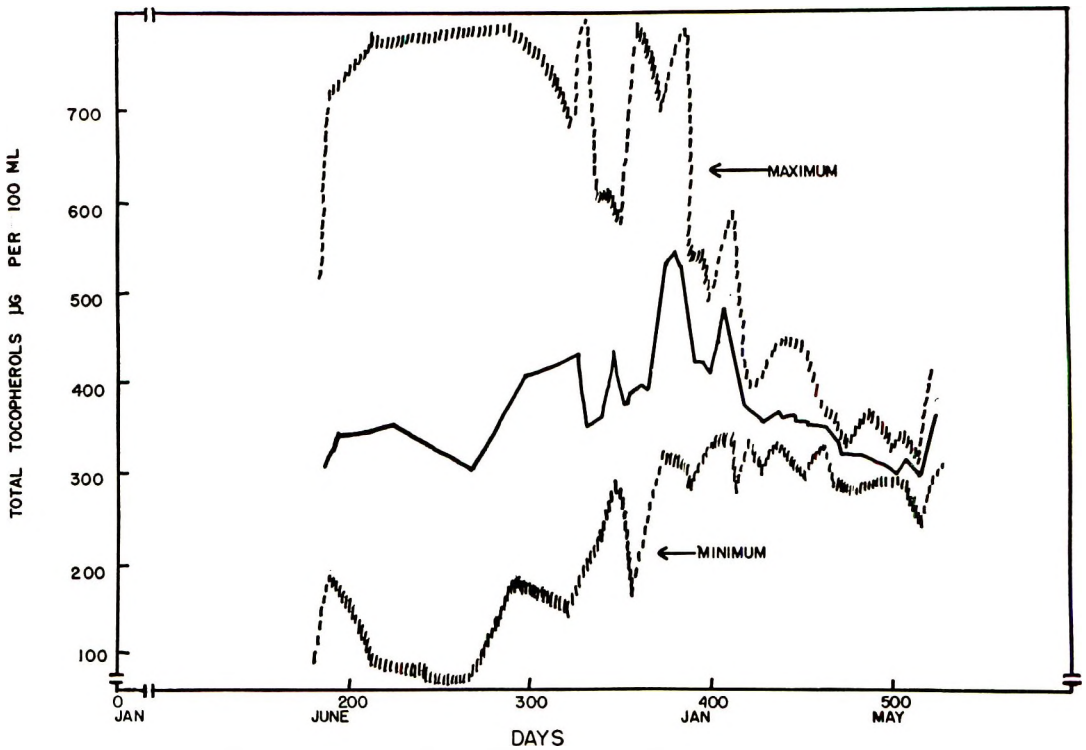

Fig. 4 Plasma total tocopherol concentration of beef calves.

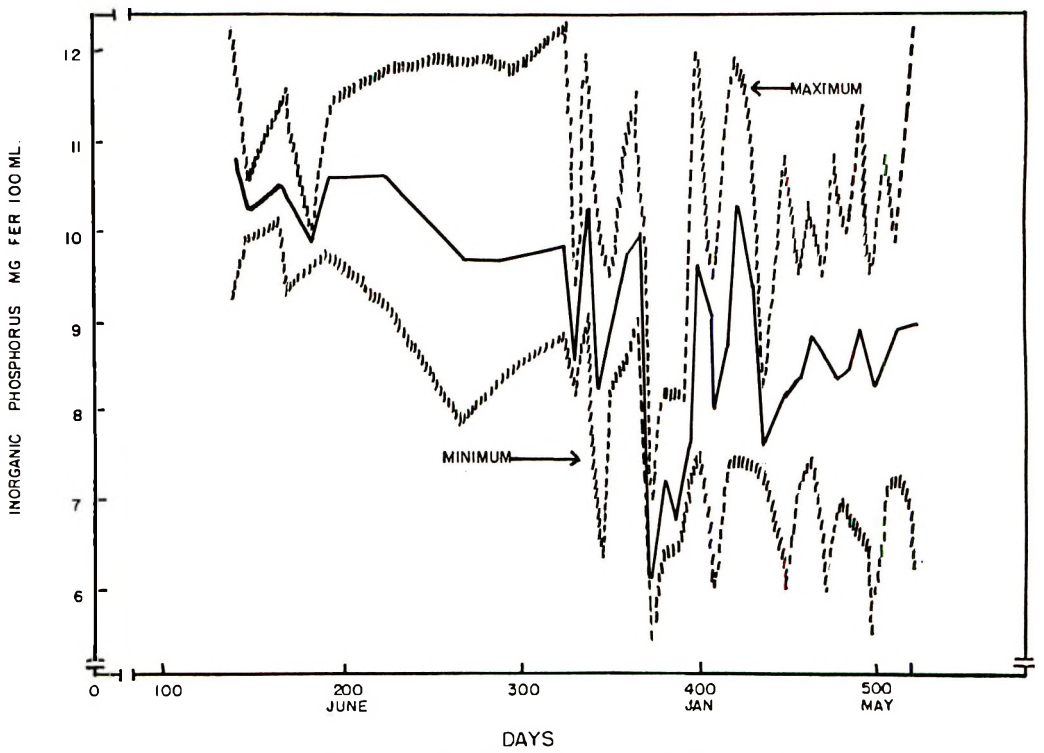

Fig. 5 Plasma acid-soluble phosphorus of beef calves.

fit them for a livestock show. When the concentrates were discontinued, the decline began again but the plasma tocopherol levels remained higher than in the previous year.

Although analyses for total tocopherol content were carried out on some of the feedstuffs in the earlier part of this work during the stable-feeding period, they are not included. Detailed investigation of the available procedures made their accuracy suspect insofar as some feedstuffs were concerned, and further research made it evident that analyses of the tocopherol content of feeds should include partition into the various forms of the vitamin. Sampling of pasture and measurement of intake also presented problems.

The plasma total tocopherol levels agree quite well with the values reported by other workers, (table 1). In the present study, Angus cows showed higher plasma tocopherol levels than the Shorthorn and Hereford cows, especially at high levels of intake, namely, while at pasture. The Herefords showed the lowest levels. Using a sequential method of testing (Snedecor, '56) there were significant differences of plasma total tocopherol levels between all breeds during periods of high tocopherol intake: Angus > Shorthorn > Hereford ($P < 0.05$). During periods of low-tocopherol intake there was little variation between breeds. A decline of plasma tocopherols associated with parturition was also observed, (fig. 2). While figure 2 shows only the plots of tocopherol values in Shorthorn cows at parturition, declines were also noted in Hereford and Angus cows. Such a decline was observed by Latschar et al. ('49) but was not found by Van der Kaay et al. ('49).

Figure 3 shows the mean inorganic phosphorus levels observed in the cows. The means have been smoothed as in figure 1. The mean plasma inorganic phosphorus value of 5.9 ± 0.08[3] mg per 100 ml when fed barn rations and 5.7 ± 0.06 mg per 100 ml on pasture are slightly higher than the mean of 4.5 ± 0.94 mg per 100 ml of serum as determined by McSherry and Grinyer ('54) on 86 Holstein cows 2 to 13 years of age. In the trials reported here approximately one third of the cows sampled were two years old at the beginning of the experimental period; the average age was 4.3 years. These animals were very well fed and mineral supplementation was at a high level. Thus the number of young cows in this group together with the feeding practices may have raised the mean plasma inorganic phosphorus levels.

Figure 4 shows the mean total tocopherol levels in the plasma of calves born to 18 cows. The calves at times showed plasma tocopherol values below 70 µg per 100 ml, lower values than are at times encountered in field cases of muscular dystrophy (Sharman, '54). Sharman stated that because of wide variation in individual samples no reliance was placed on a single sample from one animal.

Also in field outbreaks in Ontario, calves with low blood tocopherol levels, but showing no clinical signs of dystrophy, have been observed. For example, in one herd with 7 calves, two were affected with dystrophy; their plasma total tocopherol levels were zero and 50 µg per 100 ml, respectively. The clinically normal calves had plasma total tocopherol levels of 273, 165, 33, 24 and 24, respectively. This indicates that tocopherol may not be the sole nutrient concerned in the onset of dystrophy.

Figure 5 shows the mean plasma phosphorus values for the calves. The mean was not plotted until sufficient samples were available. The mean plasma inorganic phosphorus values of 8.9 ± 1.18 mg per 100 ml when fed barn rations and 9.7 ± 0.08 mg per 100 ml on pasture are somewhat higher than the normal value of 7.7 ± 1.1 mg per 100 ml of serum as determined by McSherry and Grinyer ('54) using 40 Holstein calves, 4 days to 10 months old. The high phosphorus levels and the fluctuations of phosphorus levels which occurred in the plasma of these calves are explainable, at least in part, by the diets fed and by the feeding practices, namely, fitting for a livestock show.

SUMMARY

In an attempt to establish normal values, plasma total tocopherol levels and plasma acid-soluble phosphorus levels were determined in a well-fed herd of 27 beef cows over a 520-day period. This herd

[3] Standard deviation of mean.

TABLE 1

Plasma tocopherol levels reported in the literature and found in the present study

No.	Breed	Diet	Tocopherol supplementation	Plasma tocopherol		Reference
				Range	Mean	
			gm	*μg/100 ml*		
75	Brown Swiss cows	barn rations	0		450	Harris et al., '47
		barn rations	1		1060	
15	Holstein cows	barn rations	0[1]		700	Phillips et al., '48
		barn rations	1		680	
32	Holstein, Ayrshire Jersey and Guernsey cows	pasture	0	724–1473		Latscher et al., '49
		barn rations	0.5–1	301–569[2]		
		barn rations	4	1136–1278[2]		
		barn rations	5	805–919[2]		
		barn rations	0.5–1	192–616[3]		
		barn rations	4	799–1003[3]		
		barn rations	5	396–817[3]		
48	Breed not stated, cows	pasture	0		800	Van der Kaay et al., '49
		barn rations	0	100–200		
4	Breed not stated, cows	barn rations	0		50	
			0.08[4]		80	
14	Breed not stated, dairy cows	barn rations	1/1000 pounds body weight	900–1050		Parrish, '49
		barn rations	0	625–700		
16	Holstein, Swiss and Guernsey cows	pasture	0		685	Whiting et al., '49
		pasture	1		771	
		barn rations	0		582	
		barn rations	1		735	
3	Mixed breeding cows	low in tocopherol[5]	0		249	Kachmar et al., '50
3	Mixed breeding cows	low in tocopherol[5]	5 mg/kg body weight/ day		685	

Not stated	Ayrshire cows	**Farm 1**				Blaxter, Brown and MacDonald, '53
		pasture	0		615	
		barn rations	0		1065[6]	
		Farm 2				
		pasture	0		501	
		barn rations	0		487	
		Farm 3				
		pasture	0		844	
		barn rations	0		161	
3	Red Poll Aberdeen Angus and Hereford cows	barn rations (hay only)	0		242	Safford and Swingle, '55
		pasture	0	300–1755		
3	Jersey calves	nursing above dams	0		46	
11	Not stated, cows	not stated	0		522	
8	Ayrshire, Guernsey Holstein and Jersey calves	basal (vitamin A depletion diet)	0		62	Rousseau et al., '57
			1 mg/pound body weight/day		306	
			3 mg/pound body weight/day		427	
			9 mg/pound body weight/day		478	
27	Shorthorn, Hereford and Angus cows	pasture	0	186–1446	572 ± 189[7]	Present study
		barn rations	0	93–714	360 ± 101	
	Calves, nursing above cows	pasture	0	36–780	287 ± 147	
		barn rations	0	13–792	301 ± 31	

[1] Diet estimated to provide 1 gm/cow/day.

[2] Prepartal

[3] Postpartal.

[4] One dose only intramuscularly.

[5] Diet did not contain enough tocopherol to maintain reproduction in the rat.

[6] This herd was fed high levels of grassland-conservation products.

[7] Standard deviation of the mean.

had never experienced nutritional muscular dystrophy.

There was a decline in plasma tocopherol levels of the cows during the stable feeding period. The mean plasma total tocopherol levels were 360 ± 101 and 572 ± 189 μg per 100 ml for stable and pasture feeding, respectively. The mean plasma inorganic phosphorus values were 5.9 ± 0.08 and 5.7 ± 0.06 mg per 100 ml for stable and pasture feeding, respectively.

At high levels of tocopherol intake there were significant differences between the plasma total tocopherol values of the three breeds studied.

Plasma total tocopherol and plasma inorganic phosphorus levels were determined on 18 calves born to these cows. The tocopherol levels of the calves in this herd were, at times, below the levels seen by the authors in some clinical cases of muscular dystrophy.

Nevertheless, the mean plasma total tocopherol levels of calves born to the cows fed barn rations or on pasture were 287 ± 147 and 301 ± 31 μg per 100 ml, respectively. Mean plasma inorganic phosphorus levels were 8.9 ± 1.18 and 9.7 ± 0.08 mg per 100 ml, respectively. These were in a high normal range.

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Serum Cholesterol in Acute Starvation: A Report of 20 Cases

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Although extensive studies have been made of blood lipids during the feeding of low-caloric diets, there is only limited data concerning the effects of acute starvation on serum cholesterol (Keys and others, '50). The early literature on this latter aspect is conflicting (Lennox et al., '26; Bloor, '43; Shope, '26). A recent study by Kartin, however, definitely indicated that in the acute three-to-six day starvation periods of soldier- and medical-student-subjects a significant rise in serum cholesterol occurred (Kartin et al., '44).

Shope ('26) has reported a 22-year-old female who showed a rise in total serum cholesterol from 231 to 314 mg on the 5th day of fasting. Other than this reference, there is little data available on the effect of starvation in the various age groups. Unfortunately, the age groupings of the volunteers in Kartin's study were not given.

No literature was found on the effect of acute starvation in patients with atherosclerosis.

Recently, the author investigated the effect of voluntary acute starvation on healthy males and females of various ages as well as on patients with known atherosclerosis.

METHOD

A daily intake of 163 Cal. was arbitrarily selected to define acute starvation for purposes of the study. The prescribed diet consisted of 84 Cal. of protein and 69 Cal. of carbohydrate daily (table 1). No restrictions were placed on water intake.

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TABLE 1

Experimental diet used during period of acute starvation

	Calories	Protein	CHO
		gm	*gm*
Breakfast			
Egg whites, poached, 2	30	6.6	0.4
Orange juice, 60 gm	28	0.4	6.8
Coffee, black	—	—	—
Total	58	7.0	7.2
Dinner			
Egg whites, poached, 2	30	6.6	0.4
Celery, 50 gm	9	0.6	1.8
Peaches, unsweetened, 50 gm	14	0.3	3.4
Coffee, black	—	—	—
Total	53	7.5	5.6
Supper			
Egg whites, poached, 2	30	6.6	0.4
Lettuce, 50 gm	7	0.6	1.4
Pears, unsweetened, 50 gm	15	0.2	4.1
Coffee, black	—	—	—
Total	52	7.4	5.9
Total for day	163	21.9	18.7

In general, the diet was followed strictly and in only a few instances was the intake slightly more than 163 Cal. a day. Occassionally, it was found necessary to vary the diet slightly with substitutions because a few volunteers were unable to eat poached egg whites or some of the other foods listed.

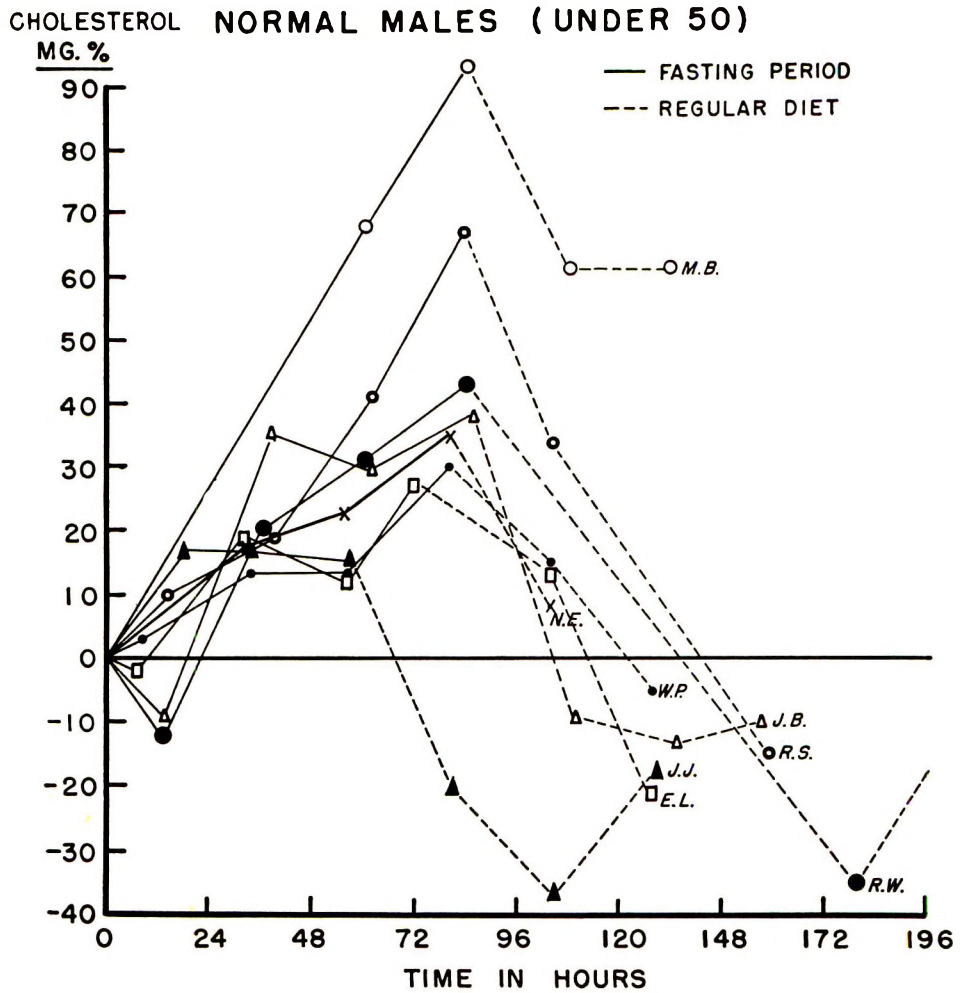

Figure 1

Subject	Race	Height	Weight	Age	Physical appearance	Weight loss during starvation	Cholesterol base line
			pounds			*pounds*	*mg*
M.B.	C	5'9"	127½	24	lean	8	182
W.P.	W	6'1"	199	29	moderately obese	7	257
J.B.	C	6'3"	152½	28	lean	7	267
R.S.	W	6'	194½	28	moderately obese	8½	263
R.W.	W	5'10½"	166½	31	normal	4½	254
J.J.	W	6'1"	208	26	moderately obese	—	205
E.L.	W	6'1"	178	21	lean	6	181
N.E.	W	6'	160	35	lean	4	225

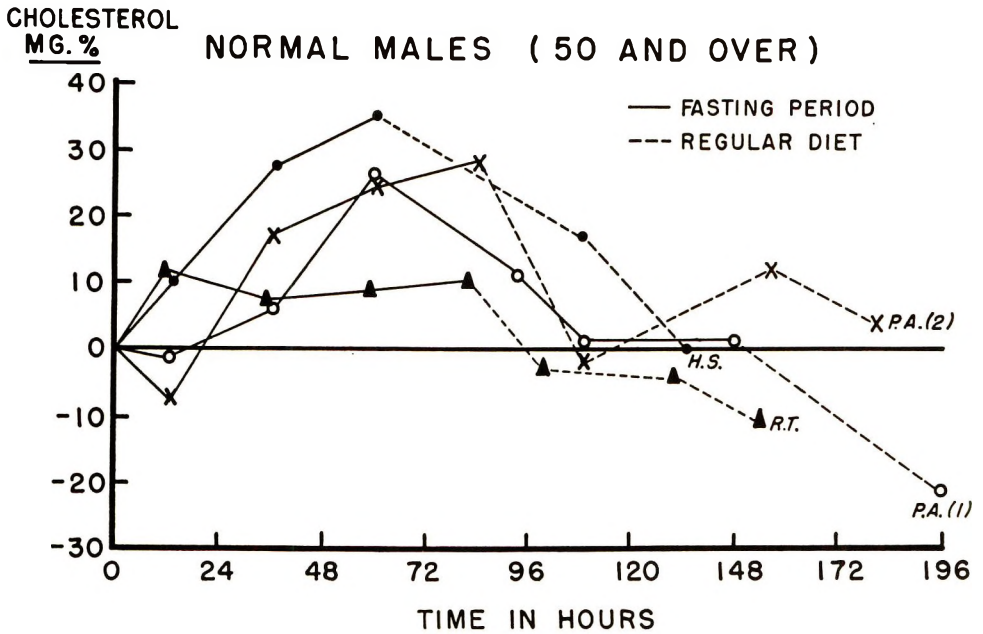

Figure 2

Subject	Race	Height	Weight	Age	Physical appearance	Weight loss during starvation	Cholesterol base line
			pounds			*pounds*	*mg*
P.A. (1)	W	6′	183	50	normal	12	316
P.A. (2)			180			5½	279
H.S.	W	5′10″	194	58	moderately obese	4	250
R.T.	W	6′	186	54	normal	5	243

During the week prior to the onset of the starvation period, the volunteers were instructed to continue their usual food intake and three random morning fasting serum specimens were collected to serve as the individual's base line. Of these volunteers, only one (S.S.—female) had changed her usual dietary intake in recent months. This woman had been following a weight-reducing diet for the past several weeks with an average daily consumption varying from 1000 to 1500 Cal.

Once the starvation period was begun, the subjects had a fasting sample of blood drawn every morning until the experiment was finished. Following the fasting period, daily specimens continued to be collected for as many as three days. The serum of all samples was separated and frozen. All specimens from a given individual were run at one time in duplicate. The method described by Pearson et al., ('53) for total serum cholesterol determination was used.

The data in figures 1–4 were calculated on the basis of milligrams per cent above or below the volunteer's base line, established previously.

The period of starvation varied. The original plan was to carry the volunteer a minimal period of 72 hours, prolonging it to 5 days if the subject were not too uncomfortable. For those volunteers, however, who complained of severe headache, generalized malaise or agitation, the starvation regimen was discontinued immediately. Although 40 to 50 hours after onset of the fast, two of the female volunteers (B.E. and L.K.) encountered considerable

difficulty and fasting was discontinued, two other females had no difficulty and one was continued through 4 days. It was necessary to discontinue fasting for one normal male (H.S.), over 50 years old, because of severe headache after 63 hours. With another male (J.J.), 28 years old, fasting was discontinued after two days because of generalized malaise. Others have noted that frequently females undergo more difficulty than males in acute starvation experiments (Deuel and Gulick, '32), an observation which appears to be borne out by the relatively small proportion of males who encountered extreme discomfort in the study reported here.

Experimental groups

Normal males. Volunteers in this group were predominantly males, members of the hospital staff personnel whose age range fell within two groups, 21 to 35 and 50 to 58 years old. Unfortunately, no males between the ages of 35 and 50 volunteered for the study. All males over 50 years of age gave no history of illness related to atherosclerosis and showed normal electrocardiograms. The subjects were working at the time of the study and were in apparent good health. Except for one participant (W.P.), who performed extremely vigorous exercise on the second day of the study, all subjects engaged in their usual activities during their fasting periods.

Normal females. The 20 to 32-year-old participants in this group were either technicians in the VA Clinical Laboratory or relatives of the staff of the faculty of Vanderbilt University. Prior to the study, one

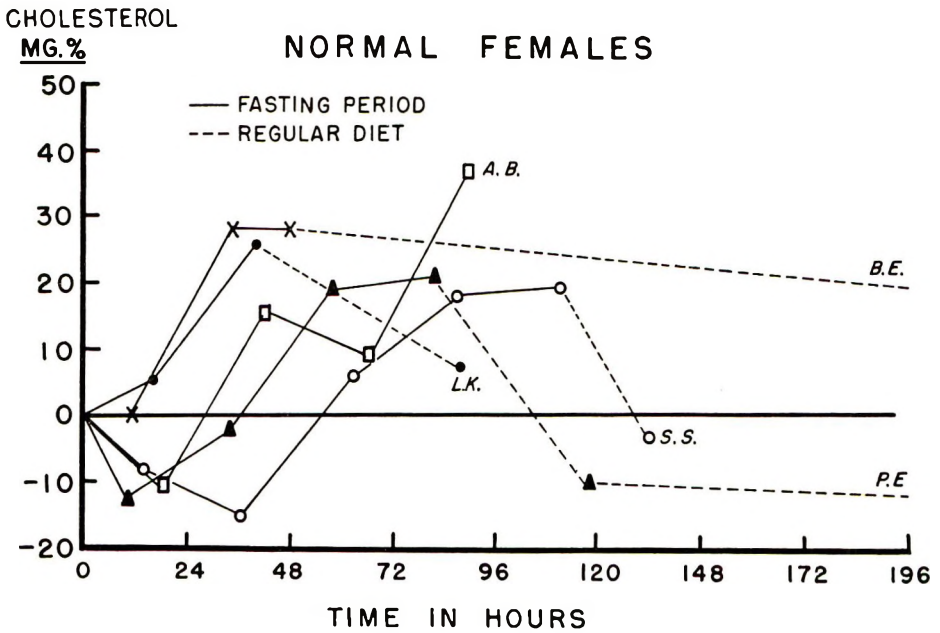

Figure 3

Subject	Race	Height	Weight	Age	Physical appearance	Weight loss during starvation	Cholesterol base line
			pounds			*pounds*	*mg*
A.B.	W	5′3¼″	114	23	normal	6	235
T.E.	W	5′4″	140	32	moderately obese	—	169
S.S.	W	5′6½″	171	22	obese	8	175
L.K.	W	5′4″	123½	21	normal	1½	226
P.E.	W	5′5″	117	20	lean	5	207

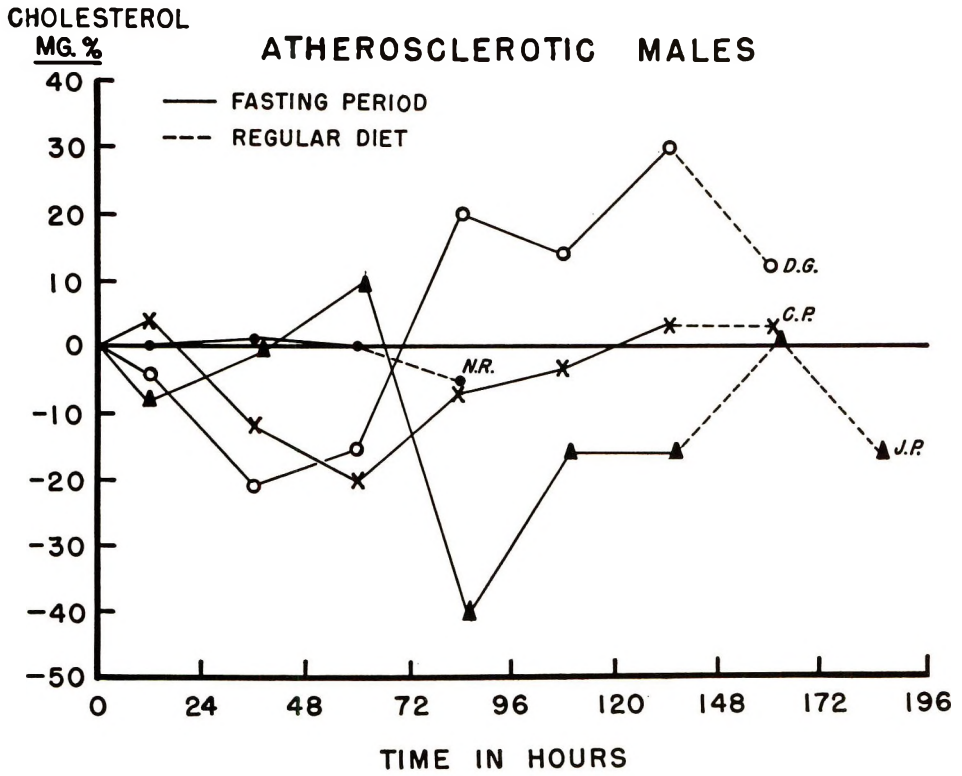

Figure 4

Subject	Race	Height	Weight	Age	Physical appearance	Weight loss during starvation	Cholesterol base line
			pounds			*pounds*	*mg*
D.G.	W	5'10"	120½	67	lean	—	283
C.P.	W	—	140	67	moderately obese	—	223
J.P.	W	5'8"	175	49	obese	10	212
N.R.	W	—	—	70	lean	—	251

volunteer had been following a weight-reducing diet which was accompanied by rather strenuous exercise and she continued her usual routine of daily exercise throughout the study (S.S.).

Atherosclerotic subjects. All volunteers who showed definite clinical manifestations of atherosclerosis were delegated to this group for purposes of discussion and comparison of results, with full recognition that many if not all the volunteers in the other groups may have had varying but undisclosed degrees of atherosclerosis. All volunteers in this group were hospitalized ambulatory patients who were not in congestive failure at the time of the study; all carried the diagnosis of generalized atherosclerosis. The age range of this group was from 49 to 70 years. All subjects in this group were males; unfortunately no females with definite clinical manifestations of atherosclerosis volunteered for the study. One patient had an abdominal aneurysm (C.P.), three had myocardial infarction (J.P., C.P., N.R.) and one patient had severe angina but no history of a definite coronary attack (D.G.). All of these patients were receiving digitalis except the last mentioned severe angina case. Liver function studies of all showed values within normal limits.

RESULTS

It was found that during the fasting period the young males and females almost universally showed an elevation in serum cholesterol. Whereas a rise in serum cholesterol occurred in normal males above the age of 50, it was less marked than that which occurred in the younger male volunteers (figs. 1 and 2).

An insufficient number of volunteers (three) in the normal older age groups made it impossible to form a definite picture. One normal volunteer over the age of 50 repeated the study with a resulting curve similar to that of his first starvation period (P. A., 1, 2).

The serum cholesterol curves in the atherosclerotic group were considerably different from those seen in normal males (fig. 4). In general, the atherosclerotic cases showed a drop in serum cholesterol with a gradual return to pre-starvation level. One subject in this group (N.R.) showed no change over a 72-hour period.

DISCUSSION

In an experiment of this nature, there are many variables which might have a bearing on the results. Some of these are the obesity of the participants, the rate and degree of weight loss during the study and the variance in activity. As far as could be determined in the data collected so far, there did not appear to be any relationship to any of these factors. Of the two young males showing the most marked rise in serum cholesterol, one was a lean, colored male carpenter (M.B.) and the other a slightly obese physician (R.S.).

It was unfortunate that the specific foods in the diet could not be rigidly adhered to, but during the stress of starvation, some items became extremely repulsive to the volunteers and substitutions had to be effected.

Considerable further study is necessary before completely evaluating the influence of all these variables.

SUMMARY

Twenty volunteers were subjected to periods of starvation ranging from two to 5 days. Four general groups were included: Young males between the ages of 21 to 35, young females between the ages of 20 to 32, males over 50 years of age, and patients with atherosclerosis from 49 to 70 years of age. In the data at present available, almost all the young males showed a rise in serum cholesterol. The older white males had less elevation than that of the younger male subjects and the atherosclerotic group showed a completely different curve from that of the other groups. Additional data will be necessary before a complete evaluation is possible.

ACKNOWLEDGMENT

The author gratefully acknowledges the assistance of Doctors Harold Roberts and Paul Anderson in obtaining volunteers for this study, Willa Robbins for technical assistance, and the many members of the staffs of Thayer VA Hospital and Vanderbilt University Hospital who undertook to forego the pleasure of eating for this study.

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The Thiamine-Sparing Action of Sorbitol in Man[1]

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Morgan and Yudkin ('57) found that white rats can live and grow while consuming a thiamine-free diet when fed sorbitol. Mehnert et al. ('58) did not confirm these results, but the results of other investigators agree with those of Morgan and Yudkin (Jones and Baumann, '58; Cremer and Hötzel, '59). Sorbitol is a natural constituent of many fruits (Täufel et al., '58) and also has been detected in animal tissues (Chino, '57). It is being used increasingly in candies and other sweets (Steiner, '57) and can be given to

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TABLE 1

One of three menus used, containing 462.5 μg of thiamine and supplying approximately 300 μg of thiamine and 2,400 Calories

Food	Quantity	Vitamin B_1	Calories
	gm	*μg*	
Breakfast, no. 1			
Rolls, hot, 2		70	235
Honey	30	—	101
Margarine	30	—	237
Sugar	10	—	40
Coffee, instant[1]		—	—
Total		70	613
Breakfast, no. 2			
Pears, canned	100	10	76
Lunch			
Rice, steamed	200	33	226
Ketchup	14	11	29.4
Flour, wheat	5	3	12
Beef, corned	100	20	240
Onions	5	1.5	1
Margarine	20	—	158
Lemon juice	10	4	4
Apricots, canned	200	40	180
Total		112.5	850.4
Mid-afternoon, 4 P.M.			
Roll, hot, 1		35	118
Honey	20	—	67
Margarine	15	—	119
Sugar	5	—	20
Coffee, instant			
Total		35	324
Supper			
Tuna fish, canned	100	40	240
Potatoes	150	150	120
Pears, canned	100	10	76
Roll, hot, 1		35	119
Margarine	10		79
Total		235	634
Total, calculated		462.5	2,497.4
Total, analyzed after daily preparation		328	

[1] Nescafé.

diabetics as a partial substitute for sugar. Thus, sorbitol is used in many human diets, and it would be of some significance if a thiamine-sparing action should take place in humans as has been described in rats.

A good indication of an increase in thiamine absorption either from the food or from intestinal synthesis, is an increase in thiamine excretion in the urine. Since any increase of thiamine supply would be better demonstrated when using a low-thiamine diet, we planned to give a diet containing as little thiamine as practicable in a human experiment. Because the vitamin-sparing effect of sorbitol on the growth of vitamin-deficient rats is demonstrable within one day, we anticipated that an effect in humans, if it should occur, also should be observed in a relatively short time—possibly within 10 days.

METHODS

Two separate experiments were performed. In experiment 1 three healthy individuals—members of the staff, two females, one male, 23 to 26 years old—were placed upon a diet rich in carbohydrates but very low in thiamine. Three menus were used alternately. The thiamine and caloric content of a typical days' diet are shown in table 1. The thiamine values in table 1 refer to the raw food. By cooking, a loss of approximately 20 to 30% can be expected. The thiamine content of the prepared diet and the excretion in the urine were determined daily (see table 2). On the 6th day each person received 35 gm of sorbitol,[2] and on the 7th to the 12th day 70 gm daily were given in smaller portions. In the two experiments sorbitol was added without caloric compensation. The average daily supply of calories was 2200 to 2500.

In experiment 2, lasting 4 weeks, 7 persons served as experimental subjects. Sorbitol was given from the 14th to the 23rd day in graded doses ranging from 20 to 70 gm daily, as shown in figure 1.

Thiamine was determined in the urine by a photographic thiochrome determination after separation by paper chromatog-

[2] Sorbitol: Karion (E. Merck A. G., Darmstadt, Germany) was used. It was not possible to detect thiamine in the sorbitol.

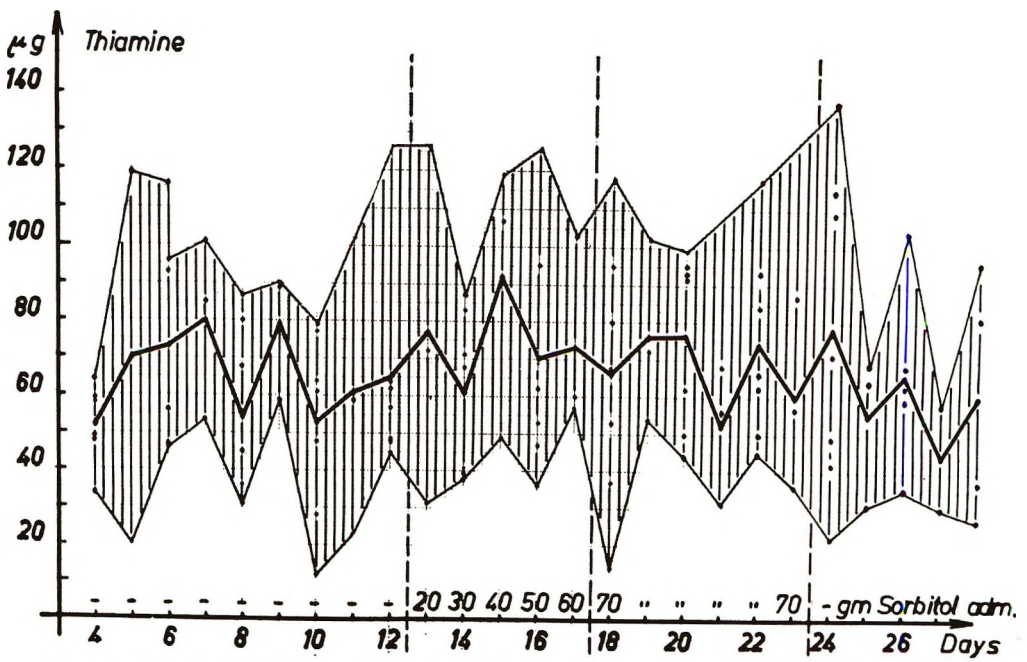

Fig. 1 Range and average of daily urinary thiamine excretion by 7 human subjects fed sorbitol.

raphy[3] (Kraut and Wildemann, '56). Since we wanted to use the same method for the thiamine determination in foodstuffs and in the complete meals, respectively, a procedure was developed to extract thiamine and separate it as much as possible from proteins, fat and polysaccharides (Neubauer, Peppler, Cremer, '60).

As this new and specific method has not been published in English, the principles are included briefly here.

Application of the method to foodstuffs: the materials are ground as small as possible in a blender in aqueous acetic acid solution of pH 3.5 under N_2. The addition of 20 to 30 ml of 6% trichloracetic acid results in partial precipitation of proteins which are removed by centrifugation or filtration if necessary. Aliquots of 60 ml are heated and treated with amyl alcohol as described in footnote 3. If starch-containing material, as well as total daily rations are analyzed, 4 parts of methanol are added slowly to the aqueous extract solution with stirring. This treatment causes the extensive precipitation of globulins, albumins and starches. After cooling, the solution is centrifuged and diluted to 50% of methanol content. Aliquots of 20 ml are concentrated to 1 ml under N_2 in a vacuum-rotation-evaporater at 30°C. Aliquots of 0.2 to 0.3 ml are used for chromatographical separation. Chromatographic paper washed in hydrochloric acid (Schleicher and Schüll no. 2043a) is used.

[3] Essentials of the Kraut (Kraut and Wildemann, '56) method. Urine is acidified with acetic acid to pH 3. Aliquots of 60 ml are heated for one minute in a water bath at 50°C. The 60-ml samples are shaken twice with equal parts of amyl alcohol (for the removal of pigments and other interfering substances). After addition of equal parts of methanol to the aqueous phase, aliquots of 20 ml are concentrated *in vacuo* under N_2 at 30°C. The residue is dissolved in 2 ml of 80% methanol. Solids (precipitated proteins) are removed by centrifuging.

Chromatography with isopropanol-water using Whatman no. 1 paper. Applied amount: aliquots of 0.2 to 0.3 ml are applied and the paper is developed in the dark.

First direction: 4 parts of isopropanol and 1 part of water pH 3. After separating, spray paper with alkaline potassium ferricyanide solution (one part 0.1% of potassium ferricyanide solution and one part of 2.2% sodium carbonate solution) in order to oxydize the thiamine to thiochrome and to destroy substances that interfere with the separation.

Second direction: 4 parts of isopropanol and one part of water pH 9. If necessary, concentrate the spot with three parts of dioxane and 7 parts of water pH 9.

The fluorescence of the thiochrome spot caused by UV-light is photographed simultaneously with a blank and a standard prepared by the same method of chromatography in a special apparatus. By means of an inverted system of lenses the blackening of the film is directly proportional to the intensity of the fluorescence and independent of size and form of the spot as well as independent of a heterogeneous distribution of thiochrome in the spot. The evaluation of the blackening is carried out photographically and photometrically according the usual methods in spectrochemical analysis. The final value is read from a standard curve.

From 0.1 to 1.0 μg of thiamine can be determined within $\pm$ 10%.

TABLE 2

Effect of sorbitol upon the excretion of thiamine by three human subjects

Day of experiment	Sorbitol administration	Intake of vitamin B_1	Vitamin B_1 excretion		
			Subject 1	Subject 2	Subject 3
	gm	*mg*	*mg*	*mg*	*mg*
1	—	0.328	0.193	0.136	0.247
2	—	0.307	0.136	0.107	0.119
3	—	0.328	0.148	0.160	0.125
4	—	0.145	0.266	0.264	0.236
5	—	0.276	0.234	0.244	0.223
Av./day (days 1–5)		0.277	0.195	0.182	0.190
6	35	0.258	0.200	0.198	0.169
7	70	0.292	0.324	0.225	0.160
8	70	0.284	0.167	0.175	0.127
9	70	0.323	0.219	0.269	0.154
10	70	0.141	0.376	0.320	0.149
11	70	0.284	0.269	0.274	0.159
12	70	0.235	0.291	0.222	0.151
Av./day (days 7–12)		0.260	0.274	0.248	0.150

RESULTS

As shown in table 2, two of the three subjects excreted more thiamine in the urine when sorbitol was given. These two subjects complained of intestinal disturbances, namely, diarrhea, whereas the third subject felt well, and did not show any increase in thiamine excretion. Also in subjects 1 and 2, the differences in thiamine excretion were too small to be considered significant.

In the second experiment, no significant increase of thiamine excretion could be demonstrated due to sorbitol, regardless of the fact that the feeding period was prolonged (fig. 1). Again, several cases of diarrhea occurred, but without any correlation between intestinal disturbances and thiamine excretion; thus, the hypothesis that sorbitol may have a thiamine-sparing action in man, as it does in rats, was not supported by our results.

DISCUSSION

After feeding sorbitol, Morgan and Yudkin ('57) and Mehnert, and co-workers ('58) observed an increased weight of the colon of rats, presumably caused by increased growth of bacteria. Possibly the slow absorption of sorbitol may be the cause of increased bacterial growth, and thus activate the synthesis of some vitamins. Rats utilize these intestinally-synthesized vitamins only when able to eat their feces; rats, being completely prevented from coprography by the tail-fixed cups described by Barnes et al. ('57), do not show any vitamin-sparing action of sorbitol as shown by Cremer and Hötzel ('59). The difference between rats and humans in the vitamin-sparing action of sorbitol may be explained on the basis of different food habits.

SUMMARY

Ten healthy individuals fed a low-thiamine diet did not show any increase in thiamine excretion in the urine after ingesting up to 70 gm of sorbitol daily for as long as 10 days. Thus the thiamine-sparing action of sorbitol, as it has been demonstrated in rats, could not be duplicated in man.

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A Mechanism for the Copper-Molybdenum Interrelationship[1]

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Molybdenum toxicity in cattle has been associated for a number of years with symptoms which respond to copper therapy (Ferguson et al., '43). Growth retardation, anemia and diarrhea are common with these species fed high molybdenum diets. Similar symptoms of molybdenum toxicity have been reported for sheep (Ferguson, '44), for rabbits (Arrington and Davis, '53) and for rats (Comar et al., '49). Although a great deal has been learned about the dietary interrelationship of molybdenum and sulfur-containing compounds, relatively little literature pertinent to the interrelationship between copper and molybdenum has appeared.

During an investigation of the effect of excessive molybdate intake upon certain enzyme systems of the rat, it was observed that the level of sulfide oxidase activity in the liver was substantially depressed (Mills et al., '58). This observation led to the suggestion that sulfide accumulation could contribute to the syndrome of molybdate toxicity.

Among the anticipated effects of sulfide accumulation in the tissues would be the loss of copper through the formation of highly insoluble cupric sulfide. The present endeavors were designed to test in part this postulated mechanism for the induction of copper deficiency by molybdenum.

EXPERIMENTAL

Young male rats of the Wistar strain[3] were used in all experiments. The basal diet used was constituted to be relatively low in both copper and inorganic sulfate. The composition of the basal diet in percentages were as follows: vitamin-free casein, 20; glucose, 70; salt mixture, 6; corn oil, 4. The following vitamin supplement was included in the diet (quantities in milligrams per kilogram of mixed diet): thiamine, 5; riboflavin, 8; niacin, 40; pyridoxin, 5; Ca pantothenate, 45; biotin, 0.4; vitamin B_{12}, 0.03; folic acid, 2; menadione, 5; inositol, 100; *p*-aminobenzoic acid, 100; and α-tocopherol, 1500 units; vitamin A alcohol, 8; vitamin D concentrate, 750 I.U. The salt mixture had the following percentage composition: $CaCO_3$, 27; K_2HPO_4, 17; Na_2HPO_4, 12.4; $Ca_3(PO_4)_2$, 23.1; NaCl, 14.6; $MgCO_3$, 4.7; $FeCl_3$, 0.18; $MnCl_2$, 0.62; KI, 0.069; and $ZnCO_3$, 0.033.

The modifications of the basal diet consisted of the addition of one or more of the following ingredients: 800 ppm of molybdenum as sodium molybdate; 15.6 ppm of copper as cupric acetate; 0.94% of L-cystine; and 0.29% of sulfate as sodium sulfate.

Precautions were taken to maintain conditions favorable for the feeding of diets low in copper. The animals were housed in individual stainless steel wire mesh cages which were washed frequently. The food was provided ad libitum in glass jars. Distilled water for drinking was dispensed from acid-cleaned glass bottles equipped with glass or stainless steel outlet tubes.

The hemoglobin measurements were made once or twice a week. The determinations were carried out upon small samples of blood collected by micropipette from minor tail incisions. After dilution of the samples to 1:251 with 0.1% of sodium

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[3] Obtained from Albino Farms, Red Bank, New Jersey.

carbonate solution, the optical density was measured at 540 mμ, using a Beckman Model DU spectrophotometer at a 0.02 mm slit width. A conversion factor based on an extinction coefficient at 540 mμ of 836 cm^2/gm was used to convert optical density to grams of hemoglobin per 100 ml of blood. Duplicate determinations were made per animal in the first two experiments and single analyses were made thereafter. Variation between duplicate determinations was less than 10%.

RESULTS AND DISCUSSION

The postulate under consideration is that the formation of copper sulfide within the tissues leads to the symptoms of copper deficiency often observed during molybdenum toxicity. This situation would result from the lowered level of sulfide oxidase observed in the tissues of rats subjected to toxic dietary levels of molybdate (Mills et al., '58). Because of the very low solubility product of cupric sulfide (3×10^{-38}), the formation of this salt is a nearly inevitable consequence of the mutual presence of cupric and sulfide ions in aqueous phase. Once formed, the cupric sulfide is difficult to dissociate, except in the presence of very concentrated acids (Carnot, '04). The properties of cuprous sulfide are similar to those of the cupric salt (Ravitz, '36; Seidell and Linke, '52).

To test this postulate, the diet used earlier (Mills et al., '58) was modified slightly to provide only a marginal satisfaction of the copper requirement of the rat. Growth of rats fed the low-copper basal diet was not detectably different from that of those fed the basal diet supplemented with cupric acetate (15.6 ppm of copper).

The direct administration of sulfide to the animals presented several technical problems. Instead, it was decided to induce an intracellular generation of sulfide by administering a dietary excess of cystine.

During the first 20 days of the experiment, molybdate drastically depressed the growth of the animals (fig. 1). Furthermore, copper had no apparent ability to alleviate growth depression. When cystine was added to the diet on the 22nd day of the experiment, the animals also receiving molybdate quickly entered a phase of rapid growth. This new growth rate was greater

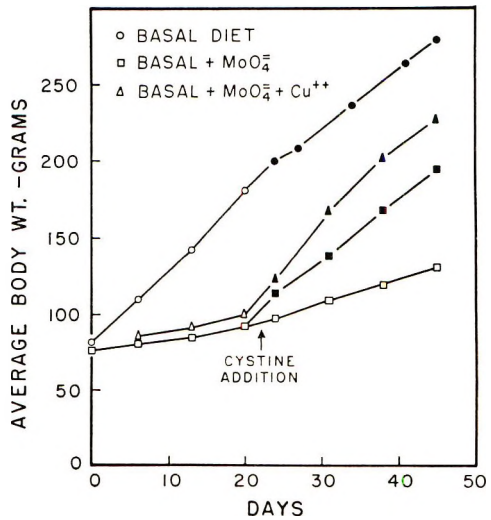

Fig. 1 *The effects of cystine and copper on the growth retardation produced by molybdate.* Solid symbols indicate that the diet was supplemented with cystine beginning on the 22nd day.

if the diet contained added copper. The upward surge in growth upon addition of cystine was anticipated in view of the observations of Van Reen and Williams ('56) that several sulfur-containing compounds, including cystine, would alleviate the toxicity of molybdate.

Although the low-copper basal diet was adequate for maintaining optimal growth rate, it led to a slight decrease in hemoglobin levels, as can be seen in the first column of table 1. The second column demonstrates that, during the progress of molybdate toxicity, hemoglobin levels fall slightly during the first two weeks and then return to normal; however, when cystine is added at the 22nd day, a substantial and prolonged drop in hemoglobin levels occurs in the animals receiving molybdate (third column of table 1). When copper supplements the molybdate diet, the response of hemoglobin to cystine is very much smaller.

The effects of cystine and copper upon the symptoms of molybdate toxicity are presented in graphic form in figure 2. The bar graphs indicate the distribution of hemoglobin levels of individual rats at various times under the several dietary regimens. Each rat is scored only in the lowest hemoglobin range achieved during that period of the experiment. In addition,

TABLE 1

Effects of cystine upon hemoglobin levels[1,2] using rats fed diets with and without molybdenum

Days on experiment (Animals per group)	Basal (9)	Basal + Mo (12)	Basal + Mo (10)	Basal + Mo + Cu (10)
0	13.0 ± 0.2	13.4 ± 0.4	12.8 ± 0.4	14.4 ± 0.4
7	10.1 ± 0.3	12.4 ± 0.7	12.6 ± 0.6	13.7 ± 0.4
14	9.6 ± 0.4	10.6 ± 1.1	11.4 ± 0.6	12.8 ± 0.6
19	9.8 ± 0.4	11.8 ± 0.6	12.4 ± 0.6	12.4 ± 0.6
22	cystine added		cystine added	cystine added
24	10.0 ± 0.4	12.8 ± 0.4	11.4 ± 0.4	12.2 ± 0.6
31	10.5 ± 0.4	12.0 ± 0.4	8.1 ± 0.6	10.0 ± 0.4
38	11.2 ± 0.4	13.4 ± 0.2	8.3 ± 0.6	10.7 ± 0.3
47	10.4 ± 0.4	12.8 ± 0.3	8.2 ± 0.4	11.4 ± 0.4

[1] Hemoglobin in gm/100 ml blood. [2] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

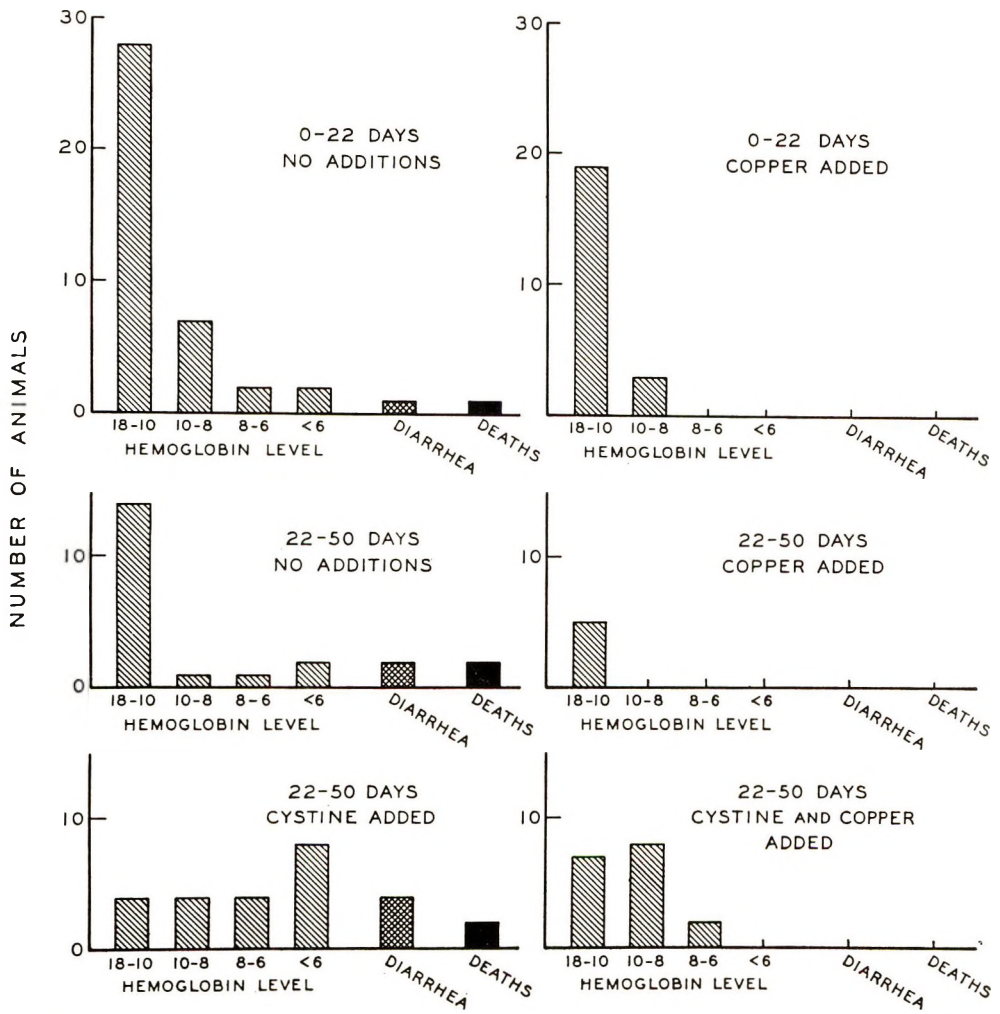

Fig. 2 *The effects of cystine and of copper on symptoms of molybdate poisoning.* The basal diet described in the text was supplemented with 800 ppm of molybdenum as Na_2MoO_4 for all experimental groups. Additions, where indicated, were L-cystine at 0.94% and copper at 15.6 ppm.

the number of animals evincing diarrhea or succumbing to the treatment is included. The diets listed at the right contained added copper, whereas those to the left did not.

It is obvious that the individual response to molybdate using the low copper diet is quite variable, and, that with time, an increasing percentage of animals acquire the severe symptoms of low hemoglobin, diarrhea and death. The largest segment of the population, however, displays none of these symptoms during the 50-day period of the experiment. When the diet was supplemented with copper, these symptoms of molybdate toxicity were nearly abolished.

The severity of the response to cystine is readily obvious, with the majority of animals experiencing anemia and a higher incidence of diarrhea and fatalities. Here, too, the presence of copper in the diet substantially prevented the onset of critical symptoms.

The symptoms of anemia, diarrhea and death, whether occurring spontaneously with the high molybdate diet or induced by cystine administration, clearly may be prevented by copper. Copper also appears to quickly reverse the symptoms of anemia and diarrhea.

An animal which experienced a precipitous fall of hemoglobin to below 6 gm % and evinced severe diarrhea was given daily intraperitoneal injections of 100 μg of copper as $CuCl_2$. The moribund animal showed immediate response to the copper injections by cessation of diarrhea, resumption of growth and hemoglobin regeneration.

A comparison of the data of figure 1 and table 1 indicated that the low-copper basal diet failed to maintain normal hemoglobin levels during the period when the animals experienced their highest rate of body weight increase. Consequently, the possibility remained that the high incidence of anemias induced by the addition of cystine to the high molybdate-low copper diet was directly related to the growth surge produced by cystine.

That this relationship does not obtain was demonstrated by comparing the effects of sulfate and of cystine as alleviators of molybdate toxicity. The experimental design was the same as the one used earlier except that only 7 days elapsed before the basal-plus-molybdenum diet was supplemented with either cystine or sulfate. From figure 3 it is apparent that, in this experiment, sulfate produced a substantially greater surge in growth than did cystine. Nevertheless, cystine produced a much higher incidence of anemia and subsequently diarrhea and death. Neither of these last two symptoms resulted from the administration of sulfate. These observations are summarized graphically in figure 4.

The results of the foregoing experiments are compatible with the hypothesis that the drop in liver sulfide oxidase resulting from molybdate toxicity leaves the animal susceptible to poisoning by sulfide generated by its own metabolism. The induction of copper deficiency by this mechanism obviously is not an inevitable consequence of an excessive dietary intake of molybdate. The precipitation of copper sulfide will be significant to the animal's overall nutritional balance only when the loss of available copper by this means exceeds the absorption of copper from the gastrointestinal tract. This situation is most likely to occur with diets relatively low in copper and relatively high in some nutrient, probably sulfur-amino acids, capable of giving rise to sulfide metabolically.

It is probable that the amount of sulfide generated in these experiments was not large, since attempts to detect sulfhemo-

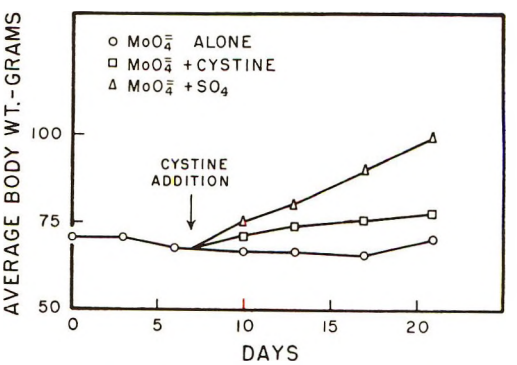

Fig. 3 *Growth response to cystine and sulfate after rats were fed 800 ppm of molybdenum for 7 days.* The original 30 animals were arbitrarily divided into three groups of 10 animals each on the 8th day. Dietary supplementations were L-cystine, 0.94%, and copper, 15.6 ppm.

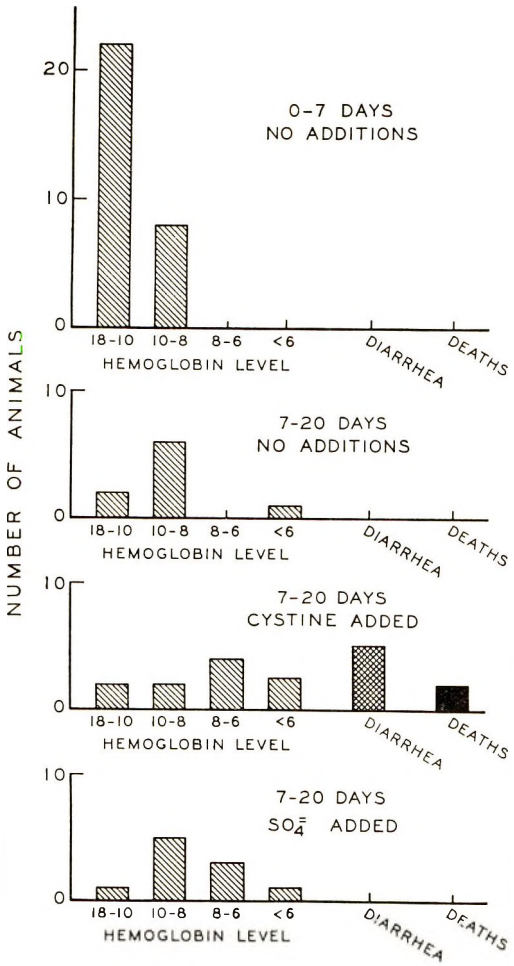

Fig. 4 *The effects of cystine and of sulfate on symptoms of molybdate poisoning.* All animals received the basal diet plus 800 ppm of molybdate. Additions to this, where indicated, were L-cystine at the 0.94% level and copper at 15.6 ppm.

globin in the circulating blood by spectrophotometric means (Israels et al., '57) yielded negative results; consequently, the sulfide toxicity encountered in these experiments is qualitatively and quantitatively different from the toxicity manifested from the ingestion of inhalation of sulfide *per se*.

The postulated mechanism for the molybdate-copper interrelationship would indicate the prediction that molybdate toxicity should lead to the precipitation of other metals, namely iron, manganese, zinc and cobalt. That deficiencies of these metals during molybdenum toxicity have never been reported is somewhat surprising. This apparent anomaly may result from the fact that CuS has by far the lowest solubility product, but more likely is explainable in terms of cell physiology or as an accident of experimental design.

SUMMARY

Excessive dietary molybdate produced a profound depression of growth and a low incidence of anemia and diarrhea in rats fed a low copper diet. Supplementing the diet with copper prevented anemia and diarrhea, but did not restore growth. Administration of excessive dietary cystine to rats fed the high molybdate-low copper diet led to critical conditions of anemia and diarrhea and to some fatalities. These toxic effects of cystine were prevented or reversed by copper, and the joint administration of cystine and copper, alleviated the growth depression due to molybdate. The observations are discussed in terms of the postulate that the reduced level of sulfide oxidase in the tissues of rats receiving excessive molybdate permits an abnormal accumulation of sulfide. This accumulation leads to the formation of copper sulfide and the subsequent appearance of symptoms of copper deficiency.

ACKNOWLEDGMENTS

The authors are indebted to Mrs. Joan Schuh and Lewis Siegel for their technical assistance in certain phases of these experiments.

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Coenzyme Q

XVI. THE ESTIMATION OF THE COENZYME Q_{10} CONTENT OF ALFALFA

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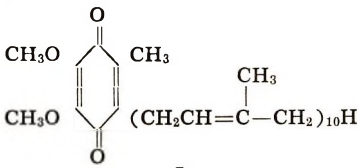
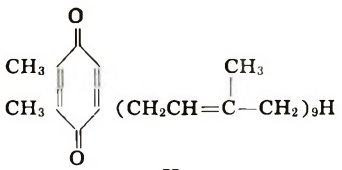
Isolated coenzyme Q_{10} has been defined (Crane et al., '57; Fahmy et al., '58), and its structure (I) has been elucidated (Wolf et al., '58; Morton et al., '58). Coenzymes Q_6 to Q_9 were also characterized (Lester et al., '58; Wolf et al., '58).

The structure (II) of plastoquinone has also been determined (Trenner et al., '59; Kofler et al., '59); this related quinone had been isolated previously (Kofler, '46; Crane and Lester, '58) from alfalfa and later from commercial alfalfa meal (Trenner et al., '59; Crane, '59a).



I



II

The distribution of coenzyme Q_{10} and plastoquinone in animal and plant tissue has been studied (Lester and Crane, '59a), and the coenzyme Q_{10} content of various dietary constituents has been determined in this laboratory (Page et al., '59) to guide the possible maintenance of animals upon a coenzyme Q-low diet.

The coenzyme Q group of compounds has been shown to be specific for restoration of cytochrome C reductase activity[1] and for restoration of succinoxidase activity[2] in inactivated systems. It appears that the redox functionality of coenzyme Q participates in mitochondrial electron transport, and that of plastoquinone participates in photosynthetic electron transport (Crane, '59b).

The reported occurrence of coenzyme Q_{10} in spinach and other white and green plant tissue (Crane, '59b) and in alfalfa meal (Lester and Crane, '59a), in addition to the presence of plastoquinone in alfalfa, seemed rather significant, because of (1) the widespread use of alfalfa and alfalfa meal in animal nutrition; (2) the widespread occurrence of coenzyme Q_{10} in mammalian tissue; and (3) the many previous studies (Vavick et al., '53; Scott et al., '53; Hill et al., '53; March et al., '55; Ershoff et al., '59; Ershoff and Hernandez, '59) which had suggested the possible presence of "unidentified factors" in alfalfa to account for the biological responses observed with alfalfa under various test conditions *in vivo*.

We wished to confirm, and have done so, the unexpected presence of both coenzyme Q_{10} and plastoquinone in alfalfa, and the apparent absence of other members of the Q group. We also examined freshly collected green alfalfa, because of the possible loss of the rather labile coenzyme Q_{10} during the commercial preparation and storage of alfalfa meal which is more commonly used in practical animal nutrition.

RESULTS

Fresh alfalfa was saponified, and the non-saponifiable lipid material was removed by extraction and purified by chro-

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[1] K. S. Ambe and F. L. Crane 1959 Coenzyme Q specific restoration of cytochrome C reductase activity. Federation Proc., *18:* 181 (abstract).

[2] S. Fleischer and R. L. Lester 1959 Restoration of succinoxidase activity by coenzyme Q in acetone-extracted mitochondria. Federation Proc., *18:* 227.

matography. The coenzyme Q_{10} was identified by means of paper chromatographic comparison with an authentic sample and estimated by spectrophotometric (Crane et al., '59; Lester and Crane, '59b; Linn et al., '59) and colorimetric assay (Koniuszy et al., '60). The coenzyme Q_{10} content of fresh alfalfa was estimated to be 3.4 mg/kg wet weight in the sample studied.

A sample of commercial alfalfa meal was extracted directly with hydrocarbon solvent and the coenzyme Q_{10} content was estimated. The value of 28 mg/kg is consistent with the value found for fresh alfalfa when adjusted for the probable water content and suggests that in the preparation of commercial alfalfa meal, the coenzyme Q_{10} content can be essentially retained.

EXPERIMENTAL

Fresh alfalfa. A sample of fresh alfalfa stems and leaves (1000 gm) was homogenized in a Waring Blendor with 200 ml of 95% ethanol by adding portions of the plant to portions of the solvent and blending for one to two minutes. To the resulting slurry in a 12-l round-bottom flask was added 45 gm of pyrogallol. The mixture was heated to boiling and 220 gm of potassium hydroxide in 400 ml of water was added during 5 minutes, with vigorous stirring. After the addition was complete, the mixture was stirred for 30 minutes under reflux, cooled to room temperature and extracted with 3 × 2000 ml portions of Skellysolve B. Additional water was added as necessary to break persistent emulsions. The combined extracts were washed with water, dried over anhydrous magnesium sulfate and evaporated *in vacuo* to 1.8 gm of orange, semi-solid residue.

A chromatographic column was prepared by adding 40 gm of dry Decalso slowly to a 2.0-cm-diameter glass tube containing Skellysolve B. To the prepared column was added the 1.8 gm of extracted solids dissolved in a minimum volume of Skellysolve B. The column was washed with 250 ml of Skellysolve B and eluted with 1% ether in Skellysolve B, collecting 20 ml fractions at a flow rate of 3 ml/minute. Fractions were assayed by spotting aliquots (10 to 20 µl) on filter paper and spraying with LMB reagent (zinc-acetic acid reduced methylene blue) (Page et al., '59) and characterized by paper chromatography on circles of Whatman no. 1 paper impregnated with vaseline and using dimethylformamide as mobile phase (Linn et al., '59; Page et al., '59).

Fractions 1 to 18 were devoid of any LMB oxidizing substances. Fractions 19 to 21 contained the bulk of the Q–254 while fractions 22 to 24 contained Q–254 and a second faster moving LMB oxidizing substance. Fractions 25 to 65 contained the coenzyme Q_{10} as well as α-tocopherol and a trace of Q–254. Fractions 65 to 93 as well as a 25% ether in Skellysolve B eluate were devoid of any LMB oxidizing substances.

Fractions 25 to 65 (0.21 gm) were dissolved in 5 ml of Skellysolve B, cooled to 0° and centrifuged. The supernate was removed and the process repeated twice. The combined supernates were evaporated *in vacuo* to 137 mg of red oil showing $E^{1\%}_{1\ cm} = 29$ at 290 mµ in ethanol but no maximum at 275 mµ, characteristic of coenzyme Q (Wolf et al., '58).

A chromatographic column was prepared by adding 10 gm of dry Decalso to a 1.0-cm-diameter buret containing i-octane. A solution of the 137 mg of red oil in a minimum volume of i-octane was added to the column and elution with 1% ether in i-octane was begun immediately, collecting 10 ml fractions at a flow rate of 2 ml/minute. The elution was followed by measuring the absorbance of each fraction at 255, 275 and 290 mµ. The elution of Q–254 was recognized in fractions 3 to 7 by the peak in 255 mµ absorbance while the elution of α-tocopherol was observed in fractions 11 to 16 by the peak in 290 mµ absorbance. The peak in 275 mµ absorbance occurred at fractions 24 to 27 which were evaporated to 11.8 mg of red oil.

The red oil was found to have an ultraviolet spectrum in ethanol nearly identical with that of coenzyme Q_{10} (Wolf et al., '58). It showed a strong LMB oxidizing spot on papergrams which was not separable from that produced by coenzyme Q_{10} and gave a positive Craven's test under the conditions used for coenzyme Q_{10}. The $\Delta E^{1\%}_{1\ cm}$ at 275 mµ obtained on reduction of an ethanol solution with sodium borohydride supported a coenzyme Q_{10} content

of 12% or a total of 1.4 mg. The amount present in neighboring fractions of the chromatogram was estimated at 1 mg from their absorbance at 275 mμ. The content of fresh alfalfa is approximately 3.4 mg/kg wet weight.[3]

Alfalfa meal. A sample (2000 gm) of commercial alfalfa meal was continuously extracted with Skellysolve B in a Soxhlet apparatus for 24 hours. The extract was evaporated *in vacuo* to a residue (77.8 gm) which was dissolved in 500 mg of Skellysolve B and filtered. The filtrate was applied to a chromatographic column prepared by adding 1500 gm of Decalso to Skellysolve B in a 7.0-cm-diameter column. The column was washed with 4 l of Skellysolve B followed by 8 l of 5% ether in Skellysolve B and eluted with 8 l of 50% ether in Skellysolve B. Removal of the solvent *in vacuo* from the eluate gave 13.5 gm of dark red residue.

A portion (1.35 gm) of the eluate residue was dissolved in 10 ml of i-octane, cooled to 0°, centrifuged and the supernate removed. The procedure was repeated and the combined supernates were applied to a 2.2-cm-diameter column of 50 gm of Decalso in i-octane. The column was washed with 25 ml of i-octane and eluted with 2% ether in i-octane, collected in 10 ml fractions at a flow rate of 1.5 ml/minute. Elution was followed by measuring absorbance at 255, 275 and 290 mμ as before. The Q–254 peak appeared at fraction 34, the α-tocopherol peak at fraction 48, and the coenzyme Q_{10} peak at fraction 96, after changing the eluting mixture to 5% ether in Skellysolve B at fraction 68. Fractions 83 to 101 were combined and evaporated *in vacuo* to 66.6 mg of red oil. This oil was dissolved in 3 ml of Skellysolve B, cooled in ice, centrifuged and the supernate separated. The procedure was repeated and the supernates combined and evaporated *in vacuo* to 37.1 mg of red oil which showed the same properties as that prepared from fresh alfalfa and assayed 15% of coenzyme Q_{10} by spectrophotometric assay. The coenzyme Q_{10} content of alfalfa meal is therefore estimated at 28 mg/kg dry weight.

SUMMARY

The coexistence of coenzyme Q_{10} with plastoquinone in commercial alfalfa meal has been confirmed, and the content of coenzyme Q_{10} was found to be *ca.* 28 mg/kg. The preparation and storage of commercial alfalfa meal can lead to retention of essentially all of the coenzyme Q_{10} of growing alfalfa since the content of coenzyme Q_{10} of the freshly collected plant was *ca.* 3.4 mg/kg; this value is reasonably consistent with the water content of the green plant.

ACKNOWLEDGMENT

Dr. Grant Richardson, Department of Agriculture, Arizona State University, Tempe, Arizona, kindly provided us with fresh alfalfa. The authors wish to acknowledge also the technical assistance of John Madas and Arthur Ritchie.

[3] A recovery of 70% is assumed. See Page et al. ('59).

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The Effect of Sources of Nonessential Nitrogen on Nitrogen Balance in Young Adults[1]

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In studies of essential amino acid requirements for nitrogen equilibrium in human subjects, glycine, urea and diammonium citrate have been used either singly or in various combinations as nitrogen sources to supplement purified essential amino acid mixtures (Rose, '49; Leverton et al., '56; Jones et al., '56). Whenever food proteins are evaluated in terms of their essential amino acid composition, however, the nonessential amino acids they contain comprise a portion of the dietary nitrogen. When under these conditions amino acid requirements are investigated at different levels of nitrogen intake, as in a recent study with egg protein (Swendseid et al., '59), it becomes particularly pertinent to question the relative effectiveness of various sources of nonessential nitrogen in maintaining nitrogen equilibrium. The present investigation was designed to obtain information as to the possible effect of the amount and source of supplemental nitrogen on nitrogen equilibrium in subjects fed near-minimal amounts of essential amino acids in the form of egg protein.

In this report the term "nonessential" nitrogen will be used interchangeably with "supplemental" nitrogen to define nitrogen-containing compounds other than the 8 essential amino acids required by man for the maintenance of nitrogen equilibrium. The nonessential nitrogen sources studied include glycine, a mixture of glycine and diammonium citrate (DAC) and a mixture of nonessential amino acids (NEAA).

EXPERIMENTAL

The comparisons of nonessential nitrogen sources were made at either 6.5 or 10.0 gm of total nitrogen intake. These amounts were selected because they have been used most frequently in studies of essential amino acid requirements (Rose, '49; Leverton et al., '56; Jones et al., '56; Swendseid et al., '56). Four college students, one man and three women, participated in the study at both total nitrogen intake levels. These subjects were in good health as determined by medical examination. The age, weight and caloric intake of each subject while on the experimental diet are given in table 1.

For each total nitrogen intake level, the subjects were placed first on a controlled diet of ordinary food with calories adjusted to need. After the subjects were established in nitrogen equilibrium (a period of at least 7 days) they were fed the isonitrogenous experimental diet contain-

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TABLE 1
Sex, age, weight and caloric intake of each subject

Subject	Sex	Age	Weight		Energy value of diet
			Initial	Final	
no.		*years*	*kg*	*kg*	*Cal./day*
1	Male	20	70.9	71.4	3200
2	Female	20	53.8	55.5	2600
3	Female	24	60.0	62.3	2400
4	Female	21	51.8	51.8	2500

ing minimal amounts of essential nitrogen in the form of egg protein and the nonessential nitrogen source to be tested. The experimental diet also contained some low-protein fruits and vegetables, centrifuged butter, sucrose and cornstarch with mineral and vitamin supplements (Swendseid et al., '56). Since these ingredients were constant throughout the study, they are referred to as the basal components. The distribution of the nitrogen in this diet when 100 gm of egg was fed is shown in table 2. An attempt was made to adjust the amount of egg for each subject to approach his minimal requirements for the essential amino acids. Whenever the amount of egg fed was reduced below 100 gm, the supplemental nitrogen was increased to maintain the total nitrogen of the diet at a constant level.

Studies were carried out first with the 6.5 gm N diet. On the experimental diet, the dietary periods comparing sources of nonessential nitrogen were 6 or 7 days.

The glycine was always administered first. With this source, the amount of whole egg was adjusted to an amount judged on the basis of urinary nitrogen values to supply near-minimal amounts of the essential amino acids. In the succeeding dietary periods, glycine was replaced by an isonitrogenous mixture of the nonessential amino acids. This mixture was composed according to the NEAA proportions in whole egg protein with the exception that tyrosine and cystine were omitted (table 2). Only L-amino acids were used. Depending upon the amount of egg the subject received, this supplemental nitrogen provided from 60 to 70% of the total nitrogen in the diet. The daily amounts of egg and the supplemental nitrogen were equally distributed among the three meals.

A 4-week interval followed during which the subjects ate ad libitum. Then they were given a controlled diet of ordinary food containing 10 gm of total nitrogen for 7 days. On the experimental diet a mixture of glycine and DAC as a source of supplemental nitrogen was given first. The amount of whole egg was the same as for the 6.5 gm N intake. The second period for all subjects was a substitution of NEAA mixture for glycine and DAC in isonitrogenous quantities. These sources of supplemental nitrogen constituted from 75 to 80% of the total nitrogen in the diet, (table 2).

Nitrogen analyses were carried out on food samples, daily urine collections, and 5- or 10-day fecal pools according to previously reported procedures (Swendseid et

TABLE 2

Sources of dietary nitrogen

Dietary component	6.5 gm N intake		10 gm N intake	
	Amount[1]	N content	Amount[1]	N content
	gm	*gm*	*gm*	*gm*
Basal components	430	0.5	430	0.5
Glycine	21.4	4.0	20.1	3.75
Diammonium citrate	—	—	30.2	3.75
(Nonessential amino acid mixture[2] replacement for glycine plus diammonium citrate during some periods)	(28.2)	(4.0)	(53.0)	(7.5)
Essential amino acids in egg/100 gm	6.45[3]	0.75	6.45	0.75
Nonessential amino acids in egg/100 gm	8.80[3]	1.25	8.80	1.25

[1] Amount varies except for basal diet. Values shown are for periods when 100 gm of whole fresh egg was fed.

[2] The percentage composition of this mixture was as follows: aspartic acid, 17.1; glutamic acid, 21.5; glycine, 6.0; proline, 8.3; serine, 15.8; alanine, 12.6; arginine·HCl, 13.4; and histidine·HCl-H_2O, 5.5. It was calculated from analytical data on whole egg furnished by Dr. M. J. Horn, Human Nutrition Research Division, Agricultural Research Service, U.S. Department of Agriculture.

[3] Calculated from data compiled by Orr and Watt ('57). Cystine and tyrosine are included with the essential amino acids.

al., '56). Nitrogen was determined according to the boric acid modification of the Kjeldahl procedure (Scales and Harrison, '20).

RESULTS AND DISCUSSION

The average daily values obtained for nitrogen balance using the different sources of nonessential nitrogen are shown in table 3. A consideration of differences in nitrogen retention for each subject when NEAA replaces glycine at 6.5 gm of total nitrogen shows an increase of 0.75 gm of nitrogen per day for subject 1, 0.53 gm for subject 3, 0.91 gm for subject 4 and an average of 0.64 gm for subject 2. These definite increases in nitrogen retention, which occur when the subject is receiving minimal or near minimal amounts of the essential amino acids, result in an average increase of 0.71 gm of nitrogen retained per day per subject. Since these changes occurred consistently with all 4 subjects, these results appear to be evidence that when glycine is the chief source of nonessential nitrogen, it is not as well utilized as a mixture of nonessential amino acids. The changes in nitrogen retention for the same subjects under the same dietary conditions, with respect to the presence of essential amino acid nitrogen but with an increase of total nitrogen to 10 gm as the result of an increase in nonessential nitrogen, are also shown in table 3. When NEAA replaces a mixture of DAC and glycine, the changes in nitrogen retention include + 0.11 gm of nitrogen per day for subject 1, −0.17 gm for subject 2, +0.38 gm for subject 3 and +0.27 gm for subject 4. The average change in nitrogen retention under these conditions is an increase of only 0.15 gm of nitrogen per day per subject and this appears to be within experimental variation. Apparently, then, a combination of glycine and DAC is as well utilized as a mixture of nonessential amino acids.

It is also possible to make a comparison of nitrogen retention in the same subjects when two levels of NEAA are fed. When the total nitrogen is increased from 6.5 gm to 10.0 gm per day by increasing the NEAA with other dietary components remaining constant, the changes in nitrogen retention are +0.07 gm nitrogen per day for subject 1, −0.22 gm for subject 2, −0.16 gm for subject 3 and −0.37 gm for subject 4. Here again the differences in nitrogen retention appear to be negligible since the average change is −0.17 gm of nitrogen per day per subject.

Since only negligible differences in nitrogen retention occur between nitrogen intake levels when NEAA is the nonessential nitrogen source, it appears possible to compare glycine and a mixture of glycine and DAC although these substances were fed at different nitrogen intake levels. The results suggest that the addition of ammonium salt to glycine results in greater nitrogen retention than when glycine alone is fed. It can be seen from table 2 that whether glycine is fed alone or in combination with DAC the amount of glycine administered is approximately the same.[2]

[2] Unpublished results from this laboratory show that the amount of urinary glycine is also the same whether the same amount of glycine is fed alone or in combination with DAC.

TABLE 3

Effect of the source of nonessential nitrogen on nitrogen balance

Subject	Amount of whole fresh egg	Average daily nitrogen balance values[1] (gm/day) on indicated source[2] of nonessential nitrogen			
		6.5 gm N intake		10.0 gm N intake	
		Glycine	NEAA[3]	Glycine + DAC	NEAA[3]
	gm				
1	100	−1.08	−0.33	−0.37	−0.26
2	80	−0.28	+0.32	+0.27	+0.10
	65	−0.47	+0.20		
3	65	+0.02	+0.55	+0.01	+0.39
4	65	−0.43	+0.48	+0.39	+0.11

[1] Average nitrogen balance values include only last 4 days of each 6 or 7 day dietary period.
[2] Amounts of nonessential nitrogen are given in table 2.
[3] Percentage composition of the nonessential amino acid mixture is given in table 2.

Apparently then, this ammonium salt is well utilized by the human body as a source of nonessential nitrogen.

The superiority of ammonium salts as compared with glycine has been shown in a number of growth experiments with rats (Rose et al., '48; Lardy and Feldott, '50; Rechcigl et al., '57).

SUMMARY

Four young adult subjects were maintained on an experimental diet wherein the amount of whole egg was adjusted to supply near-minimal amounts of the essential amino acids. Supplemental nitrogen provided from 60 to 80% of the total dietary nitrogen and was varied as to source. When a nonessential amino acid mixture replaced glycine at a total nitrogen intake of 6.5 gm, there was an average increase in nitrogen retention of 0.71 gm per day per subject. When the nonessential amino acid mixture replaced a mixture of glycine and DAC at a total nitrogen intake of 10 gm there was an average increase in nitrogen retention of 0.15 gm per day per subject. A comparison of nitrogen balance when the nonessential amino acid mixture was fed at the two levels of nitrogen intake shows that increasing the amount of nonessential amino acids resulted in an average decrease in nitrogen retention of 0.17 gm per day per subject. These results were taken as evidence that, under the experimental conditions, glycine alone as a source of supplemental nitrogen is not as well utilized as a mixture of nonessential amino acids nor as an ammonium salt and glycine mixture when this mixture is fed at a higher nitrogen level. The combination of ammonium salt and glycine appeared to be as effective as a mixture of the nonessential amino acids in maintaining nitrogen equilibrium.

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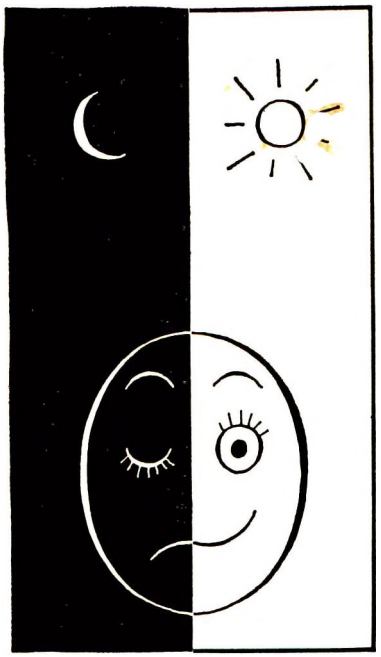

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