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*(1883 — 1956)*

Louis Harry Newburgh

# Louis Harry Newburgh — A Biographical Sketch

*(June 17, 1883 — July 17, 1956)*

A long career of clinical investigation into problems of fundamental concern in medicine and in nutrition came to an end on July 17, 1956, when Louis Harry Newburgh died at Escondido, California.

His publications, authored alone or with his associates, numbered approximately one hundred and represent sound extension of knowledge in areas where speculation, rather than fact, had particularly annoyed him. He sought painstakingly the truth through exquisitely designed fundamental research.

Louis Harry Newburgh was born June 17, 1883, in Cincinnati, Ohio. His father, Henry Newburg, was born in Chicago, Illinois, but lived most of his life in Cincinnati, and his mother, Laura Mack Newburgh, was born in Cincinnati. Henry Newburgh graduated from the College of the City of New York and hoped to become a doctor. Since his family could not afford to provide further education, he returned to Cincinnati to enter the business world. His careers in a clothing concern and in the wholesale tobacco business provided financial security to assure his son the medical education he had longed for in his own youth. His long membership on the board of directors of one of the Cincinnati banks terminated with his death at the age of ninety-two.

A sister, Emily Newburgh Freiberg, four years younger than Louis Harry, lives in Cincinnati. She recalls with gratitude, that their father instilled in the two children a love for learning at an early age. The family was devoted and shared their common interest in reading and good conversation, whenever they could be together. The need for and love of close family ties was also apparent in the home which Louis Harry established.

Louis Harry Newburgh attended public schools in Cincinnati until high school age, when he was enrolled in the Franklin Preparatory School. He entered Harvard University in 1901, receiving his A.B. degree in 1905 and his M.D. in 1908. He served a medical internship for 16 months at the Massachusetts General Hospital, and then studied a year abroad, chiefly in Vienna and Berlin. There he admired the discipline and excellence of work in the German laboratories but found intolerable the ritual of clinical dogma which failed to recognize patients as human beings in need of sympathy and concern.

On Dr. Newburgh's return to the United States, he entered the private practice of medicine in Cincinnati, with the late Dr. Frederick Forcheimer, then one of the leading internists in the United States. Dr. Forcheimer was his father's closest friend and may have been influential in encouraging the brilliant student to pursue a career in medicine. Dr. Newburgh soon discovered that the private practice of medicine was not for him. His absorbing interests were in the field of clinical research and in teaching. Inspiring young doctors, medical students, and others associated with him became a primary mission in life. He returned to Harvard and began there his long career of clinical investigation. He served as an assistant in internal medicine at the Harvard Medical School until 1915.

While in Boston, he married Irene Haskell, a graduate nurse of Montreal, Canada, and Henry, the first son, was born in 1915. Dr. Newburgh worked with Dr. David L. Edsall, Jackson Professor of Clinical Medicine and later Dean of the Medical School at Harvard.

Dr. Newburgh, at this stage of his career, would have preferred to spend all

his time in clinical research and teaching. However, financial support for such a career was practically nonexistent at that time. Consequently, he found it necessary to supplement his very modest stipend with a minimal amount of private practice.

The problem of pneumonia was a most distressing one, and Dr. Newburgh's publications during these years at Harvard attest to his need to assure himself through the avenue of careful investigation that the symptomatic treatment was, in fact, based on an understanding of physiology of the disease under consideration. Investigations were carried out in the wards of the Massachusetts General Hospital and in the physiological laboratory of Professor William Townsend Porter at the Harvard Medical School. Studies on the cardiac output of normal subjects and patients with heart disease were reported jointly with W. T. Porter and J. H. Means.

Throughout Dr. Newburgh's long career in clinical investigation, he was ever grateful to Dr. W. T. Porter, who must have been a great inspiration and example to him as he developed his own scientific approach to problems.

Having launched his lifelong career in clinical investigation in Boston, Dr. Newburgh accepted an invitation from the University of Michigan Medical School to work full time in medicine with ample opportunity for clinical research. He was appointed Assistant Professor of Internal Medicine, and in 1916 at the age of 33, became the assistant of Dr. Nellis B. Foster, then Professor of Medicine and Chairman of the Department. When Dr. Foster decided to contribute his services to the United States Army, he left Dr. Newburgh with the responsibility of Acting Head of the Department of Medicine. His own application for service in the Medical Corps was refused and he was "ordered" to remain at the Medical School to train young doctors for the army.

In 1917 Dr. Newburgh found himself with an insatiable zeal to investigate, but with literally no facilities and with a good bit of his time devoted to administrative matters. Although he was serving as Acting Head of the Department of Medicine, his appointment as Assistant Professor of Medicine not only permitted but obligated him to teach in the Medical School. He served as Acting Head of the Department until 1922, having been promoted to Associate Professor in 1918, and to Professor of Clinical Investigation in 1922. This title was created for Dr. Newburgh and has never been granted to anyone else in this Medical School.

Dr. Newburgh's second son, John David, was born in 1921.

Throughout Dr. Newburgh's career, he was particularly interested in metabolic and kidney diseases. The first of several publications on the use of a high-fat diet in the treatment of diabetes mellitus was published in 1920. His rather revolutionary approach to the feeding of patients with diabetes was a significant contribution, for insulin was not then available. The diet was high in fat, low in protein and low in carbohydrate. In 1923 Marsh and Waller described the application of this diet to a farmer who had severe diabetes. The initial diet was one which involved partial starvation of the patient. It contained 20 grams of protein, 85 grams of fat and only 14 grams of carbohydrate. As was routine for several years, the initial diet for a diabetic patient contributed about 900 calories. The diet was very gradually increased until it provided 43 grams of protein, 230 grams of fat, 25 grams of carbohydrate, and about 2350 calories. At this time, Newburgh and Marsh prescribed for every diabetic patient admitted to the wards of the hospital at Ann Arbor an initial diet contributing about 900 calories. Dr. Newburgh reported that this type of a diet produced the same fall in basal metabolism as the then popular Allen fasting treatment did, it was more rapid in eliminating sugar, and he felt it was far less dangerous than fasting. When one of the authors (A.M.B.) became one of Dr. Newburgh's "own dietitians" in 1932, the procedure for feeding patients with diabetes was still a very rigid one. As a matter of historical interest, the purpose of the diabetic diets he prescribed at that time was "to furnish an *adequate* diet in which the total available glucose is low enough to avoid glycosuria and the fatty acid-glucose ratio low enough to avoid ketosis." His diets were designed so as 1) to provide two-thirds to

one gram of protein per kilogram of body weight; 2) to yield a fatty acid-glucose ratio which routinely did not exceed 2.5:1; 3) to supply dietary fat by the generous use of 40 per cent cream, butter, and salad oils; and 4) to provide the limited amount of carbohydrate permitted through the selection of vegetables and fruits very low in carbohydrate content.

Dr. Newburgh's diet prescriptions were interpreted to mean a permissible variation of plus or minus one gram of protein, fat or carbohydrate. A variation of two or three grams, on occasion, was permissible when the total available glucose was calculated within one gram of the prescribed amount. The diets were weighed and replacements made, as necessary, for the patients who were receiving insulin.

The high-fat diets required that dietitians be most creative in devising ways to serve large amounts of whipped cream and butter in forms palatable enough for the patient with diabetes to be able to consume high amounts of fat week in and week out. Ice cream became frozen whipped cream with just a suggestion of artificial or, on rare occasion, fruit flavor. Agar agar, brilliantly colored red or green, provided a strange source of volume to the patient who longed for an additional quantity of food.

Dr. Newburgh's interest in the comfort of his patients, his deep compassion for people, and his dedication to exactness, required that he surround himself with dietitians knowledgeable in metabolism and expert in the feeding of his patients. The first dietitian was hired by the University Hospital in 1902, but dietetics truly flourished during the years when Dr. Newburgh lent his support to the education and training of the dietetic interns. The first dietitians to join the staff for an "apprenticeship" remained for six months in 1924. In 1930, the period was extended to nine months and in 1931 the current plan of a twelve-month internship was established. Dr. Newburgh had a firm conviction that dietitians must be given the opportunity to obtain the type of experience and graduate study that would prepare them to be effective colleagues of clinical investigators and practicing physicians.

As early as 1919, Dr. Newburgh demonstrated his interest in diseases of the kidney in a publication entitled, "The Production of Bright's Disease by Feeding High Protein Diets." His last publications dealt also with kidney mechanisms. Interest in this area continued for a span of 32 years.

Dr. Newburgh's extensive and intensive research in diseases of the kidney, electrolyte and water balance, obesity and energy metabolism is amply documented in the literature. It might be mentioned, however, that the Newburgh-Marsh high-fat diet for diabetes was a blessing and literally a life-saver for diabetics just prior to the discovery of insulin. His studies on water balance had wide implications in the whole field of medicine and surgery. His metabolic studies and his simplification of the concept of obesity brought common sense to the management of this condition and removed it from the realm of the mystical.

His publications speak for the man as a scientist. It is fitting that the influence which he exerted on those who knew him best should be described.

Perhaps the characteristic that stands out in the memory of the hundreds who came under Dr. Newburgh's influence is the brilliance of his mind. A student is fortunate indeed to observe the workings of the brain of just one teacher as brilliant as was Dr. Newburgh. His lectures were like a symphony in logic and clarity. He loved to teach and to lead complacent students to question, to wonder and to be skeptical. He did not tolerate a "spoon feeding" approach to the students. He indicated that they were capable of obtaining information available from all the books and journals in the library. It was his purpose to so stimulate the brain and arouse the curiosity of young people, that they would dedicate their lives to seeking the truth. He agreed fully with Carl Von Voit that "the results of a properly conducted and properly appreciated experiment can never be annulled, whereas a theory can change with the progress of science." When a co-worker raised a question, Dr. Newburgh would gently lead the young worker into a research design to seek the answer. He was always generous in giving credit to his co-workers, al-

ways making certain that the younger members of his staff, in particular, received recognition for work accomplished.

As a scientist, he was, of course, his own severest critic. Students, however, who displayed a careless or lazy approach to a problem were reprimanded kindly but very firmly. "The boss," as he was affectionately called, could not tolerate anything less than the nearest approach to perfection that a human being could produce.

Although he displayed great kindness to the patients in his care, one always sensed that even while he was at the bedside of a patient, he was impatient to hurry back to the laboratory to determine the basis for the chemical phenomena operative in the disease under study. His enthusiasm for metabolic diseases was contagious, and his influence with students as he tried to demonstrate "the way of science" cannot be measured. Much of his success as an investigator can be attributed to a persistent, compulsive drive to seek the answer once he had formulated the problem.

Dr. Newburgh devoted his life to clinical investigation during the years when funds were very short for equipment, supplies, and personnel. He was forced to seek funds personally from individuals and from foundations, a job he especially abhorred. This actually consumed much time and often led to deep disappointments so that he was often disturbed and worried about providing for his staff and the continuance of his research. This problem was not peculiar to this Medical School but nationwide. Such a situation is hard to picture now, when money for research is so readily available from many sources. As for his personnel, however, many dedicated young people worked with him gladly for the privilege of learning in the old-fashioned arena of little money, makeshift equipment, but with curiosity and a passionate desire to learn that could not be contained.

Dr. Newburgh was a very humble man. He asked little for himself. His home, his wife, and his two sons brought him as much peace and quiet as a restless brain could ever hope to find. His home and magnificent gardens were open to all of his friends. He traveled extensively in the presentation of papers, but he was truly happiest when his hours were divided between his home and his laboratory.

During World War II, Dr. Newburgh was asked to serve on the Committee for Clinical Investigation of the National Research Council. In his Harvard Fiftieth Anniversary Report he wrote, "I devoted my whole strength to the many problems that needed the most rapid solution. When it was over I found myself exhausted and anxious to retire." Perhaps, Dr. Newburgh was remembering what Pasteur had suggested to his students many years before. "Live in the serene peace of laboratories and libraries. Say to yourselves first: What have I done for my instruction? And, as you gradually advance, what have I done for my country?" As the nature of the problems brought before the Committee for Clinical Investigation evolved, he was asked to serve on a Committee of the Office of Scientific Research and Development on Clothing for the Armed Forces. He was eminently qualified for this assignment because of his long research on channels of heat loss from the human body. Dr. Newburgh was tired but devoted himself to the service of his country, and for this work he received a Certificate of Merit. In January of 1944 he returned to the University Hospital and carried on his teaching responsibilities and research until he retired in 1951.

Dr. Newburgh served as a member of the Editorial Board of the *Journal of Nutrition* from 1936 to 1940. Membership in professional organizations included: American Diabetes Association, American Institute of Nutrition, American Medical Association, American Society for Clinical Investigation, Association of American Physicians, Central Society for Clinical Research, Society for Experimental Biology and Medicine, Fellow of the American College of Physicians since 1930, and a Diplomate of the American Board of Internal Medicine.

On retirement, Dr. and Mrs. Newburgh moved to Valley Center, California, where they could live in close proximity to their older son, Henry, an electrical engineer, who at that time was with the United States Naval Ordnance Test Station, China Lake, California. Henry holds a Bachelor

of Science degree in Engineering from the University of Michigan (1939) and now lives with his mother in Valley Center, California, where he is carrying on independent research in electrical engineering and physics.

A great tragedy in Dr. Newburgh's life was the death of his brilliant son, David, in 1953. David had a precocious knowledge of mathematics and chemistry, and throughout his younger years, Dr. Newburgh would talk with him for hours on problems in chemistry and mathematics that to the outsider would have seemed far beyond the grasp of a young lad. But Dr. Newburgh recognized and nurtured this child who was so precocious. David was ill for many years, but in spite of ill health completed work towards his Ph.D. degree in mathematics from the University of Michigan in 1947. He held appointments in mathematics successively at Massachusetts Institute of Technology, the Institute for Advanced Studies at Princeton, and Tulane University. David's death was a shock to which Dr. Newburgh never really adjusted.

In June, 1956, Dr. Cecil Striker, a one-time student and a long-time friend of Dr. Newburgh, accepted the Banting Medal on behalf of Dr. Newburgh.

Dr. Newburgh was a man of deep convictions and deep loyalties. Nothing was too much for him to undertake either in support of a theory or of an individual he deemed worthy. It can be easily understood that these loyalties and convictions sometimes led to controversy, which at times unfortunately became bitter. To those of us who knew him best this was but a manifestation of the single-hearted devotion to what he believed was the truth.

His humility and kindness, his devotion to his work, his brilliant contributions and his complete loyalty to his friends will ever be remembered. Many of his students attempt each day of their lives to pay tribute to the memory of this great man through a determined effort to pass on to another generation of students a little of the honesty, the devotion and the insatiable scientific curiosity which Dr. Newburgh demonstrated. His keen intellect, his strength of character, his analytical way of addressing himself to a problem and his insatiable search for truth affected all who had the privilege of working with him.

Perhaps, herein lies the greatest contribution of this devoted man of science, his influence on the individuals who were privileged to work with him in his laboratory (medical students, dietitians, biochemists, physiologists, internists, surgeons), many of whom have distinguished themselves in their respective fields. In their continuing contributions to teaching and investigation his work goes on. Thus his influence will continue, for the personal and professional characteristics he exemplified represent the fabric of the dedicated clinical investigator and teacher.

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# Delay of Sexual Maturity of the Female Rat by *Sterculia foetida* Oil [1,2]

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*ABSTRACT* Groups of weanling 21-day-old female albino rats of the Sprague-Dawley strain were fed 4% safflower or corn oil diets or these diets partially substituted with *Sterculia foetida* oil, for periods of 16 or 20 weeks to determine the influence of this rich (35%) source of the cyclopropenoid fatty acid, sterculic, on sexual maturity of the female rat. Oil diets containing 1% corn or safflower and 3% *S. foetida* oil significantly delayed opening of the vagina, increased the length and decreased the frequency of estrous cycles as compared with 4% corn or safflower oil diets. Comparison of ad libitum with paired-feeding indicated that rate of growth was not a factor under the conditions used. At termination of the experiments highly significant differences in body, liver and ovaries-oviduct-uterus weights were observed in a comparison with the groups pair-fed 1% safflower-3% *S. foetida* oil and 4% safflower oil.. Heart, kidney and brain weights were not significantly different. Feed utilization of groups fed *S. foetida* oil was consistently poorer with both ad libitum and paired-feeding.

Early reports of certain biological effects attributed to cottonseed oil or to cottonseed products containing cottonseed oil were explained recently by the identification of cyclopropenoid fatty acids as the causative agents (1–3).

Effects of cyclopropenoid fatty acids on sexual maturation and reproduction have been reported only for the avian species. Almquist et al. (4) reported finding several birds with small ovaries among pullets fed cottonseed oil rations. Schneider et al. (5) reported that feeding of *Sterculia foetida* oil, a rich source of the cyclopropenoid fatty acid, sterculic, to pullets greatly retarded growth of ovaries and oviducts and, consequently, inhibited egg production. Supplementing the diet of laying hens with 25 mg *S. foetida* oil daily increased mortality of developing embryos to over 80% by the last day of incubation (6).

The present report describes an investigation of the effect of *S. foetida* oil on sexual maturation of the female rat. Age of the rat at the time the vagina opened and regularity and length of consecutive estrous cycles were used as criteria.

## MATERIALS AND METHODS

Weanling 21-day-old female albino rats of the Sprague-Dawley strain were fed a standard laboratory diet for 24 hours and then distributed into comparable groups of ten according to weight. Each group was fed a specific 4% oil diet and water was supplied ad libitum. The rats were housed individually in hanging galvanized screen-bottom cages in a controlled temperature room (26.5 ± 1°).

The composition of the basal diet is shown in table 1. The diets were stored in polyethylene bags at 4°. In all experiments diets were weighed into individual feed cups and replaced with fresh diets daily for both paired-feeding and ad libitum-feeding experiments. Both of these feeding techniques were used to determine the influence of rate of growth on sexual maturity. Records were kept of the daily feed consumption and weekly weight of each rat for 16 weeks.

The control oils were commercial corn or safflower oils. The test oil was *S. foetida* oil which was extracted from *S. foetida* seeds.[3] The seeds were ground in a large Waring Blendor and extracted with Skellysolve F (3 liters/kg). The extracted oil was separated from the solvent in a

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[1] Arizona Agricultural Experiment Station Technical Paper no. 923.

[2] This investigation was supported in part by Public Health Service Research Grant no. EF-00509 from the National Institutes of Health, Division of Environmental Engineering and Food Protection.

[3] *Sterculia foetida* seeds were obtained from Mr. Zoilo C. Fraga, Forestry College, Laguna, Philippines.

TABLE 1

*Composition of basal diet*

| | % |
|---|---|
| Casein [1] | 18.0 |
| Fat | 4.0 |
| Glucose monohydrate [2] | 69.62 |
| Non-nutritive fiber [3] | 4.0 |
| Salt mixture [4] | 4.0 |
| Vitamin mixture [5] | 0.38 |
| Total | 100.0 |

[1] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] Cerelose 2001, Corn Products Company, New York.
[3] Solka-Floc, Brown Company, Berlin, New Hampshire.
[4] Jones and Foster (7) with NaF added to give 10 ppm in salt mixture.
[5] Vitamin mixture supplied/100 g diet: (in milligrams) thiamine·HCl, 0.4; riboflavin, 0.5; niacinamide, 5.0; pyrodoxine·HCl, 0.25; Ca pantothenate, 2.0; choline bitartrate, 200.0; inositol, 100.0; p-aminobenzoic acid, 10.0; folic acid, 0.2; biotin, 0.02; vitamon $B_{12}$ (0.1% trit. in mannitol), 10.0; menadione, 0.2; α-tocopherol, 5.0; and vitamin A (PGB-10, Distillation Products Industries, Rochester, N. Y.), 1000 USP units; and vitamin $D_3$ (Super Nopdex-30, Nopco Chemical Company, Richmond, Cal.), 120 USP units.

rotary vacuum evaporator. The oil contained 35% cyclopropenoid fatty acids determined as sterculic acid by a modification of the Halphen reaction (8). The diets contained 4% control oil, 2% control oil — 2% *S. foetida* oil, or 1% control oil — 3% *S. foetida* oil. The oil levels were calculated to supply a minimum of 95 mg of linoleic acid/10 g of the 1% corn oil — 3% *S. foetida* oil diet and 115 mg of linoleic acid/10 g of the 1% safflower — 3% *S. foetida* oil diet. According to Hansen et al. (9), the daily requirement of linoleic acid for the growing rat is 80 mg/day.

The sexual maturity of the rats was determined by observing the age of the rat at which degeneration of the membrane covering the vagina occurred and by the length of the estrous cycle, according to the vaginal smear technique of Long and Evans (10). The rats were observed daily for the disappearance of the vaginal membrane, and then a vaginal smear was made daily for each rat. The length of the estrous cycle was determined by counting the number of days between the presence of the greatest number of cornified cells on the daily vaginal smear slide.

Since onset of sexual maturity and ovulation in the rat may be subject to variations in environmental conditions, certain environmental factors such as temperature, light, position of cage and rack, and handling were controlled as uniformly as possible.

## RESULTS AND DISCUSSION

*Feed utilization.* During a period of 16 weeks the groups fed 4% corn oil and 4% safflower oil showed no significant differences in efficiency of feed utilization regardless of whether they were pair-fed or fed ad libitum (table 2).

Comparable groups fed ad libitum 4% oil diets in which one-half of the control oil was replaced with *S. foetida* oil gained significantly less weight and utilized feed less efficiently than their respective controls ($P < 0.05$). When the proportion of *S. foetida* oil was increased to three-

TABLE 2

*Comparison of feed utilization of female rats fed various oil diets ad libitum or by paired-feeding for 16 weeks*

| Oil in diet | No. of rats | Avg initial wt | Avg wt gain | Avg feed intake | Feed utilization [1] |
|---|---|---|---|---|---|
| | | *g* | *g* | *g* | % |
| Fed ad libitum | | | | | |
| 4% Corn | 10 | 51 | 190 | 1314 | 14.5 |
| 4% Safflower | 9 | 51 | 191 | 1305 | 14.6 |
| 2% Safflower + 2% *S. foetida* | 9 | 51 | 157 | 1219 | 12.9 |
| 1% Safflower + 3% *S. foetida* | 10 | 51 | 127 | 995 | 12.8 |
| Pair-fed | | | | | |
| 4% Corn | 9 | 50 | 187 | 1245 | 15.0 |
| 1% Corn + 3% *S. foetida* | 8 | 50 | 157 | 1193 | 13.2 |
| 4% Safflower | 10 | 50 | 194 | 1258 | 15.4 |
| 2% Safflower + 2% *S. foetida* | 10 | 50 | 184 | 1253 | 14.7 |
| 1% Safflower + 3% *S. foetida* | 9 | 50 | 159 | 1153 | 13.8 |

[1] Grams gained/gram feed intake (× 100).

fourths of the total oil in the diet (3% *S. foetida* oil), growth gains and feed utilization were decreased further ($P < 0.01$).

A similar comparison of pair-fed groups showed that 2% *S. foetida* oil lowered the weight gains and feed utilization slightly, but 3% *S. foetida* oil significantly lowered both weight gains and feed utilization ($P < 0.01$).

Control oil groups gained weight equally with safflower or corn oil when pair-fed or fed ad libitum; feed utilization was slightly better under paired-feeding.

*Organ weights.* At termination of the experiments highly siginficant differences in body ($P < 0.001$), liver ($P < 0.001$) and ovaries-oviduct-uterus weights ($P < 0.003$) were found in a comparison of the pair-fed 1% safflower — 3% *S. foetida* oil and the 4% safflower oil groups (table 3). These results are in agreement with those reported by Schneider et al. (5) for the chicken. Heart, kidney and brain weights were not significantly different.

*Sexual maturity.* Comparison of growth of rats on 4% oil diets with and without *S. foetida* oil are shown graphically in figure 1. In the ad libitum feeding experiment the average age at the time the vagina opened was 37 days with a range of 31 to 46 days for the 4% corn oil or 4% safflower oil group (fig. 1). This agrees with the report of Long and Evans (10) that in albino rats the vagina opens between 32 and 109 days of age. The 1% safflower — 3% *S. foetida* oil group had an average age of 68 days with a range of 53 to 81 days at the time the vagina opened. The average body weights when the vagina opened were 101 g for the control group and 126 g for the 3% *S. foetida* oil group.

In the paired-feeding experiment the growth of the control group was slower than in the ad libitum feeding experiment, since the control rats were allowed only as much diet as the 1% safflower — 3% *S. foetida* oil rats had ingested on the pre-

TABLE 3

*Comparison of organ weights of female rats pair-fed oil diets with and without Sterculia foetida oil for 20 weeks*

| Oil in diet | 4% Corn | 1% Corn + 3% *S. foetida* | 4% Safflower | 2% Safflower + 2% *S. foetida* | 1% Safflower + 3% *S. foetida* |
|---|---|---|---|---|---|
| No. of rats | 10 | 8 | 10 | 10 | 9 |
| Body wt, g | 243.9 ±12.9 [1] | 219.1 ±17.7 | 254.1 ±16.6 | 238.1 ±14.9 | 215.7 ±16.5 |
| Liver | | | | | |
| Wt, g | 5.71± 0.94 | 10.94± 0.94 | 6.22± 1.41 | 11.78± 1.59 | 11.03± 1.76 |
| % of body wt | 2.33± 0.29 | 5.03± 0.69 | 2.47± 0.62 | 4.98± 0.55 | 5.13± 0.86 |
| Ovary-oviduct-uterus | | | | | |
| Wt, g | 0.71± 0.26 | 0.42± 0.07 | 0.50± 0.06 | 0.46± 0.09 | 0.36± 0.12 |
| % of body wt | 0.29± 0.09 | 0.19± 0.04 | 0.20± 0.02 | 0.20± 0.03 | 0.17± 0.05 |
| Heart | | | | | |
| Wt, g | 0.84± 0.10 | 0.80± 0.06 | 0.80± 0.05 | 0.83± 0.10 | 0.82± 0.09 |
| % of body wt | 0.34± 0.03 | 0.37± 0.04 | 0.33± 0.03 | 0.35± 0.04 | 0.38± 0.03 |
| Kidney | | | | | |
| Wt, g | 1.44± 0.13 | 1.50± 0.09 | 1.46± 0.09 | 1.63± 0.10 | 1.49± 0.25 |
| % of body wt | 0.59± 0.03 | 0.68± 0.05 | 0.58± 0.04 | 0.69± 0.05 | 0.69± 0.07 |
| Brain | | | | | |
| Wt, g | 1.79± 0.09 | 1.79± 0.08 | 1.76± 0.12 | 1.80± 0.27 | 1.87± 0.29 |
| % of body wt | 0.74± 0.03 | 0.82± 0.07 | 0.70± 0.05 | 0.76± 0.05 | 0.87± 0.15 |

[1] SD.

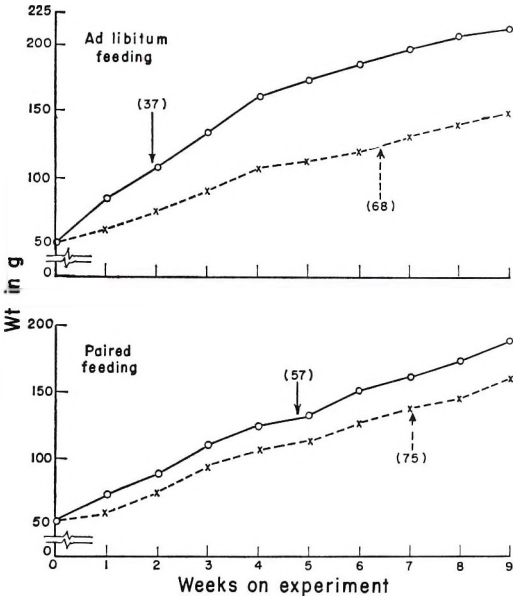


Fig. 1 Growth curves of female rats fed diets with and without *Sterculia foetida* oil by ad libitum and paired-feeding. Average age of rats at time vagina opened is indicated by arrows. ○———○, 4% control oil diet; ×-----×, 1% control + 3% *S. foetida* oil diet.

vious day. In the pair-fed comparisons of the time of opening of the vagina, the average age of the control group was 57 days with a range of 43 to 86 days and of the group fed 1% safflower — 3% *S. foetida* oil, 75 days with a range of 53 to 101 days. At the time the vagina opened the average body weight was 129 g for the control group and 139 g for the 1% safflower — 3% *S. foetida* oil group. In both the pair-fed and the ad libitum-fed experiments the age at the time the vagina opened was significantly greater for the 1% safflower — 3% *S. foetida* oil group than the control group, but the body weight at that time was not significantly different in either case and, therefore, rate of growth, to the extent that it was measured here, appeared not to be a factor in sexual maturity of the female rat.

There was greater variation and a definite increase in length of the estrous cycle of the rats fed *S. foetida* oil (table 4). The sequence in days of the consecutive estrous cycles of one rat chosen at random from each group on the pair-fed experiment is shown in table 5. Similar results were observed for ad libitum feeding. Both

TABLE 4

*Comparison of sexual maturation of female rats pair-fed oil diets with and without Sterculia foetida oil for 20 weeks*

| Oil in diet | Mean age at time vagina opened | Total no. estrous cycles | Mean length of estrous cycles | % of 4 to 6-day estrous cycles |
|---|---|---|---|---|
| | *days* | | *days* | |
| 4% Corn | 55 | 22 | 5.0 | 93 |
| 1% Corn + 3% *S. foetida* | 74 | 12 | 7.0 | 61 |
| 4% Safflower | 60 | 20 | 5.4 | 89 |
| 2% Safflower + 2% *S. foetida* | 68 | 18 | 5.6 | 78 |
| 1% Safflower + 3% *S. foetida* | 77 | 13 | 7.6 | 51 |

TABLE 5

*Comparison of number and length of consecutive estrous cycles of typical individual female rats pair-fed oil diets with and without Sterculia foetida for 20 weeks*

| Oil in diet | Length of estrous cycles | No. of cycles |
|---|---|---|
| | *days* | |
| 4% Corn | 5–5–5–5–5–5–5–5–5–5–5–4–9–4 | 15 |
| 1% Corn + 3% *S. foetida* | 14–7–11–16–5–4–6 | 7 |
| 4% Safflower | 6–5–6–5–5–5–5–5–5–5–5–4–5–5–5–5 | 17 |
| 2% Safflower + 2% *S. foetida* | 12–5–5–5–5–5–14–5–4 | 9 |
| 1% Safflower + 3% *S. foetida* | 13–8–8–4 | 4 |

the 4% corn oil and 4% safflower oil groups showed uniformly regular consecutive estrous cycles of 5 days. This agrees with the report of Long and Evans (10) who noted an average cycle length of 5 days for normal albino rats. When 2% safflower oil — 2% *S. foetida* oil was fed the cycles were slightly longer and tended to be irregular. When 1% safflower oil— 3% *S. foetida* oil was fed, the average cycle length was 7.6 days and very irregular. Therefore 3% *S. foetida* oil in the diet, which supplied approximately 105 mg of sterculic acid per rat per day (based on 10 g of feed intake daily), definitely delayed sexual maturity of the female rat as determined by the criteria of age at the time the vagina opened and the regularity and length of consecutive estrous cycles.

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# Magnesium Requirement of the Baby Pig [1,2]

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*ABSTRACT* The magnesium requirement of baby pigs receiving a purified casein-glucose diet containing 0.8% of Ca, 0.6% of P and 1800 IU of vitamin $D_3$/kg was studied in two 5-week trials using levels of dietary Mg from 25 to 825 ppm. Pigs receiving less than 125 ppm of Mg exhibited poor growth, poor food utilization, weakened pasterns, stepping syndrome, tetany and death. Pigs receiving 125 ppm of Mg exhibited all of these symptoms except tetany and death. Pigs receiving 225 ppm of Mg or more exhibited no overt symptoms of magnesium deficiency. Serum and bone Mg concentrations were reduced in all pigs receiving 225 ppm or less of dietary Mg. Values of serum Ca and Mg concentration were significantly related. Values of serum and bone Mg concentration were also positively related. A dietary Mg level of 325 ppm was adequate to meet all criteria and under the conditions of this study is judged to be the minimal magnesium requirement of the baby pig.

Studies by Mayo et al. (1) with pigs weaned at 3 and 9 weeks of age indicated magnesium requirements for maximal growth rate of 334 and 241 ppm, respectively, and 400 and 505 ppm, respectively, for the prevention of deficiency symptoms. On the basis of balance studies with pigs 3 to 5 weeks of age, Bartley et al. (2) recommended a dietary magnesium concentration of 400 ppm. Estimated magnesium requirements of 400 and 505 ppm are listed by the NRC (3) and Lucas and Lodge (4), respectively, for pigs weighing over 4.5 kg. Studies have not been reported of determinations of the magnesium requirement of pigs weaned prior to 3 weeks of age.

The present studies were conducted to determine the magnesium requirement of baby pigs weaned at one day of age and reared with a purified diet. Criteria of dietary magnesium adequacy were similar to those used in previously reported studies of mineral requirements (5–7).

## MATERIALS AND METHODS

Two trials were conducted using 36 Yorkshire-Hampshire crossbred pigs of either sex. Pigs were taken from the sow during the first day of life and reared in individual metal cages. Adjustment of the pigs to a purified diet in dry meal form was facilitated by means described earlier (7) and pigs were assigned to experimental levels of dietary magnesium at one week of age. The purified diet was very similar to that used in earlier studies (7) and distilled water was measured and supplied ad libitum as the only source of drinking water. This seemed advisable because the magnesium concentration in tap water at our laboratory is about 25 ppm and the distilled water contained less than 1 ppm. The purified diet consisted of 30% of casein,[3] 5% of lard, 5% of cellulose,[4] 54% of glucose,[5] 6% of minerals (table 1) and vitamins (8). During the final 2 weeks of each of the 5-week trials, casein was reduced to 20% of the diet and glucose was increased to 64% of the diet. Dietary calcium and phosphorus levels were maintained at 0.8 and 0.6%, respectively, by adjusting the mineral mixture ingredients as shown in table 1. The basal purified diet contained 25 ppm of magnesium and supplemental levels of zero, 100, 200 and 400 ppm of magnesium were supplied in the first trial and 50, 200, 300, 400 and 800 ppm of added magnesium in the second trial. All supple-

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[2] Presented in part before the meeting of the American Society of Animal Science, Chicago, November, 1962 (J. Animal Sci., *21*: 1006, 1962, abstract).

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

[4] Solka Floc, Brown Company, Berlin, New Hampshire.

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

TABLE 1

*Composition of basal mineral mixture*

| | % |
|---|---|
| KCl (0.002% I) | 10.0 |
| KI | 0.002 |
| $FeSO_4 \cdot 2H_2O$ | 0.7 |
| $CuSO_4$ | 0.1 |
| $CoCO_3$ | 0.1 |
| $MnSO_4 \cdot H_2O$ | 0.2 |
| $ZnSO_4 \cdot H_2O$ | 0.4 |
| $NaHCO_3$ | 25.0 |
| $CaCO_3$ | 12.3( 8,1) [1] |
| $CaHPO_4 \cdot 2H_2O$ | 36.2(42.0) [1] |
| $MgSO_4 \cdot 7H_2O$ | 0.0 |
| Glucose [2] | 15.0(13.4) [1] |

[1] Changed to this value during final 2 weeks of each trial.
[2] Cerelose, Corn Products Company, Argo, Illinois.

mental magnesium was supplied by USP grade magnesium sulfate in the mineral mixture to supply the appropriate dietary level.

All animals were weighed weekly. Blood was withdrawn from the anterior vena cava on 3 occasions (initial, 3 weeks and final) during either trial for the determination of levels of serum calcium, phosphorus, and magnesium by the methods of Mori (9), Gomori (10), and Orange and Rhein (11), respectively. Serum alkaline phosphatase concentration was determined in the first trial by the method of Bessey et al. (12). Pigs were killed at the end of each trial and determinations of bone composition and strength were made by methods used in previous studies (5–7). Bone magnesium concentration was determined gravimetrically as magnesium pyrophosphate as described by Kolthoff and Sandell (13). Statistical analyses of data were performed with application of the multiple range test of Duncan (14).

## RESULTS AND DISCUSSION

*Trial 1.* Growth rate and food consumption of all pigs receiving 100 ppm or less of supplemental magnesium were depressed within one week after the initiation of the trial. Pigs receiving the basal diet containing 25 ppm of magnesium began exhibiting the stepping syndrome described by Mayo (1) during the second week of the trial. Two of these pigs went into tetany at the end of the second week and died. The 2 remaining pigs died in tetany during the fourth week. Pigs receiving 125 ppm of dietary magnesium began exhibiting the stepping syndrome (fig. 1) during the third week of the trial and this neuromuscular anomaly persisted in these pigs throughout the remainder of the trial. One of these pigs went into tetany but recovered and survived to the end of the trial. All of the pigs receiving 125 ppm of dietary magnesium had extremely weakened or relaxed carpo-meta-

Fig. 1 Magnesium-deficient pig exhibiting the stepping syndrome. Pig stepped almost continuously while standing. Weakness of pasterns is apparent. This pig, from trial 2, received a purified diet containing 0.8% Ca, 0.6% P, 1800 IU of vitamin $D_3$/kg and 75 ppm of Mg.

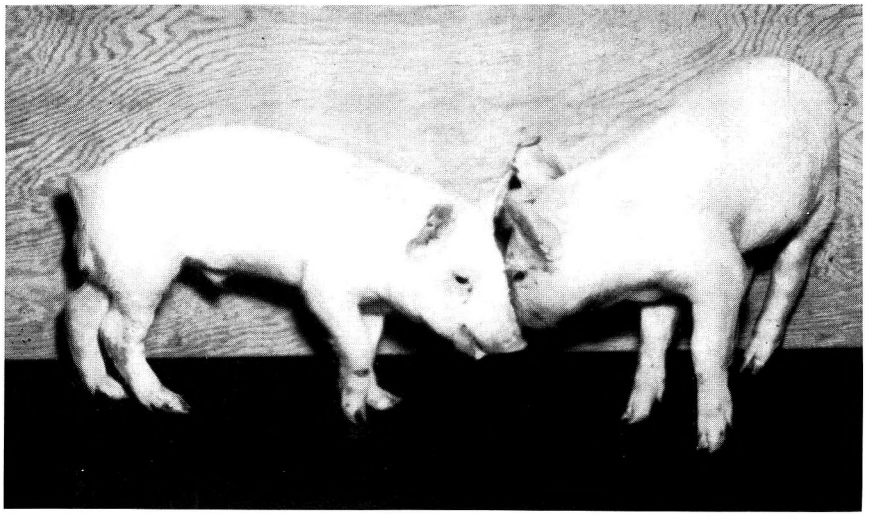

Fig. 2 Magnesium-deficient pig on left with littermate receiving 325 ppm of Mg on right. Compare the extreme pastern weakness of the pig on the left with the firmly extended phalanges of the pig on the right.

carpo - phalangeal and tarso - metatarso-phalangeal joints causing the phalanges to be hyperextended (figs. 1 and 2). This has been observed in dogs receiving a diet low in magnesium (15). Pigs receiving 125 ppm of dietary magnesium consumed a greater quantity of water relative to their food intake than the other pigs (table 2). Pigs receiving 225 ppm or more of dietary magnesium did not exhibit any overt abnormal symptoms. Rates of growth and dietary intake of pigs receiving 225 ppm of dietary magnesium were normal and did not differ significantly from those of pigs receiving 425 ppm of dietary magnesium (table 2).

Serum calcium level was decreased after 3 weeks of the trial in pigs receiving less than 225 ppm of dietary magnesium. At the end of the trial, pigs receiving 225 ppm or less had significantly lower serum calcium levels than the animals receiving 425 ppm. Serum inorganic phosphorus level was not significantly altered by level of dietary magnesium.

Serum magnesium concentration decreased precipitously in pigs receiving no supplemental magnesium. Serum magnesium concentration also decreased very rapidly in pigs receiving dietary magnesium levels of 125 or 225 ppm. The stepping syndrome appeared when the serum magnesium concentration fell to about 1.0 mg/100 ml. When serum magnesium level fell to near 0.5 mg/100 ml, extended tetany occurred. The one pig receiving 125 ppm of dietary magnesium which exhibited tetany was given a 10-ml intraperitoneal injection of a glucose-saline solution containing 60 mg of magnesium. This pig quickly recovered from tetany and did not exhibit tetany again, although the stepping syndrome reappeared within a few days and persisted for the remainder of the trial. Serum magnesium concentration of pigs receiving 225 ppm of dietary magnesium was significantly lower than that of pigs receiving 425 ppm of dietary magnesium after 3 weeks of the trial but did not diminish sufficiently to initiate the stepping syndrome, tetany or growth depression during the trial. Serum magnesium levels of pigs receiving 425 ppm of dietary magnesium ranged between 2 and 3 mg/100 ml at the end of the trial. This appears to be a normal level for pigs of this age (2, 7), calves (16, 17), infants (18) and older human subjects (19), chicks (20), lambs (21), and rats (22). Normal values are somewhat lower for the dog (23, 24) and are somewhat higher for rabbits (25) and guinea pigs (26).

TABLE 2

*Growth, serum analyses and skeletal development of baby pigs fed at different levels of magnesium (trial 1)*

| | Dietary Mg level, ppm | | | |
|---|---|---|---|---|
| | 25 | 125 | 225 | 425 |
| No. of pigs | 4 | 4 | 4 | 4 |
| Initial weight, kg | 2.0 ±0.1 [1] | 2.0 ±0.1 | 2.1 ±0.1 | 2.0 ±0.1 |
| Daily gain, kg | 0.10±0.01 | 0.15±0.02 | 0.26±0.03[bb] | 0.31±0.01[bb] |
| Daily food intake, kg | 0.19±0.01 | 0.24±0.01 | 0.36±0.01[bb] | 0.40±0.01[bb] |
| Gain/food | 0.54±0.05 | 0.62±0.03 | 0.73±0.03[aa] | 0.78±0.03[bb] |
| Daily water intake, kg | 0.63±0.02 | 0.95±0.02[aa] | 0.91±0.02[aa] | 1.04±0.02[aa,b] |
| Water/food | 3.3 ±0.1 [b] | 4.0 ±0.1[bb,c] | 2.5 ±0.1 | 2.6 ±0.1 |
| Mortality | 100 | 0 | 0 | 0 |
| Serum Ca, mg/100 ml | | | | |
| Initial | 10.8 ±0.4 | 11.0 ±0.3 | 10.5 ±0.3 | 11.0 ±0.5 |
| 3 weeks | 9.0 ±0.1 | 9.4 ±0.3 | 11.4 ±0.1 | 11.9 ±0.2[a] |
| 5 weeks | — | 8.9 ±0.5 | 10.6 ±0.2[a] | 12.2 ±0.2[aa,b] |
| Serum inorganic P, mg/100 ml | | | | |
| Initial | 9.0 ±0.4 | 9.4 ±0.7 | 9.0 ±0.5 | 9.4 ±0.1 |
| 3 weeks | 9.5 ±0.7 | 8.5 ±0.4 | 7.9 ±0.3 | 8.9 ±0.3 |
| 5 weeks | — | 7.9 ±0.6 | 7.6 ±0.3 | 8.2 ±0.4 |
| Serum Mg, mg/100 ml | | | | |
| Initial | 2.8 ±0.5 | 2.8 ±0.5 | 3.0 ±0.5 | 3.2 ±0.5 |
| 3 weeks | 0.8 ±0.3 | 1.5 ±0.2 | 1.7 ±0.1 | 3.2 ±0.6[aa,c] |
| 5 weeks | — | 0.7 ±0.2 | 1.4 ±0.1[aa] | 2.3 ±0.1[bb] |
| Serum alkaline phosphatase, Bessey-Lowry units | | | | |
| Initial | 14.8 ±0.9 | 18.8 ±2.8 | 16.0 ±2.2 | 16.8 ±0.4 |
| 3 weeks | 8.9 ±0.2 | 14.0 ±2.0 | 10.0 ±0.7 | 10.2 ±0.1 |
| 5 weeks | — | 9.3 ±1.4 | 7.3 ±0.5 | 8.4 ±0.9 |
| 8th rib analyses (dry, fat-free basis) | | | | |
| Ash, % | 51.6 ±1.0 | 51.9 ±1.2 | 52.7 ±1.0 | 52.0 ±0.5 |
| Ca, % | 18.8 ±0.3 | 19.2 ±0.6 | 19.1 ±0.3 | 19.0 ±0.1 |
| P, % | 9.6 ±0.1 | 9.6 ±0.1 | 9.9 ±0.1 | 9.8 ±0.1 |
| Mg, % | 0.21±0.06 | 0.18±0.03 | 0.34±0.03[b] | 0.59±0.03[cc] |
| Femur strength | | | | |
| Breaking load, kg | — | 94±8 | 108±2 | 115±10 |
| Bending moment, kg-cm | — | 173±28 | 213±14 | 239±7 |
| Moment of inertia, $cm^4$ | — | 0.23±0.05 | 0.15±0.03 | 0.23±0.05 |
| Breaking stress, $kg/cm^2$ | — | 580±2 | 1100±93 | 850±160 |
| Young's modulus of elasticity, 1000 $kg/cm^2$ | — | 20.0 ±1.4 | 113.0 ±5.0[aa,b] | 61.3 ±4.1 |

[1] Mean ± SE.
[a] Significantly greater than least value ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly greater than least 2 values ($P < 0.05$); [bb] $P < 0.01$.
[c] Significantly greater than least 3 values ($P < 0.05$); [cc] $P < 0.01$.

There was a significant positive relationship between serum magnesium and calcium concentrations at the end of the trial in the present study. Correlation coefficients were 0.39, 0.36 and 0.88 ($P < 0.01$) for these values from initial, 3-week, and 5-week measures, respectively. Apparently, the relationship of serum calcium and serum magnesium concentration is positive but low in animals receiving adequate dietary magnesium levels, but the positive relationship becomes high when serum magnesium levels are depressed. In young animals skeletal magnesium can be mobilized for soft tissue growth during magnesium deficiency and calcium ions apparently replace magnesium ions in bone (27). Probably the calcium ions for this replacement have come from the extracellular fluid and perhaps this was responsible for the concurrently reduced serum calcium level.

The serum alkaline phosphatase level becomes elevated in baby pigs with dietary deficiencies of calcium (5), phosphorus (6) or vitamin D (7). Data in table 2 indicate that magnesium deficiency does not significantly influence serum alkaline phosphatase activity in the baby pig. Griffith et al. (28) also observed no influence of dietary magnesium upon plasma alkaline phosphatase activity in the chick.

Ash analyses of ribs from pigs at the end of the trial indicate no significant effect of dietary magnesium upon the percentage of ash, calcium or phosphorus. There was, however, a significant effect of dietary magnesium upon rib magnesium concentration. Rib magnesium concentrations were significantly related to final serum magnesium concentrations with a correlation coefficient of 0.94 ($P < 0.01$) and the equation of the line of regression of rib magnesium concentration (%) on serum magnesium concentration (mg/100 ml) was $Y = 0.062 + 0.206\,X$. Similar relationships of bone and serum magnesium concentrations have been observed in calves (17) and rats (29).

Measurements of strength of femurs from pigs receiving 125, 225 or 425 ppm of dietary magnesium did not indicate any significant effect of dietary magnesium except upon elasticity. Femurs from pigs receiving dietary magnesium at the 2 higher levels exhibited greater elasticity. There was also a statistically non-significant trend toward greater breaking load, bending moment and breaking stress values for femurs from these pigs.

No gross pathological lesions were observed at necropsy in pigs receiving 225 ppm or more of dietary magnesium. Vascular dilation and hemorrhage in the thoracic cavity, as well as rectal and colonic hemorrhage, was evident in pigs which died in tetany. There was excess pericardial fluid with some adhesions between the pericardium and the heart. There were several single fractures of ribs in these pigs, which may have occurred during the tetanic convulsions. Mineral deposits were observed in the lumenal wall of the dorsal aorta of pigs receiving 125 ppm of dietary magnesium. This was similar to that observed in magnesium-deficient dogs (15). Analysis of sections of the dorsal aorta indicated an eightfold increase in calcium concentration above that measured in similar tissues from pigs receiving 225 or 425 ppm of dietary magnesium. Similar observations have been reported for other experimental animals (15, 30, 31).

Judging from the information obtained from trial 1, it can be concluded that 225 ppm of dietary magnesium is adequate to prevent gross pathologic lesions, to achieve near optimal rates of body weight gains and economy of food utilization and to support optimal rate of skeletal development. However, it can also be concluded that this level of dietary magnesium does not support optimal levels of serum calcium and magnesium and bone magnesium concentration. A dietary magnesium level of 425 ppm is adequate to meet all of these criteria.

*Trial 2.* Results of the second trial are presented in table 3. Three of the pigs receiving 75 ppm of dietary magnesium exhibited tetany and died during the trial. The remaining pig did not exhibit tetany and survived to the end of the trial but exhibited the stepping syndrome during the final week of the trial. As in the first trial, no overt deficiency symptoms were observed in any of the pigs receiving 225 ppm or more of dietary magnesium. Likewise, rate and efficiency of gain were not significantly improved by increasing the dietary magnesium concentration beyond 225 ppm.

Serum calcium and magnesium levels decreased rapidly in pigs receiving 75 ppm of dietary magnesium. Serum magnesium but not serum calcium concentrations were significantly decreased at the end of the study in pigs receiving 225 ppm of dietary magnesium. Normal values of serum magnesium concentration were present in all pigs receiving 325 ppm or more of dietary magnesium. Again, serum phosphorus levels were not significantly affected by dietary magnesium levels. Values of simultaneous concentrations of serum calcium and magnesium were significantly related with correlation coefficients of 0.49 ($P < 0.05$), 0.84 ($P < 0.01$) and 0.71 ($P < 0.01$) for initial, 3-week, and 5-week measures, respectively.

TABLE 3

*Growth, serum analyses and skeletal development of baby pigs fed at different levels of magnesium (trial 2)*

| | Dietary Mg level, ppm | | | | |
|---|---|---|---|---|---|
| | 75 | 225 | 325 | 425 | 825 |
| No. of pigs | 4 | 4 | 4 | 4 | 4 |
| Initial weight, kg | 2.7 ±0.5 | 2.7 ±0.2 | 2.8 ±0.4 | 2.8 ±0.5 | 3.0 ±0.3 |
| Daily gain, kg | 0.14 [1] | 0.26±0.01[aa] | 0.26±0.01[aa] | 0.29±0.01[aa] | 0.26±0.01[aa] |
| Daily food intake, kg | 0.36 [1] | 0.40±0.01[a] | 0.40±0.01[a] | 0.41±0.01[a] | 0.41±0.01[a] |
| Gain/food | 0.38 [1] | 0.64±0.01[aa] | 0.65±0.02[aa] | 0.71±0.01[aa,b] | 0.63±0.02[aa] |
| Mortality, % | 75 | 0 | 0 | 0 | 0 |
| Serum Ca, mg/100 ml | | | | | |
| Initial | 11.0 ±0.3 | 11.8 ±1.2 | 11.1 ±0.1 | 10.8 ±0.2 | 11.1 ±0.1 |
| 3 weeks | 7.4 ±0.7 | 11.2 ±0.3[aa] | 11.4 ±0.3[aa] | 11.2 ±0.3[aa] | 12.0 ±0.4[aa] |
| 5 weeks | 7.6 [1] | 10.7 ±0.3[aa] | 11.0 ±0.2[aa] | 10.8 ±0.2[aa] | 11.1 ±0.2[aa] |
| Serum inorganic P, mg/100 ml | | | | | |
| Initial | 8.5 ±0.5 | 7.8 ±0.9 | 7.6 ±0.8 | 7.2 ±0.3 | 7.8 ±0.9 |
| 3 weeks | 10.3 ±1.1 | 9.4 ±0.4 | 9.8 ±0.4 | 8.6 ±0.8 | 9.6 ±0.8 |
| 5 weeks | 10.0 [1] | 8.3 ±0.4 | 9.1 ±0.4 | 9.1 ±0.1 | 9.2 ±0.2 |
| Serum Mg, mg/100 ml | | | | | |
| Initial | 2.3 ±0.2 | 2.6 ±0.2 | 2.3 ±0.3 | 2.4 ±0.3 | 2.1 ±0.3 |
| 3 weeks | 1.2 ±0.2 | 2.2 ±0.1[aa] | 2.6 ±0.1[aa] | 2.9 ±0.3[aa,b] | 2.7 ±0.1[aa] |
| 5 weeks | 0.5 [1] | 1.3 ±0.2[aa] | 2.0 ±0.1[bb] | 2.2 ±0.2[bb] | 2.3 ±0.1[bb] |
| Humeral analyses (dry, fat-free basis) | | | | | |
| Ash, % | 46.4 ±0.8 | 50.0 ±0.2[a] | 48.4 ±0.3 | 47.5 ±0.3 | 47.8 ±1.3 |
| Ca, % | 16.6 ±0.4 | 17.8 ±0.3[a] | 17.3 ±0.1 | 16.8 ±0.2 | 16.7 ±0.3 |
| P, % | 8.5 ±0.1 | 9.3 ±0.1 | 9.1 ±0.1 | 8.9 ±0.2 | 9.0 ±0.2 |
| Mg, % | 0.21±0.01 | 0.33±0.06 | 0.50±0.08[aa,b] | 0.42±0.04[aa] | 0.42±0.02[aa] |
| Specific gravity | | | | | |
| Femur | 1.20±0.0 | 1.21±0.1 | 1.19±0.1 | 1.19±0.1 | 1.19±0.1 |
| Humerus | 1.20±0.1 | 1.24±0.1 | 1.22±0.1 | 1.20±0.1 | 1.22±0.1 |
| Femur strength | | | | | |
| Breaking load, kg | 78±12 | 104±10 | 119±9[a] | 115±7[a] | 98±10 |
| Bending moment, kg-cm | 162±30 | 222±19 | 258±22[a] | 250±16[a] | 206±22 |
| Moment of inertia, $cm^4$ | 0.19±0.03 | 0.19±0.02 | 0.15±0.01 | 0.18±0.01 | 0.13±0.01 |
| Breaking stress, $kg/cm^2$ | 630±54 | 850±29 | 1230±127[a] | 1020±63 | 1050±100 |
| Young's modulus of elasticity, 1000 $kg/cm^2$ | 18.3 ±2.5 | 31.2 ±2.4 | 42.2 ±2.3[a] | 32.8 ±2.2 | 42.7 ±5.0[a] |

[1] Value for one 75 ppm pig surviving to end of trial. Skeletal analyses include values on bones from this pig and another which died near end of trial.
[a] Significantly greater than least value ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly greater than least 2 values ($P < 0.05$); [bb] $P < 0.01$.

Humeral analyses showed slightly reduced ash and calcium values for pigs which were severely magnesium-deficient. On the other hand, slightly elevated ash and calcium values were noted in the mildly deficient pigs receiving 225 ppm of dietary magnesium. Humeral magnesium concentrations were reduced in both the severely and mildly deficient pigs. Since magnesium is withdrawn from bone in an attempt to maintain plasma magnesium level, then if magnesium ions are replaced by calcium ions in bone, this may account for the slightly elevated bone calcium in the mildly deficient pigs. Values for specific gravity of femur and humerus paralleled the values of humeral ash analysis. Dietary magnesium level did not significantly alter values of bone specific gravity. Strength of femurs was reduced in pigs receiving 75 ppm of dietary magnesium; however, the reduced strength measures could be largely accounted for by the reduced weight of femurs in the severely deficient pigs. Bone magnesium concentrations were positively related to final serum magnesium concentrations in this trial also. The correlation coefficient was 0.70 ($P < 0.01$) and the regression equation was $Y = 0.128 + 0.143\ X$.

The results of trial 2 substantiated the conclusions of trial 1. A dietary magnesium level of 225 ppm was adequate to meet most of the criteria; however, this dietary level did not support normal levels of serum and bone magnesium concentration. A dietary magnesium level of 325 ppm was adequate to meet all of the criteria. This level is similar to that calculated by Bartley et al. (2) to provide a retention of magnesium needed to maintain normal tissue levels of magnesium. The higher requirement value obtained by Mayo et al. (1) may be due to source of dietary protein. Chicks have a somewhat higher dietary magnesium requirement when isolated soybean protein is the dietary protein source than when casein is used (32). The magnesium concentration in sow's milk is 0.012 to 0.022% (33–35). This constitutes a concentration of 600 to 1100 ppm in the dry matter. Hence, there appears to be little possibility of magnesium deficiency in nursing pigs receiving adequate milk from their dam for normal growth rate.

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# Effect of Thiamine Deficiency and Thiamine Repletion on Neutral Glyceride, Total and Free Cholsterol, Phospholipids, and Plasmalogens in Rat Liver [1]

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*ABSTRACT* Intraperitoneal injection of 1 mg of thiamine in severely thiamine-deficient rats is followed by a 3- and 20-fold elevation of liver total cholesterol and neutral glycerides, respectively. These elevations continue up to 8 and 20 times normal after a second thiamine injection.

Phospholipid levels per gram of liver or per 100 g of body weight fall to below normal during development of thiamine deficiency. Little change in plasmalogens occurred, however, when expressed in either manner although there was a tendency for them to fall slightly below the ad libitum-fed control level. After a single thiamine injection, phospholipids tend to return to normal, whereas plasmalogens increase to far above normal levels. A second injection of thiamine decreases phospholipids to below normal, which then return nearly to normal. Plasmalogens return nearly to normal without fluctuating after a second thiamine injection.

During thiamine deficiency the ratio of free to total cholesterol is significantly higher than normal, suggesting either a breakdown in the cholesterol esterification system or a severe deficiency of fatty acids available for esterification. After thiamine repletion the ratio rapidly falls to well below normal.

After thiamine repletion fine droplet lipidosis and a striking increase in glycogen in the portal areas precedes the massive paracentral fatty change which is reflected biochemically. When massive fatty deposition occurs, stainable glycogen decreases. Cellular regenerative activity after thiamine repletion is slight, indicating that the changes observed with thiamine repletion are unassociated with cell destruction and subsequent cell proliferation.

Although oral or parenteral administration of thiamine generally produced similar effects on liver lipids, the magnitude of the effects on total cholesterol and plasmalogens was much greater with parentral administration.

In an earlier paper in which the effect of thiamine deficiency on rat liver lipids was presented (1), total lipid, phospholipid, and plasmalogen levels were followed throughout the development of the deficiency and for 48 hours after thiamine repletion by intraperitoneal injection of the vitamin. Total lipids and phospholipids were lost from the liver as the deficiency progressed. Plasmalogens, however, were not greatly affected by the deficiency. Total cholesterol was higher after 40 days of depletion than in the controls. It was also shown that between 4 and 48 hours after repletion of thiamine, total lipids rebounded to above normal levels, whereas phospholipids and plasmalogens appeared simply to return to normal. Cholesterol did not return to normal during this period, but rather increased even further above normal.

The purpose of this report is to present a detailed study of the histological changes and the changes in the various lipid fractions both during development of the thiamine deficiency and particularly after intraperitoneal injections of thiamine in the deficient rats. This study of the lipid changes occurring in the liver after thiamine repletion in deficient rats was con-

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sidered to be important since in the earlier study (1) data were not obtained more than 48 hours after repletion. The very unusual appearance of the livers at this time (light tan color, greatly enlarged, and friable) indicated that the changes taking place were incomplete after 48 hours.

## EXPERIMENTAL

Male, weanling rats of the Sprague-Dawley strain were divided into 2 groups One group of 29 rats received ad libitum a casein-sucrose-corn oil ration (20:69:5) supplemented with the minerals and vitamins known to be essential for the rat (1). A comparable group of 88 rats received the same diet from which thiamine was omitted. Twelve animals were pair-fed with the rats receiving the thiamine-deficient diet (average daily food consumption). At zero time and at intervals thereafter rats from each group were decapitated. The livers were removed and 3 portions were fixed for histological study: the first in buffered (pH 7.0) 10% formalin, the second in absolute ethanol, and the third in osmium tetroxide (2). Frozen sections, as well as sections from paraffin and methacrylate-imbedded tissue were examined by a variety of techniques and special stains. Lipids were extracted from the remaining liver with alcohol-petroleum ether by the method of Rice et al. (3). Total lipid, total cholesterol, phospholipids, and plasmalogens were determined as previously described (1). Neutral glycerides were calculated as tripalmitin according to the following formula:

$$\mu\text{moles Neutral glyceride} = [\mu\text{g Total lipid} - (\mu\text{moles Phospholipid} \times 761) - (\mu\text{moles Total cholesterol} \times 387)] \div 807.$$

Free cholesterol was precipitated as the digitonide according to the method of Schoenheimer and Sperry (4) prior to determination.

When thiamine deficiency was well established (35 days), 64 of the deficient rats were given 1 mg of thiamine hydrochloride by intraperitoneal injection. Another group of 12 deficient rats was given 1 mg of thiamine by gastric tube to determine whether a different route of administration of thiamine produced any variation in the results. At the time of thiamine administration, 10 rats were killed and groups of 10 animals were killed at 24-hour intervals for up to 72 hours. At 72 hours 20 of the remaining repleted rats were given a second 1-mg thiamine injection and groups of 5 animals were killed at 24 hour intervals for the next 96 hours. A group of 4 rats was, however, not given the second thiamine injection and was killed 96 hours after initiation of thiamine repletion. Of the animals given thiamine orally, groups of 5 rats were killed at 48 hours and 96 hours after administration, and finally 2 animals were killed at 144 hours.

## RESULTS

*Histological studies.* Histological examination of livers from thiamine-deficient rats 7 days before repletion and from rats at the time of thiamine injection (each group of 5 rats showing, respectively, 18.9 mg and 21.1 mg total lipid/g liver) revealed negligible lipid stainable by Flaming red. Animals fed the complete purified diet for a similar period (6 rats at the time of thiamine injection to deficient animals, with 27.6 mg total lipid/g liver) showed a trace-to-slight lipidosis of the small droplet variety. The distribution of this lipid was distinctly portal. By 24 hours after thiamine injection (5 rats showing 23.7 mg total lipid/g liver) fine droplets of neutral lipid were observed to accumulate in the portal areas of the liver lobules. This was associated with a striking increase in glycogen in this area as shown by Best's Carmine stain. The pattern observed at 48 hours (5 rats with 63.2 mg total lipid/g liver) was strikingly different in that the lipid droplets were of the large variety and clustered about central veins. A concomitant decrease in Best's Carmine stainable glycogen was noted. This pattern progressed until the lobules were diffusely involved by large-type lipid vacuoles at 96 hours (4 rats showing 65.1 mg total lipid/g liver). At this point the fine-droplet portal pattern was almost completely obscured. Periodic acid Schiff (PAS)-positive material, which as far as we know, consists of water-insoluble glycolipids and glycoproteins under the conditions of fixation and staining, showed no consistent change other

than that ascribed to associated lipid vacuolation. There was a progressive loss of cytoplasmic argyrophilia and an increase in nuclear argyrophilia in the animals given the thiamine supplement, indicating changes in structural proteins. These changes became more marked in the thiamine-injected group than in the group given the oral supplement. Cellular regenerative activity as reflected in terms of binucleation, hyperchromatism, and hypertrophy was inconspicuous.

*Chemical studies.* The results are presented in figures 1 to 6. Lipid components were calculated on 2 bases: per gram of fresh liver and per 100 g body weight. The latter basis allows adjustment of the lipid component to equivalent body weight since during the experiment the average body weight of the deficient group was much lower than that of the controls. To indicate the significance of differences observed in the results, values of $P$ based on the $t$ test are included.

*Neutral glycerides.* Neutral glycerides (fig. 1) decreased during development of the thiamine deficiency. Although not apparent because of the scale of figure 1, the difference is significant ($P < 0.05$). Neutral glyceride quantitatively changes with total lipid. After thiamine administration, the bulk of lipid is neutral glyceride rather than the phospholipid-cholesterol combination as in livers of the normal and the thiamine-deficient rat. After a single intraperitoneal injection of thiamine the neutral glyceride increased to about 5 times normal (per 100 g rat) in 48 hours ($P < 0.001$ for either per gram of liver or per 100 g rat). Omitting a second thiamine injection at 72 hours, there was a tendency for total lipid to drop by 96 hours to the low level of the controls. However, a second thiamine injection at 72 hours was followed by a large increase in neutral glyceride to a value about 22 times normal at 144 hours. As early as 24 hours after the initial injection the livers were frequently greyish pink and friable as compared with the red resilient livers of control and non-injected thiamine-deficient rats.

*Cholesterol.* Total cholesterol concentration in the livers of thiamine-deficient rats closely paralleled that in the control animals during the first 35 days of the ex-

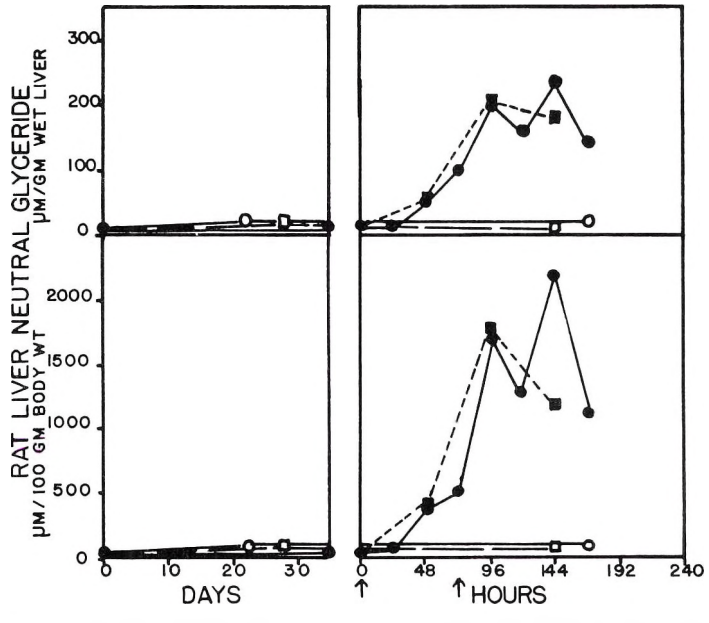


Fig. 1 Changes in neutral glyceride in control and thiamine-deficient rat liver. Thiamine given deficient rats ↑ ; injections on thirty-fifth and thirty-eighth day ●——; oral administration ■ – – –. Pair-fed control □ – – –; ad libitum control ○——.

periment (fig. 2). After injection of thiamine there was an almost immediate increase in cholesterol when expressed either in terms of 100 g body weight or per gram of wet liver ($P < 0.01$ at 72 hours). There was a slight lag when expressed per gram of wet liver. A second thiamine injection 72 hours after the initial repletion produced an even greater increase followed by fluctuations at a high level. Oral administration of thiamine was followed by only a moderate increase in liver cholesterol.

Free cholesterol per gram of liver tended to increase in the livers of thiamine-deficient rats as compared with that in animals receiving thiamine (fig. 3). After the first injection of thiamine there was an increase in free cholesterol/100 g body weight ($P < 0.01$). The second thiamine injection at 72 hours was followed by a further increase at 144 hours. In contrast, expressed per gram of wet liver, there was a moderate decrease in free cholesterol during the post-injection period ($P < 0.05$ at 48 hours). By 72 hours, free cholesterol per gram of liver began to increase but again was immediately depressed by the second thiamine injection. By the third day after the second injection (144 hours) free cholesterol per gram of liver started to increase and continued to do so for the next 24 hours although a significant difference from the control value could not be proved for this point. However, even after 7 days of thiamine repletion both free and total cholesterol were still significantly higher than normal when based on body weight ($P < 0.05$ and $P < 0.001$, respectively).

Examination of figure 4 reveals an initial elevation of the free-to-total cholesterol ratio in the liver during development of thiamine deficiency. The initial thiamine administration was followed by a large decrease in the ratio. After the second thia-

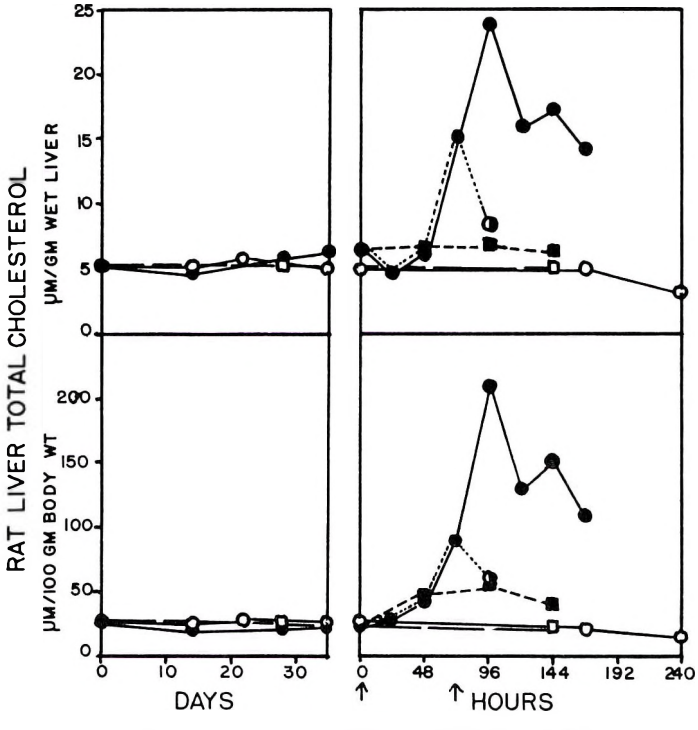


Fig. 2 Changes in total cholesterol in control and thiamine-deficient rat liver. Thiamine given deficient rats ↑ ; injection only on thirty-fifth day ◑ - - - -; injections on thirty-fifth and thirty-eighth days ●——; oral administration ■ – – –. Pair-fed controls □ – – –; ad libitum controls ○——.

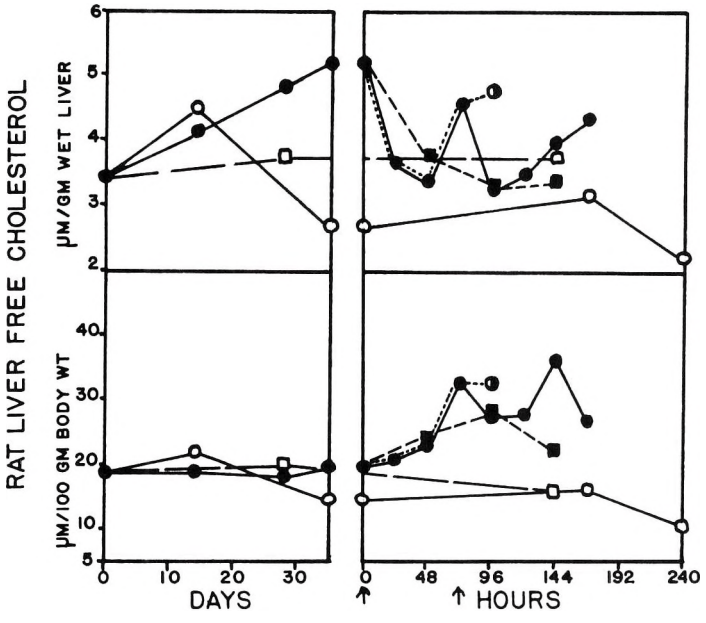


Fig. 3 Changes in free cholesterol in control and thiamine-deficient rat liver. Thiamine given deficient rats ↑ ; injection only on thirty-fifth day ◑ - - - -; injections on thirty-fifth and thirty-eighth days ●——; oral administration ■ – – –. Pair-fed controls □ – – –; ad libitum controls ○——.

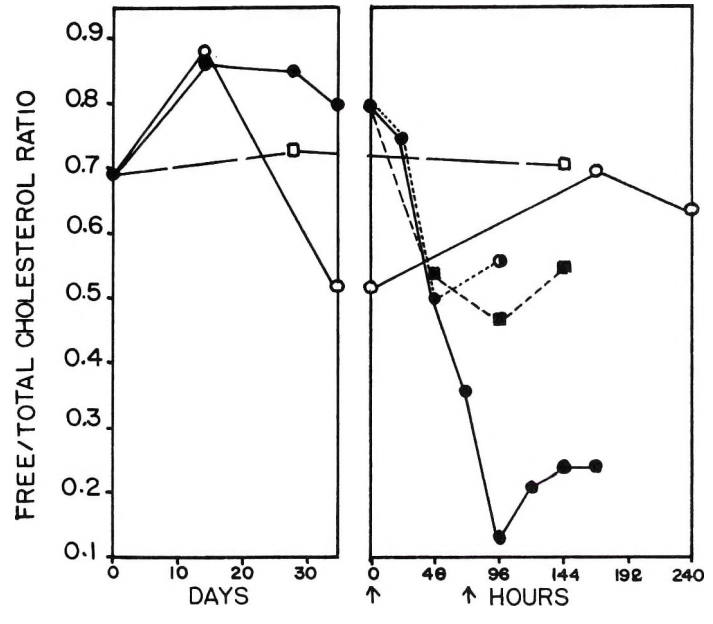


Fig. 4 Changes in free-to-total cholesterol ratios in control and thiamine-deficient rat liver. Thiamine given deficient rats ↑ ; injection only on the thirty-fifth day ◑ - - - -; injections on thirty-fifth and thirty-eighth days ●——; oral adminstration ■ – – –. Pair-fed control □ – – –; ad libitum control ○——.

mine injection there was a further marked decline in the ratio which leveled off 48 hours later, far below normal ($P < 0.02$).

*Phospholipids.* Phospholipids became depleted during development of thiamine deficiency (fig. 5), which is similar to the results of Williams and Anderson (1). Injections of thiamine produced results similar to those described above for free cholesterol. On the basis of per gram of liver the phospholipid values remain significantly lower ($P < 0.01$) in the thiamine-treated rats than in the control rats except after 72 hours when the second thiamine injection was given. Based on 100 g body weight the values remain lower ($P < 0.001$) until after 96 hours when the values become significantly higher than the values for the control rats ($P < 0.001$).

*Plasmalogens.* Liver plasmalogen concentrations (fig. 6) in thiamine-deficient rats decreased to about the same extent as those in the pair-fed controls, suggesting that the lowered food intake was primarily responsible for their loss. The initial injection of thiamine was followed by an increase in plasmalogen concentration to a maximum by 72 hours ($P < 0.001$ for both bases). A second thiamine injection at 72 hours was followed by a decrease in plasmalogen concentration reaching a plateau in 48 hours at about twice the value observed in the control rats given the thiamine supplement, on the basis of 100 g body weight ($P < 0.05$).

Intraperitoneal thiamine injections given to thiamine-supplemented control rats were followed by no significant changes in liver lipids. Pair-feeding the thiamine-supplemented control rats with the thiamine-deficient animals produced slight deviations from normal liver lipid values only in free cholesterol and plasmalogens.

Oral administration of thiamine to deficient rats was followed by much the same general pattern of liver lipid change as when thiamine was administered by intraperitoneal injection. However, the changes were in general not as great after oral administration.

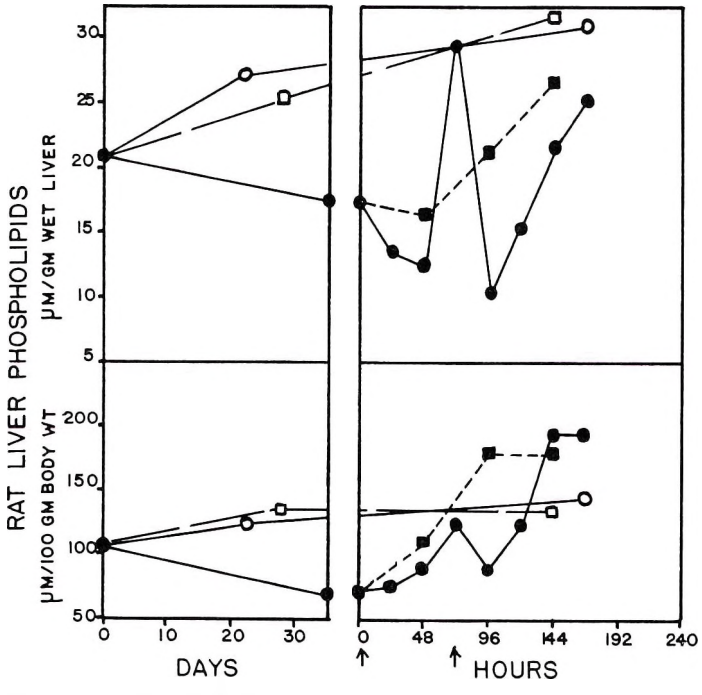


Fig. 5 Changes in phospholipids in control and thiamine-deficient rat liver. Thiamine given deficient rats ↑ ; injections on thirty-fifth and thirty-eighth day ●——; oral administration ■ – – –. Pair-fed control □ – – –; ad libitum control ○——.

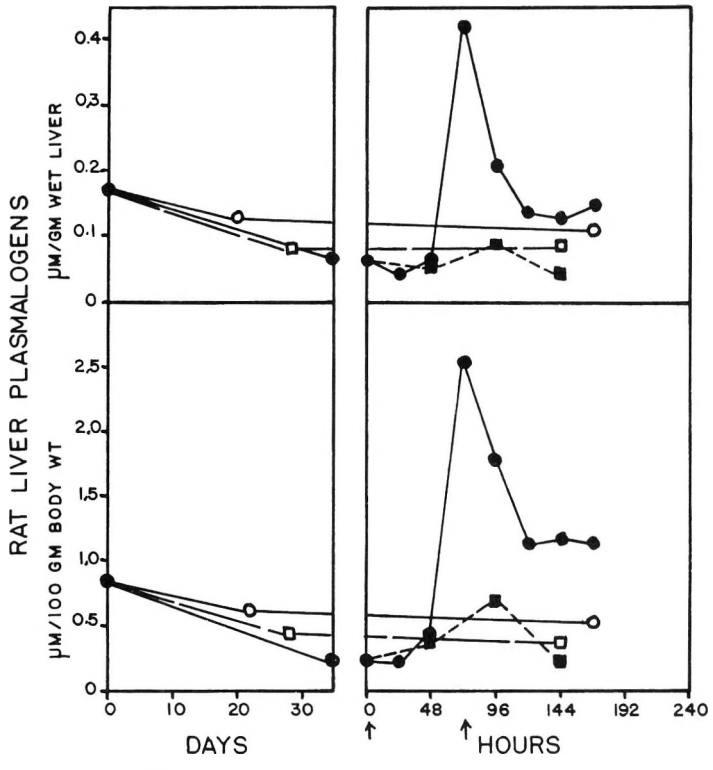


Fig. 6 Changes in plasmalogens in control and thiamine-deficient rat liver. Thiamine given deficient rats ↑ ; injections on thirty-fifth and thirty-eighth day ●——; oral administration ■ – – –. Pair-fed control □ – – –; ad libitum control ○——.

## DISCUSSION

As shown previously (1, 5, 6) rapid lipid accumulation in the liver may be produced in the severely thiamine-deficient rat by a sudden repletion of thiamine.

The observations presented show acute thiamine deficiency in the rat after being supplied with the deficient diet for 35 days. This differs with the time reported by Williams and Anderson (1) in that the earlier work was terminated by thiamine repletion at 49 days after the first rat died of thiamine deficiency on the forty-sixth day. In one experiment following the work cited, the deficiency period could be extended only to the fortieth day and in 3 subsequent experiments the deficient rats died in such numbers that thiamine had to be given on the thirty-fifth day if the rats were to survive. The cause of this time difference is unknown and can only be suggested by the use of a different lot of thiamine-free casein and greater efficiency of personnel in diet preparation and rat care. However, since three different thiamine deficiency experiments had to be terminated after 35 days, the data for the present paper were taken from appropriate experiments in these groups.

Total cholesterol values in the rats receiving a thiamine-deficient diet remained close to those for the livers from animals fed a thiamine-supplemented diet. Although the cholesterol values at the termination of thiamine deficiency are close to those of the previous paper (1) when based on liver weight, they are reversed when presented per 100 g body weight. Upon thiamine injection changes in values follow the same direction as those shown in the earlier paper.

Although in the control rats the proportion of free cholesterol was from 50 to 70% of the total, the proportion of free cholesterol during development of thia-

mine deficiency increased to 70 to 90% of the total. It appears that as the deficiency state developed there was a decreased esterification of cholesterol due possibly either to a failure of esterification mechanisms or to a lack of supply of fatty acids for esterification. Layne et al. (7) have reported an increased rate of cholesterol synthesis and a decreased rate of fatty acid synthesis in pigeon liver when glycolysis was minimal. This condition was attributed to a reduced supply of hydrogen for reduction reactions, favoring the synthesis of cholesterol over that of fatty acids. An analogous scheme would help to explain the results observed in the thiamine-deficient rat liver. The large decrease in the ratio of free-to-total cholesterol after thiamine injection might be explained by a superabundant fatty acid production and cholesterol esterification while the liver has not yet adjusted to metabolism and transport of large amounts of lipid.

The continued accumulation of all lipid fractions except plasmalogens after the second thiamine injection suggests that after an initial increase, enough plasmalogen has been produced and is maintained to serve its metabolic function. Yarbro and Anderson (8, 9) showed an increased concentration of plasmalogen to precede lipid mobilization and deposition in the rat. It is possible that this has also happened in the livers of thiamine-repleted rats and that if examination of lipid values were continued beyond that period observed in this report, the values would very soon return to normal levels.

The changes observed histologically in argyrophilia of liver cells and reticulum suggest that thiamine administration to rats deficient in thiamine may create a borderline protein deficiency not unlike that which may be reversed by lysine and tryptophan supplementation of the diet (10). However, this is not reflected in the biochemical parameters studied in the present paper. Such a pattern was soon overshadowed by central fatty changes indistinguishable from those associated with choline deficiency (11). The involvement of a choline deficiency in the observed lipid accumulation was suggested by Longnecker et al. (6) but, according to Engel and Phillips (5), choline had no effect on the accumulation of liver lipid after thiamine administration to deficient rats. The purified diet used in the present study contained adequate amounts of choline and methionine.

ACKNOWLEDGMENTS

The authors wish to thank David P. Henry, John Burgess, and Roger White for assistance in preparation of diets and in the feeding and care of the rats used.

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# Protein Levels in Growing Diets and Reproductive Performance of Cockerels [1,2]

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*ABSTRACT* Two experiments were conducted with Single Comb White Leghorn cockerels to determine the effect of dietary protein during the growing period on sexual maturity and various reproductive characteristics. Dietary protein levels of 16.0, 9.0, 6.75 and 4.5% were fed beginning when the cockerels were 9 weeks of age and 7 weeks of age in experiments 1 and 2, respectively. A 17% protein breeder diet was fed from 23 weeks until 37 weeks of age. Body growth was depressed with each decrease in dietary protein. The 4.5% protein level approximated a maintenance diet. Sexual maturity was delayed in the low protein groups, without apparent permanent damage to the reproductive system. After the recovery diet had been fed for 7 weeks, there was little difference between dietary groups in the number of birds producing sperm or in semen scores. By the end of the recovery period the low protein groups had higher sperm concentrations and greater testes weights. Fertility was higher in the 9.0 and 6.75% protein groups than in the 16.0 and 4.5% protein groups at the end of the recovery period. Hatchability of fertile eggs was not affected by protein levels during the growing period.

Recent reviews indicate that a marginal level of protein in the diet of laboratory animals may delay or inhibit the development of the male reproductive system and depress fertility (1, 2). However, relatively little work has been reported concerning the relationship between nutrition and reproduction in male chicks. Parker and Arscott (3) have shown that energy restriction of adult male chickens reduced volume and fertilizing capacity of semen, and reduced body and testes weights. Parker and McSpadden (4) demonstrated that feed restriction resulted in decreased fertility, semen volume, sperm concentration and motility in adult male chickens. A recent report by Arscott and Parker (5) indicated that dietary protein levels of 10.7 and 6.9% fed to adult males had no detrimental effect on fertility or hatchability as compared with the 16.9% level. In fact, fertility was higher in the 6.9% group than in the 16.9% group. Protein restriction has been reported to delay sexual maturity in females (6). This study was conducted to determine the effects of low levels of dietary protein during the growing period on the reproductive performance of cockerels.

## EXPERIMENTAL

*Experiment 1.* Seventy-two Single Comb White Leghorn (July-hatched) cockerels were placed at random into 12 replicates of 6 birds each. Three replicates were fed each experimental diet. Four experimental diets were fed which were calculated to contain 16.0, 9.0, 6.75 and 4.5% protein, respectively. The composition of the 16.0 and 9.0% protein diets is shown in table 1. The other 2 diets were formed from the 9.0% diet by dilution with glucose monohydrate.[3] When dilutions were made, increased levels of ground limestone, defluorinated phosphate and the micro-ingredient premix were added to maintain constant levels of calcium, phosphorus, and supplemental vitamins. The cockerels were placed in finishing batteries at 9 weeks of age and fed the experimental diets and water ad libitum for 14 weeks. Prior to being given the experimental diets, the cockerels had been

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[1] Florida Agricultural Experiment Stations Journal Series no. 1900.

[2] Presented in part before the meeting of the Poultry Science Association, August, 1963 (Poultry Sci., 42: 1319, abstract), and August, 1964 (Poultry Sci., 43: 1377, abstract).

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

TABLE 1

*Composition of basal diets*

| | 16% protein diet | 9% protein diet |
|---|---|---|
| | % | % |
| Yellow corn | 49.3 | 67.8 |
| Ground oats | 16.7 | 22.0 |
| Soybean meal (50% protein) | 18.8 | — |
| Animal fat | 3.8 | 0.4 |
| Oat hulls | 5.5 | 3.7 |
| Alfalfa meal (17% protein) | 2.0 | 2.0 |
| Ground limestone | 0.9 | 0.9 |
| Defluorinated phosphate (34% Ca + 18% P) | 2.0 | 2.2 |
| Iodized salt | 0.4 | 0.4 |
| Micro-ingredients [1] | 0.6 | 0.6 |

[1] Supplied per kg of diet: (in milligrams) niacin, 39.6; riboflavin, 4.4; Ca pantothenate, 19.8; choline, 770.0; iron, 19.8; copper, 2.0; $MnSO_4$, 220.0; ethoxyquin (Santoquin, Monsanto Chemical Co., St. Louis), 125.4; (in micrograms) vitamin $B_{12}$, 22.0; zinc, 99.0; and vitamin A, 6930 IU and vitamin $D_3$, 1320 ICU.

full-fed a grower diet containing 21% protein. The birds received natural autumn daylight (13 to 11 hours) until 21 weeks of age and then 24-hour lights for 2 weeks. Body weight measurements were made when the birds were 9, 12, 16, 20 and 37 weeks of age.

At 23 weeks of age 3 groups of 5 cockerels were randomly selected from each treatment group and each group of 5 was placed in a litter-bottom floor pen containing 40 hens and fed a breeder diet containing 17% protein, hereafter referred to as the recovery diet. This diet contained vitamins and minerals in amounts adequate to meet the breeder hen requirements as suggested by the NRC (7). Eggs were collected from the hens when the cockerels were 25, 29, 33 and 37 weeks of age, incubated for one week, and hatchery fertility determined by macroscopic examination of the broken-out egg.

*Experiment 2.* The experimental design and treatment diets were the same as those used in experiment 1. In this experiment the cockerels were hatched in March, placed in individual wire cages at 7 weeks of age and received the experimental diets for 16 weeks. The lighting consisted of natural daylight with artificial light to give a minimum of 13 hours of light per day. Body weights were obtained biweekly. The cockerels were ejaculated weekly beginning at 14 weeks of age and the ejaculate examined for sperm and scored according to relative concentration and motility (4, good sperm concentration and swirling; 3, good concentration, poor motility; 2, poor concentration, good motility; 1, poor concentration, poor motility; and zero, no sperm).

At 23 weeks of age all cockerels were fed the 17% protein breeder diet. From one to three hens per male were artificially inseminated biweekly with 0.05 $cm^3$ undiluted semen and hatchery fertility and hatchability data obtained. Two sets of hens were used for the inseminations; therefore, each set was inseminated every fourth week. Fertility was determined by candling after one week incubation and macroscopic examination of broken-out apparent non-fertile eggs. Hatchability was determined as percentage of fertile eggs hatched by the 22nd day of incubation. Sperm concentrations were determined by diluting semen 1:200 with distilled water in an RBC diluting pipette, and counting in an AO Spencer hemacytometer. Testes weights were obtained from 7 males per treatment at 40 weeks of age.

## RESULTS AND DISCUSSION

*Experiment 1.* Feeding of low protein diets resulted in decreased growth rates (fig. 1). The 4.5% protein level appeared to approach the level required for maintenance as body weight remained almost constant. Cockerels fed the low protein diets increased weight rapidly when fed the 17% protein recovery diet and by 37 weeks of age the body weights were 2225, 2055, 2118 and 1922 g for the 16.0, 9.0, 6.75 and 4.5% protein groups, respectively. This type of compensatory growth has been discussed in detail in a recent review (8).

Fertility by natural mating, determined after the recovery diet had been fed for 2 weeks (25 weeks of age), showed a marked reduction in the low protein treatment groups (fig. 2). After the recovery diet had been fed for 14 weeks, fertility was 85% or more in all treatments. The decrease in fertility of the 9.0% group between 10 and 14 weeks was partially due to a rather severe decrease in egg production in one pen.

These data indicate that the growth retardation and delay of sexual maturity

from feeding at the low protein levels during the growing period did not result in permanent damage to the reproductive system of the bird. Mortality during the treatment period was 1, 1, 2 and zero birds for the 16.0, 9.0, 6.75 and 4.5% protein groups, respectively, and apparently was not due to treatment.

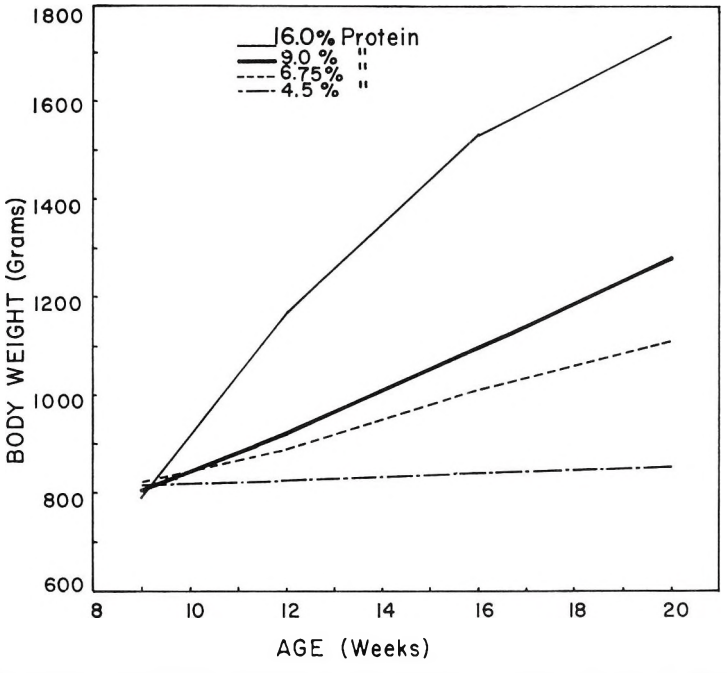


Fig. 1 Growth rate of White Leghorn cockerels fed at various levels of dietary protein during the growing period (exp. 1).

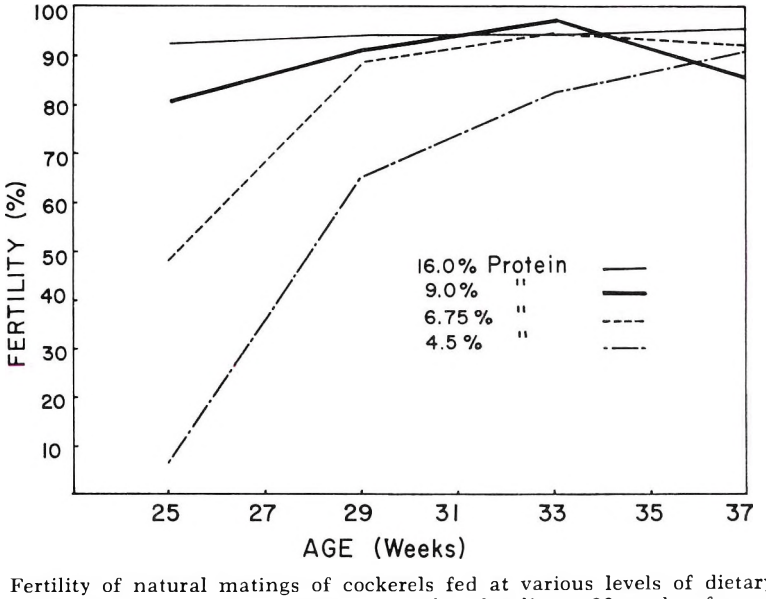


Fig. 2 Fertility of natural matings of cockerels fed at various levels of dietary protein during the growing period and then a 17% protein breeder diet at 23 weeks of age (exp. 1).

*Experiment* 2. The growth curves observed in this experiment (fig. 3) followed closely those in experiment 1. The 4.5% protein level again appeared to be very close to a maintenance diet as indicated by a fairly constant body weight. The low protein groups showed a very rapid increase in growth rate after being fed the recovery diet (fig. 4). It was noted that the low protein groups did not replace their chick feathers until they were given the recovery diet, after which feather growth progressed rapidly. This is in general agreement with the work of Gericke and Platt (9) on feather development.

The effect of low protein diets during the growing period on sexual maturity is shown by the percentage of the birds producing sperm at various ages (fig. 5). At least 80% of the cockerels had reached sexual maturity by the age of 16 weeks in the 16.0% protein group and 28 weeks (5 weeks with recovery diet) in the 9.0, 6.75 and 4.5% groups. Weekly semen scores followed a very similar pattern (fig. 5). A decrease was noted in the period 28 to

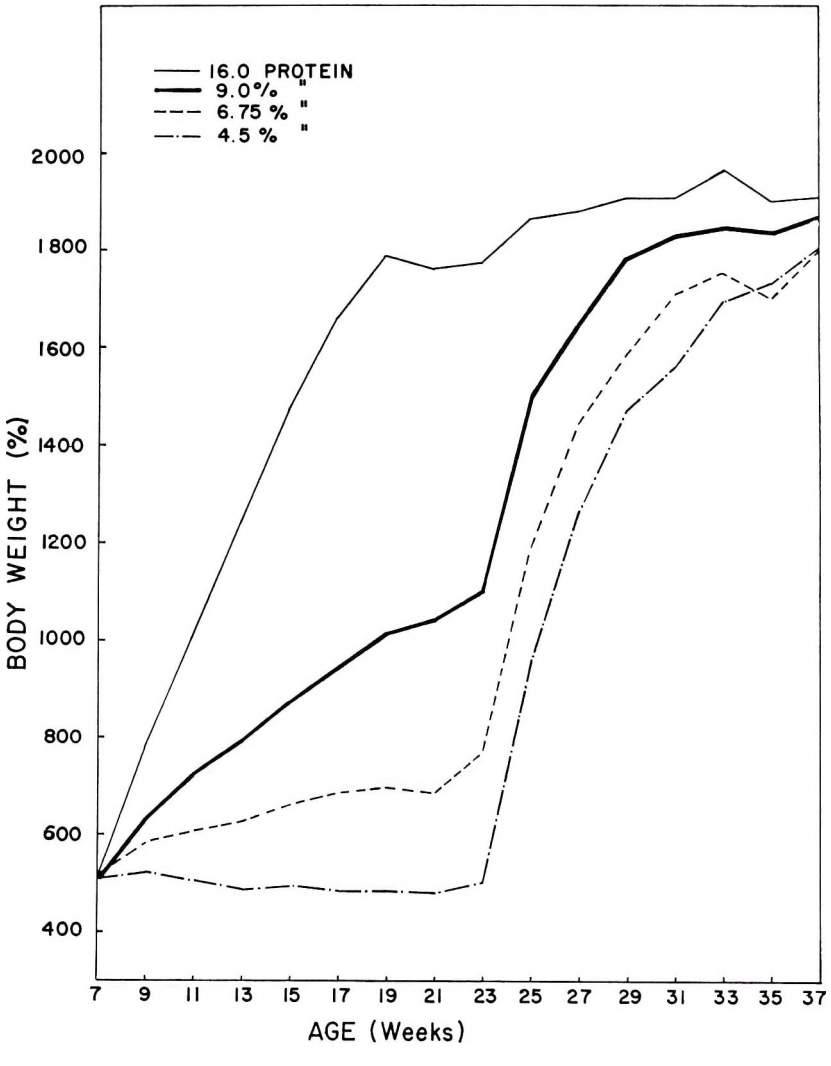


Fig. 3 Growth rate of White Leghorn cockerels fed at various levels of dietary protein during the growing period (exp. 2).

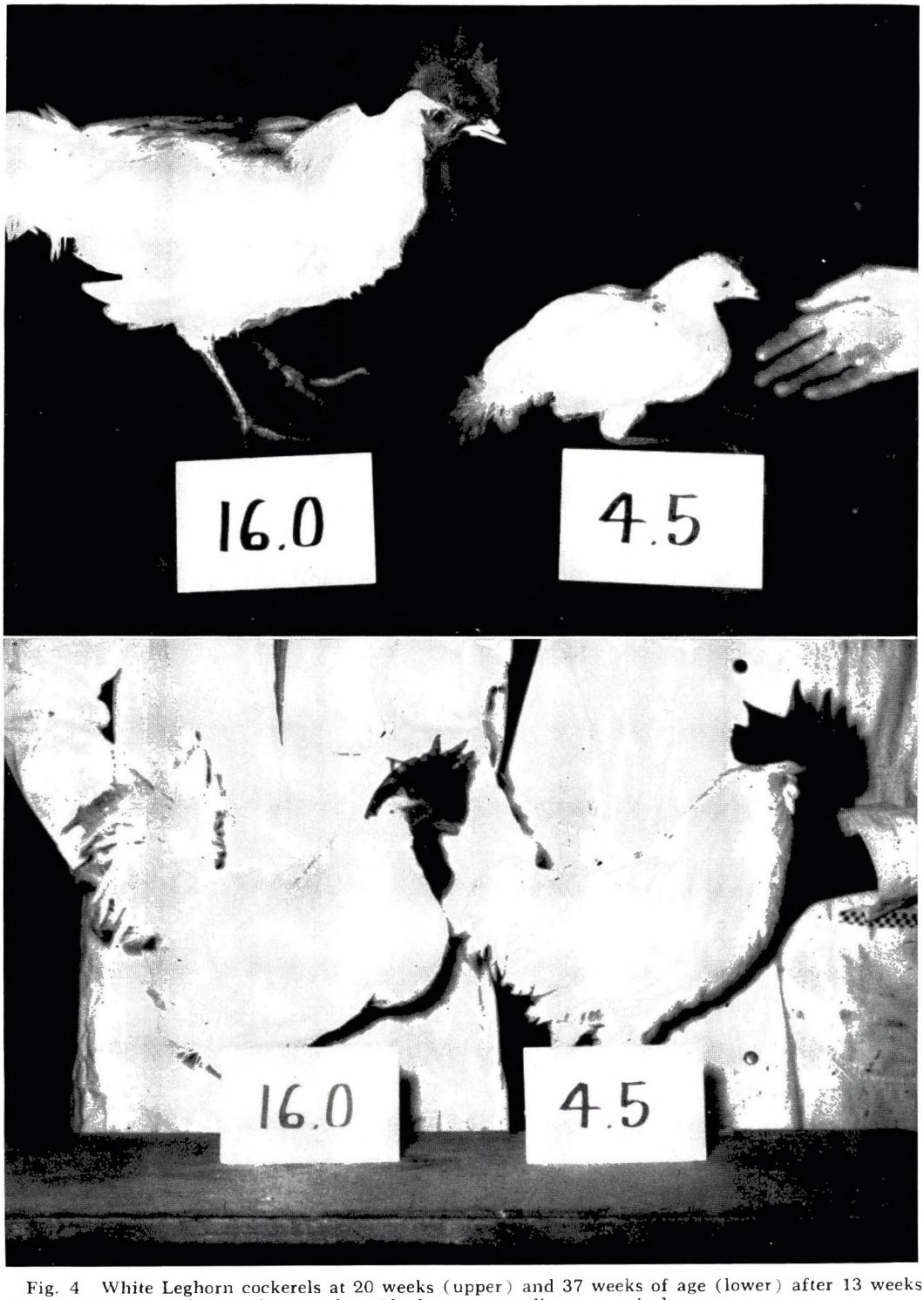


Fig. 4 White Leghorn cockerels at 20 weeks (upper) and 37 weeks of age (lower) after 13 weeks with the treatment diets and 14 weeks with the recovery diet, respectively.

33 weeks in the number of males producing sperm and in the semen scores, especially in the 16.0% protein group. During this time, 3 birds of the 16.0% protein group were losing weight and producing few or no sperm. However, no overall health problem was observed nor was one indicated by body weights (fig. 3).

After 2 weeks of feeding the recovery diet fertility was significantly lower ($P < 0.05$) in the low protein groups than in the 16.0% group (fig. 5). Fertility in the 4.5% group was still significantly lower after 4 weeks with the recovery diet. From week 10 with the recovery diet to the termination of the experiment there were no significant differences in fertility. Fertility following recovery was not impaired by feeding the low levels of protein during the growing period. However, it appears

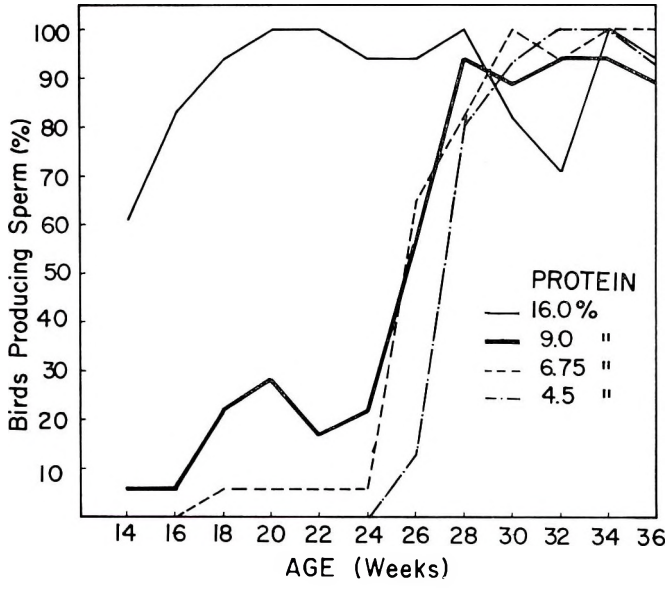


Figure 5a

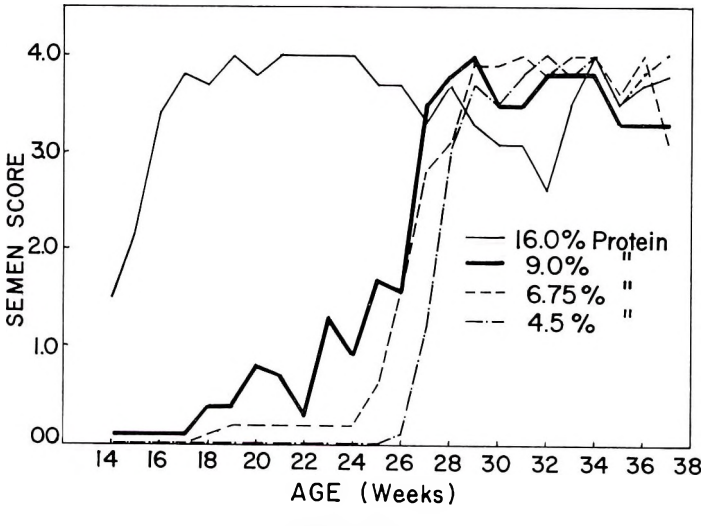


Figure 5b

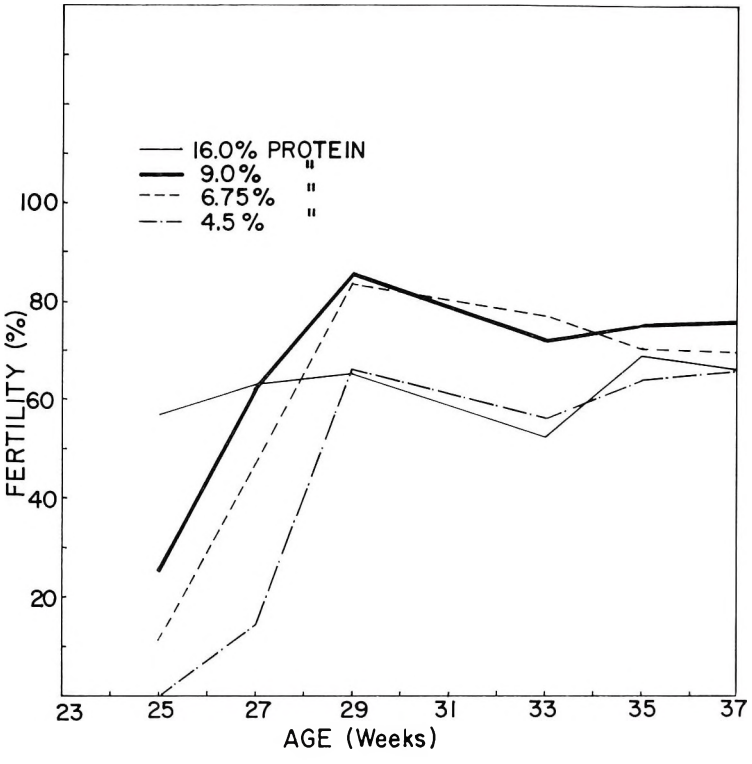


Figure 5c

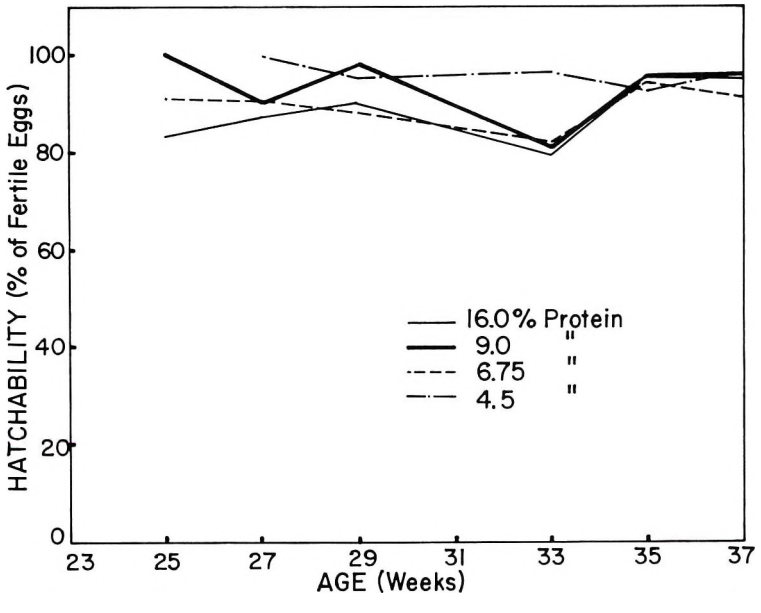


Figure 5d

Fig. 5 Influence of feeding dietary protein at various levels during the growing period, on sexual maturity, semen scores, fertility, and hatchability.

TABLE 2

*Effect of low protein grower diets on sperm concentration (exp. 2)*

| Protein | Sperm concentration | | | | |
|---|---|---|---|---|---|
| | 20 weeks | 22 weeks | 28 weeks | 30 weeks | 36 weeks |
| % | million/$mm^3$ | million/$mm^3$ | million/$mm^3$ | million/$mm^3$ | million/$mm^3$ |
| 16.0 | 2.76(17) [1] | — | 3.32(2) | 2.40(5) | 2.96(6) |
| 9.0 | — | 0.52(5) | 3.04(3) | 4.04(6) | 3.22(6) |
| 6.75 | — | — | 1.41(2) | 4.15(6) | 3.37(6) |
| 4.5 | — | — | 0.43(2) | 3.40(6) | 3.93(6) |

[1] Numbers in parentheses indicate the number of birds represented by the data.

from these data that 16.0 and 4.5% protein during the growing period gave less than maximal reproductive performance. Hatchability of fertile eggs was not affected by protein levels (fig. 5).

When semen was examined for sperm concentration it was found that as the birds came into production the concentration was low (table 2). After the recovery diet had been supplied for 5 weeks, the sperm concentration varied directly with the protein level from 3.32 million/$mm^3$ in the 16.0% protein group to 0.43 million/$mm^3$ in the 4.5% group. Thirteen weeks later, at 36 weeks of age, the pattern had reversed and sperm concentration increased with each decrease in protein level.

Testes weights were greater in the low protein groups at 40 weeks of age (table 3). This phenomenon has been reported recently in pigs (10). Lower sperm concentration in the 16.0% protein group may reflect this testes size difference, although earlier work (11) has indicated no relationship between testes size and sperm concentration. The testes weights of the 16.0% group were lower than might have been expected on the basis of previous reports (12) which indicated that after 24 weeks of age the testes weights of White Leghorns make up about 0.80% of the body weight.

TABLE 3

*Effect of low dietary protein during the growing period on testes weights at 40 weeks of age (exp.2)*

| Protein | Testes wt [1] | % of body wt |
|---|---|---|
| % | g | |
| 16.0 | 5.8 ± 1.7 [2] | 0.29 |
| 9.0 | 14.1 ± 3.8 | 0.68 |
| 6.75 | 11.8 ± 2.9 | 0.62 |
| 4.5 | 16.5 ± 1.0 | 0.77 |

[1] Average weight of both testes of 7 males.
[2] SE of mean.

Although low dietary protein during the growing period caused a delay in sexual maturity as well as a reduction of body growth, it did not appear to cause any permanent damage to the reproductive system. In comparison with the 16.0% protein group, the low protein groups had as many birds producing sperm, with comparable semen scores, higher sperm concentration, better fertility, and greater testes weights after recovery. This indicates that diets in the range of 7 to 9% protein for the growing period (7 to 23 weeks) might be more desirable for cockerels that are to be used for reproductive purposes than a higher protein diet.

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# Effect of Age and Dietary Fat on the Lipids of Chicken Muscle [1]

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*ABSTRACT* The influence of age, and type and level of dietary fat on muscle lipids of young chickens was studied. Total lipid and phospholipid levels in breast muscle were not significantly influenced by dietary fat, but were each significantly lowered with age. Phospholipids were generally higher in amounts of stearic acid and long-chain polyunsaturated fatty acids than neutral lipids, whereas neutral lipids contained higher quantities of oleic and linoleic acids. Each lipid fraction tended to assume the fatty acid pattern of the dietary fat with neutral lipids being affected far more than phospholipids. Age had comparatively little effect on the fatty acid content of neutral lipids, but tended to reduce the level of linoleic acid in phospholipids while increasing levels of polyunsaturated fatty acids having 20 or more carbons. The concentration and fatty acid composition of thigh muscle lipids showed approximately the same dietary-induced changes as those noted for breast muscle lipids.

Lipid metabolism studies with chickens have shown that age and dietary factors such as level of protein and fat have a pronounced effect on the level and composition of liver lipids (1–4). Similar studies with rats have also demonstrated that fatty acids of tissues such as heart, liver, and testes may be greatly altered by dietary fatty acids (5). Less attention has been directed toward factors that might influence the lipid composition of skeletal muscles of animals, although feeding different dietary fats to chickens (6) and to rats (7) resulted in a deposition of fatty acids in skeletal muscles resembling those in the dietary fats. Furthermore, it has been noted that the fatty acids of tissue phospholipids in rats are modified less than triglycerides by different fats in the diet.[2]

The present study was initiated to investigate the influence of dietary fats and age on the level and composition of lipids in skeletal muscles of chickens. The effect of feeding an unsaturated oil (corn oil) and a more saturated fat (beef tallow) at different dietary levels on the amount and composition of neutral lipids and phospholipids of chicken muscle at various ages was studied.

## EXPERIMENTAL

Five groups of broiler-strain White Plymouth Rock cockerels were reared in electrically heated battery brooders from one day of age. Each group was fed ad libitum one of the following diets: 1) basal with no added fat; 2) basal plus 6% corn oil; 3) basal plus 12% corn oil; 4) basal plus 6% beef tallow; or 5) basal plus 12% beef tallow. The basal diet was composed of ground yellow corn, soybean oil meal (50% protein), and levels of minerals and vitamins to meet established requirements for growing chicks. The basal diet contained 3.10% ether extractable materials and was of the same composition as that used by Marion and Woodroof (6) except that fish meal was excluded from the diet and replaced by corn. The fats were substituted for corn in the diet on an equal weight basis. The fatty acid composition, by analysis, of the basal diet and dietary fats is presented in table 1.

A minimum of 3 chickens in each group was killed at 21, 42, and 63 days of age, and samples of breast muscle excised for analysis. Thigh muscle samples were also taken at 63 days. Each sample was stored at − 29° until analyzed. Samples of 25 to 50 g were weighed and the lipids extracted with chloroform and methanol by the

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[1] Journal Series Paper no. 492, Georgia Experiment Station, Experiment, Georgia.

[2] Turchetto, E., M. Proja and M. G. Gandolfi 1963 Fatty acids of different tissue lipids in rats fed on diets quantitatively different in lipid composition. Biochem. J., *89:* 22 p (abstract).

TABLE 1

*Fatty acids of basal diet and dietary fats*

| Fatty acid | Basal diet | Corn oil | Beef tallow |
|---|---|---|---|
| | % | % | % |
| 12:0 [1] | — | — | 0.3 |
| 14:0 | 0.2 | — | 6.2 |
| 14:1 | — | — | 1.7 |
| 16:0 | 12.4 | 11.1 | 25.3 |
| 16:1 | 1.9 | 0.5 | 6.7 |
| 16:2 | — | — | 0.6 |
| 18:0 | 15.7 | 1.7 | 19.9 |
| 18:1 | 32.0 | 26.7 | 32.8 |
| 18:2 | 36.4 | 59.0 | 5.1 |
| 18:3 | 1.4 | 1.0 | 1.4 |

[1] Carbon chain length:number of double bonds. Each fatty acid expressed as a percentage of total fatty acids.

method of Bligh and Dyer (8). The extracted lipids were isolated and dried as outlined previously (9). Total lipids were then applied to thin-layer chromatography plates coated with silica gel G (250 μ thick) in amounts up to 100 mg per each 20 × 20 cm plate. Separation into neutral lipid and phospholipid fractions was accomplished with a 100% chloroform solvent system. Fractions on the chromatoplates were located and recovered as described by Privett et al. (10), and weighed. After weighing, each fraction was refluxed with 5% $H_2SO_4$ in anhydrous methanol to hydrolyze the lipids and methylate the fatty acids. The methyl esters were then recovered as outlined previously (9) and fractionated with an F & M Model 1609 flame ionization unit attached to a Model 720 gas chromatograph. Injection port, column chamber and detector temperatures of 245°, 225° and 260°, respectively, were maintained. Copper columns (0.635 × 152 cm) were used, packed with 15% liquid phase on chromosorb W. Diethylene glycol succinate polyester (DEGS) was used as a liquid phase for all analyses, and silicone oil and apiezon L were used as liquid phases to aid in the identification of fatty acid peaks. In contrast with DEGS columns, apiezon columns reverse the order of appearance of unsaturated members in a carbon series, whereas silicone oil columns give no separation due to unsaturation. Relative retention times for the following National Heart Institute Fatty Acid Standards (mixtures and individual acids included 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 20:0, 20:1, 20:4, 20:5, 22:0, 22:6, 24:0 and 24:1) were obtained on the 3 liquid phases used and compared with retention times of unknown fatty acids when plotted on semilogarithmic paper. For identification purposes, this provides essentially the same information as that obtained by the "carbon-number" technique of Woodford and van Gent (11).

## RESULTS

The content of total lipids and phospholipids observed in breast muscle of chickens at 21, 42, and 63 days, and in thigh muscle at 63 days is shown in table 2. Neither amount nor type of dietary fat significantly influenced the content of total lipids and phospholipids in breast and thigh muscles. However, total lipid and phospholipid levels in breast muscle decreased with age ($P < 0.01$). The mean

TABLE 2

*Effect of age and dietary fat on total lipid and phospholipid levels of muscle tissues of chicks*

| Tissue | Age of chicks | Lipid | Added dietary fat | | | | | Avg |
|---|---|---|---|---|---|---|---|---|
| | | | Zero | Corn oil, % | | Beef tallow, % | | |
| | | | | 6 | 12 | 6 | 12 | |
| | *days* | | *% of wet tissue* | *% of wet tissue* | *% of wet tissue* | *% of wet tissue* | *% of wet tissue* | % |
| Breast | 21 | Total [1] | 1.07 | 1.31 | 1.06 | 1.15 | 1.22 | 1.16 |
| | | Phospholipid [1] | 0.56 | 0.61 | 0.50 | 0.51 | 0.54 | 0.54 |
| | 42 | Total | 1.02 | 1.08 | 1.10 | 1.00 | 1.09 | 1.06 |
| | | Phospholipid | 0.52 | 0.49 | 0.42 | 0.46 | 0.55 | 0.49 |
| | 63 | Total | 0.76 | 0.90 | 0.82 | 0.85 | 0.73 | 0.81 |
| | | Phospholipid | 0.41 | 0.42 | 0.35 | 0.50 | 0.35 | 0.41 |
| Thigh | 63 | Total | 2.01 | 2.58 | 2.41 | 1.90 | 1.91 | 2.16 |
| | | Phospholipid | 0.35 | 0.46 | 0.43 | 0.35 | 0.52 | 0.42 |

[1] Lipid and phospholipid levels were influenced significantly ($P < 0.01$) only by age in breast tissue.

phospholipid content at 63 days was practically the same in breast and thigh muscles, whereas a much higher quantity of total lipids was found in thigh muscle.

The fatty acid composition of neutral lipids and phospholipids of breast muscle of chickens at different ages and from different diets is shown in tables 3 and 4. Generally, phospholipids contained higher levels of stearic acid and long-chain polyunsaturated fatty acids (20:4 through 22:6) and lower levels of oleic and linoleic acids than neutral lipids.

The results of an analysis of variance for the major fatty acids of each lipid fraction are presented in table 5. In general, the fatty acids of each fraction tended to assume the pattern of fatty acids in the diet with the neutral lipids being influenced to a much greater extent than phospholipids by diet. These changes are presented more specifically in the following sections.

*Dietary fats.* Feeding corn oil resulted in an increased deposition of linoleic acid (18:2) in neutral lipids of breast muscle with an accompanying decrease in palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acids. Feeding beef tallow resulted in higher levels of 18:1 and in lower levels of 18:2 being deposited in neutral lipids. The most pronounced changes in fatty acids occurred when 6% of each fat was added to the diet, whereas further changes due to the addition of more fat were relatively small. Changes induced in the 18-carbon fatty acids of phospholipids by feeding corn oil and beef tallow were generally similar to those noted in neutral lipids although the changes were not consistent and were of much less magnitude. In phospholipids, the levels of 20:4 and 24:1 appeared to be higher when corn oil was fed, whereas the levels of 22:5 and 22:6 were slightly higher when beef tallow was fed. The fatty acids of neutral lipids and phospholipids of thigh muscle (table 6) at 63 days of age showed approximately the same dietary-induced changes as those noted for breast muscle lipids.

*Age.* The influence of age on the fatty acids of neutral lipids of breast muscle was slight with the exception of 16:0 and 16:1. The fatty acid content of phospho-

TABLE 3

*Effect of age and dietary fat on fatty acids of breast muscle neutral lipids*

| Age, days | 21 | | | | | 42 | | | | | 63 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Added fat, % | | Corn oil | | Beef tallow | | | Corn oil | | Beef tallow | | | Corn oil | | Beef tallow | |
| | 0 | 6 | 12 | 6 | 12 | 0 | 6 | 12 | 6 | 12 | 0 | 6 | 12 | 6 | 12 |
| Fatty acid [1] | % *total fatty acids* | | | | | | | | | | | | | | |
| 14:0 | 2.2 | 1.2 | 2.3 | 2.2 | 2.6 | 1.0 | 0.8 | 1.3 | 3.2 | 2.2 | 0.8 | 0.5 | 0.2 | 1.8 | 2.0 |
| 14:1 | 0.5 | 0.3 | 0.8 | 0.9 | 1.1 | 0.6 | 0.2 | 0.2 | 0.9 | 0.9 | 0.3 | 0.1 | — | 0.7 | 0.7 |
| 16:0 | 26.4 | 17.6 | 15.3 | 23.6 | 27.6 | 25.6 | 16.6 | 14.2 | 22.8 | 24.6 | 28.8 | 18.5 | 15.0 | 31.2 | 27.1 |
| 16:1 | 6.7 | 2.5 | 3.2 | 6.0 | 6.0 | 6.0 | 2.4 | 1.9 | 4.5 | 5.9 | 6.0 | 2.2 | 1.5 | 4.9 | 4.8 |
| 16:2 | 0.5 | 0.7 | 0.8 | 0.8 | 0.7 | 0.4 | 0.4 | 0.5 | 0.4 | 0.7 | 0.1 | 0.3 | 0.4 | 0.3 | 0.9 |
| 18:0 | 7.0 | 5.3 | 3.0 | 7.1 | 7.4 | 6.0 | 4.1 | 3.4 | 7.3 | 6.8 | 7.2 | 5.0 | 3.5 | 9.1 | 7.2 |
| 18:1 | 35.6 | 27.5 | 23.6 | 39.9 | 41.7 | 34.4 | 28.1 | 26.6 | 36.3 | 39.7 | 35.6 | 29.3 | 26.7 | 34.2 | 39.5 |
| 18:2 | 19.0 | 41.7 | 47.2 | 17.1 | 14.7 | 22.6 | 44.6 | 48.8 | 20.3 | 15.5 | 19.1 | 41.2 | 50.3 | 15.5 | 15.9 |
| 18:3 | 1.4 | 1.4 | 1.6 | 1.5 | 1.6 | 1.6 | 1.6 | 1.4 | 2.1 | 1.8 | 1.3 | 1.2 | 0.9 | 1.1 | 0.9 |
| 20:3 [2] | 0.7 | 0.9 | 0.6 | 0.1 | 0.1 | 0.4 | 0.3 | 0.2 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 |
| 20:4 | 0.4 | 0.8 | 1.0 | 0.7 | 0.5 | 1.3 | 0.9 | 1.0 | 1.7 | 1.2 | 0.5 | 0.9 | 0.9 | 0.7 | 0.7 |
| 20:5 | — | 0.2 | 0.1 | 0.2 | — | — | 0.1 | 0.3 | — | 0.2 | 0.2 | 0.3 | 0.1 | 0.4 | — |

[1] Carbon chain length:number of double bonds.

[2] Contains 20:3 and 22:0 under the same peak.

TABLE 4

*Effect of age and dietary fat on fatty acids of breast muscle phospholipids*

| Age, days | 21 | | | | | 42 | | | | | 63 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Added fat, % | | Corn oil | | Beef tallow | | | Corn oil | | Beef tallow | | | Corn oil | | Beef tallow | |
| | 0 | 6 | 12 | 6 | 12 | 0 | 6 | 12 | 6 | 12 | 0 | 6 | 12 | 6 | 12 |
| Fatty acids [1] | % *total fatty acids* | | | | | % *total fatty acids* | | | | | % *total fatty acids* | | | | |
| 14:0 | 0.8 | 0.6 | 0.7 | 0.9 | 0.8 | 0.5 | 0.9 | 0.6 | 1.1 | 0.6 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 |
| 14:1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | — | 0.1 | 0.1 |
| 14:2 | 1.4 | 1.7 | 2.3 | 2.8 | 1.6 | 1.6 | 4.0 | 0.6 | 4.4 | 3.1 | 0.3 | 2.9 | 5.2 | 4.2 | 1.7 |
| 16:0 | 23.7 | 22.0 | 24.2 | 23.0 | 20.6 | 24.6 | 24.0 | 21.4 | 22.2 | 24.6 | 24.4 | 23.9 | 24.6 | 21.5 | 24.0 |
| 16:1 | 1.8 | 0.6 | 0.6 | 1.2 | 1.3 | 1.4 | 0.6 | 0.8 | 0.8 | 1.1 | 2.0 | 0.8 | 0.9 | 1.4 | 1.1 |
| 16:2 | 0.8 | 0.7 | 0.8 | 1.3 | 1.2 | 0.6 | 1.2 | 0.3 | 1.6 | 1.4 | 0.2 | 0.6 | 1.3 | 1.3 | 1.2 |
| 18:0 | 11.9 | 15.0 | 12.3 | 11.8 | 12.8 | 10.5 | 10.7 | 10.4 | 11.2 | 11.2 | 9.1 | 8.6 | 10.4 | 11.0 | 10.1 |
| 18:1 | 21.1 | 12.4 | 12.9 | 18.8 | 21.4 | 19.2 | 13.8 | 15.0 | 16.6 | 18.1 | 19.8 | 16.8 | 16.1 | 21.3 | 20.8 |
| 18:2 | 20.7 | 22.5 | 25.4 | 20.5 | 21.2 | 18.1 | 20.9 | 23.8 | 16.3 | 18.5 | 18.9 | 19.1 | 18.5 | 17.0 | 13.0 |
| 18:3 | 0.8 | 0.4 | 0.2 | 0.6 | 0.5 | 0.4 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.8 | 0.1 | 0.4 | 0.2 |
| 18:4 | 0.2 | 0.2 | 0.6 | 0.3 | 0.3 | 0.7 | 0.9 | 1.2 | 0.6 | 0.3 | 0.6 | 0.6 | 0.5 | 0.2 | 0.2 |
| 20:3 [2] | 1.8 | 1.4 | 1.1 | 1.5 | 1.6 | 1.8 | 1.1 | 1.0 | 1.6 | 1.6 | 1.5 | 0.9 | 0.5 | 1.2 | 0.8 |
| 20:4 | 9.4 | 15.0 | 12.9 | 9.6 | 9.9 | 12.6 | 13.7 | 15.6 | 12.3 | 13.5 | 12.5 | 15.9 | 13.7 | 12.0 | 15.2 |
| 20:5 | 0.4 | 0.2 | 0.2 | 0.5 | 0.6 | 0.5 | 0.2 | 0.3 | 0.4 | 0.5 | 0.5 | 0.2 | 0.1 | 0.2 | 0.3 |
| 24:1 | 2.0 | 3.6 | 3.0 | 1.8 | 1.6 | 2.8 | 3.6 | 4.0 | 2.5 | 1.8 | 2.5 | 3.7 | 3.3 | 2.2 | 2.4 |
| 24:2 | 0.8 | 1.2 | 1.2 | 1.4 | 0.9 | 1.4 | 2.0 | 1.6 | 1.8 | 1.5 | 1.5 | 1.5 | 1.2 | 1.2 | 1.6 |
| 22:5 | 1.0 | 0.8 | 0.6 | 1.5 | 1.5 | 1.2 | 0.7 | 1.0 | 2.1 | 2.3 | 2.6 | 1.5 | 1.2 | 2.0 | 2.7 |
| 22:6 | 1.2 | 0.8 | 0.7 | 2.5 | 2.2 | 1.8 | 1.3 | 1.8 | 3.5 | 4.1 | 2.0 | 1.6 | 1.3 | 2.3 | 4.1 |

[1] Carbon chain length:number of double bonds.
[2] Contains 20:3 and 22:0 under the same peak.

TABLE 5

*Statistical significance of age and dietary-induced changes in muscle fatty acids*

| Fraction | Fatty acid [1] | Source of variation | | | |
|---|---|---|---|---|---|
| | | Breast muscle | | | Thigh muscle |
| | | Dietary fat | Age | Interaction | Dietary fat |
| Triglyceride | 16:0 | ** [2] | ** | * | ** |
| | 16:1 | ** | ** | N.S. | ** |
| | 18:0 | ** | N.S. | N.S. | ** |
| | 18:1 | ** | N.S. | N.S. | ** |
| | 18:2 | ** | N.S. | N.S. | ** |
| | 20:4 | N.S. | N.S. | N.S. | N.S. |
| Phospholipid | 16:0 | N.S. | N.S. | N.S. | ** |
| | 16:1 | ** | N.S. | N.S. | * |
| | 18:0 | N.S. | ** | N.S. | N.S. |
| | 18:1 | ** | ** | * | ** |
| | 18:2 | ** | ** | ** | ** |
| | 20:4 | ** | ** | * | N.S. |
| | 24:1 | ** | * | N.S. | * |
| | 24:2 | * | * | N.S. | N.S. |
| | 22:5 | ** | ** | ** | ** |
| | 22:6 | ** | ** | * | * |

[1] Carbon chain length:number of double bonds.
[2] N.S. = not significant; * = significant at $P < 0.05$; ** = significant at $P < 0.01$.

TABLE 6

*Effect of diet on fatty acids of thigh muscle lipids*

| Fraction | Neutral lipids | | | | | Phospholipids | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Added fat, % | | Corn oil | | Beef tallow | | | Corn oil | | Beef tallow | |
| | 0 | 6 | 12 | 6 | 12 | 0 | 6 | 12 | 6 | 12 |
| Fatty acid [1] | % total fatty acids | | | | | % total fatty acids | | | | |
| 14:0 | 1.1 | 0.7 | 0.3 | 2.2 | 2.2 | 0.4 | 0.6 | 0.3 | 0.4 | 0.4 |
| 14:1 | 0.4 | 0.1 | 0.1 | 0.9 | 0.9 | 0.2 | 0.5 | — | 0.1 | 0.1 |
| 14:2 | — | — | — | — | — | 4.2 | 4.1 | 1.0 | 1.2 | 1.1 |
| 16:0 | 28.1 | 18.6 | 15.2 | 23.4 | 22.6 | 20.7 | 18.0 | 18.6 | 18.9 | 17.6 |
| 16:1 | 8.5 | 3.2 | 2.1 | 5.5 | 5.3 | 1.7 | 0.9 | 0.8 | 1.5 | 1.4 |
| 16:2 | 0.2 | 0.5 | 0.5 | 0.5 | 0.5 | 1.6 | 0.9 | 0.3 | 0.7 | 0.7 |
| 18:0 | 4.8 | 4.0 | 3.4 | 8.4 | 7.9 | 14.8 | 16.0 | 15.0 | 14.6 | 15.4 |
| 18:1 | 34.3 | 27.2 | 28.2 | 40.0 | 42.4 | 15.8 | 13.1 | 14.7 | 19.9 | 22.2 |
| 18:2 | 20.8 | 43.3 | 48.2 | 16.9 | 16.2 | 19.2 | 21.2 | 25.8 | 18.9 | 15.7 |
| 18:3 | 1.1 | 1.1 | 1.3 | 1.2 | 1.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 |
| 18:4 | — | — | — | — | — | 0.3 | 0.6 | 0.5 | 0.2 | 0.2 |
| 20:3 [2] | 0.2 | 0.1 | 0.2 | — | — | 1.0 | 0.4 | 0.4 | 0.7 | 0.5 |
| 20:4 | 0.5 | 0.7 | 0.6 | 0.8 | 0.6 | 13.3 | 15.7 | 14.4 | 13.5 | 14.9 |
| 20:5 | 0.2 | 0.5 | — | — | — | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 |
| 24:1 | — | — | — | — | — | 3.1 | 3.5 | 3.1 | 2.9 | 2.3 |
| 24:2 | — | — | — | — | — | 1.0 | 1.4 | 1.5 | 1.4 | 1.2 |
| 22:5 | — | — | — | — | — | 1.1 | 1.1 | 1.1 | 2.0 | 2.7 |
| 22:6 | — | — | — | — | — | 1.1 | 1.8 | 1.6 | 2.7 | 3.0 |

[1] Carbon chain length:number of double bonds.
[2] Contains 20:3 and 22:0 under the same peak.

lipids, however, was influenced noticeably by age. In phospholipids, the content of 18:2 generally decreased with age, whereas the quantities of 20:4, 24:1, 24:2, 22:5 and 22:6 generally increased.

*Diet and age interaction.* Statistically significant interactions ($P < 0.05$ or 0.01) for age and diet were noted only for 16:0 in neutral lipids, and for 18:1, 18:2, 20:4, 22:5, and 22:6 in phospholipids, indicat-

ing that the response of these particular fatty acids to diet is mediated by age of the bird.

## DISCUSSION

The amount and type of dietary fat did not significantly influence the quantity of total lipids and phospholipids in breast and thigh muscles in the present study. These results do not agree with those presented by Miller et al. (12) which showed that breast muscle, and especially thigh muscle, of chickens responded to high levels of dietary fat by depositing more fat. In the present experiment, adipose tissue deposits were carefully trimmed from all muscle samples taken, which may account for part of the differences between values observed in the present work and for those in the literature. The level of total lipids and phospholipids of breast muscle decreased with age. This same pattern has been observed in lipids of chicken livers during similar age periods although changes in the liver were much more pronounced (1, 2).

The specific differences in fatty acid composition of neutral lipids and phospholipids noted in this study agree closely with those reported previously for chicken liver (3), chicken muscle (9), and muscle tissues of other animals (13).

From previous studies of fatty acid metabolism in chickens (1, 2, 4, 6) and rats (5, 7), it was expected that the fatty acid composition of total lipids of skeletal muscles would tend to resemble the fatty acid composition of the dietary fat. The results obtained confirmed this and further demonstrated that most of the changes in 16- and 18-carbon fatty acids were primarily localized in the neutral lipids. This observation supports that of Turchetto and co-workers [3] which showed that phospholipids of tissues are modified less than triglycerides by dietary fats.

With the exception of 16:0 and 16:1, the fatty acids in neutral lipids were not significantly influenced by age, although all of the major fatty acids of this fraction were very markedly altered by dietary fats. In contrast, the fatty acids of phospholipids were influenced less by diet than neutral lipid fatty acids, and were nearly all significantly affected by age. These results suggest that living tissues have definite requirements for phospholipids with fairly closely defined fatty acid configurations, and that very drastic dietary changes must be imposed before appreciable changes in phospholipid level or composition can be expected. The fact that total phospholipids decreased with age while undergoing changes in fatty acid composition could mean that tissues of chickens at different ages may differ in phospholipid fatty acids required for normal function, or that all phospholipids present are not needed for metabolic purposes; some may simply serve as depot reserves.

The results of this investigation may be of value in explaining the role of fatty acids in the oxidation of meat tissue lipids. A correlation between unsaturation of chicken tissue fatty acids and rate of oxidation development exists (14), although oxidation rates, with other factors approximately equal, cannot be predicted from fatty acid composition, or degree of unsaturation alone.[4] Evidence has been presented (13) indicating that the phospholipids of tissues are more unstable than neutral lipids, and are closely involved in the development of oxidation products. Since neutral lipids are apparently of secondary importance in tissue oxidation, it appears that a wide variation in neutral lipid fatty acids could be induced by dietary means without markedly influencing phospholipid fatty acids or without significantly affecting the rate at which tissue oxidation would occur.

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[3] See footnote 2.

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# Effect of Pyridoxine Deficiency upon Polysomes and Messenger RNA of Rat Tissues [1]

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*ABSTRACT* Relation of the role of pyridoxine in nucleic acid metabolism to its function in protein biosynthesis has been investigated. Sedimentation patterns of RNA pulse-labeled with orotic-6-$C^{14}$ acid have been determined in a linear sucrose gradient. With this procedure, a decreased incorporation of labeled orotic acid into the 29S and 18S components of ribosomal RNA and into messenger RNA was observed in liver of pyridoxine-deficient rats. The decreased synthesis of these components of RNA, particularly messenger RNA, was related to the decrease in number of polysomes per unit weight of liver and spleen observed in this deficiency and the resultant decreased capacity of ribosomal preparations from these deficient tissues to incorporate labeled leucine and valine in vitro. These observations on the mode of action of pyridoxine in protein biosynthesis formed the basis for an explanation of the adverse effects of a pyridoxine deficiency upon the development of immune phenomena.

It is now recognized that the structures engaged in protein biosynthesis in the cell cytoplasm are the polysomes (ergosomes). The polysomes are ribosomal aggregates of varying size consisting of individual 73S ribosomes bound by a strand of messenger RNA (m-RNA) which carries the genetic message determining sequential arrangement of amino acids in polypeptides (1–3). The genetic information is translated to a co-linear polypeptide chain by movement of m-RNA relative to the ribosomes (tape mechanism, 4–6). Each ribosome of a polysome constitutes a condensing site through which the m-RNA passes with consecutive exposure of codons and at which a polypeptide chain is growing.

Previous investigations from this laboratory have demonstrated a decreased incorporation of injected L-valine-1-$C^{14}$ into proteins of liver, spleen and serum and into subcellular fractions of liver and spleen in the pyridoxine-deficient rat (7). Rate of incorporation was increased by administration of pyridoxine shortly before injection of labeled valine. Further studies utilizing labeled precursors of nucleic acids revealed that the synthesis of DNA and RNA in liver and spleen was diminished in this deficiency state (8). Pyridoxine-deficient rats possessed fewer cells and a correspondingly decreased content of DNA per milligram of splenic tissue (8). These adverse effects of pyridoxine deficiency upon biosynthesis of nucleic acids and cell multiplication were attributed to the function of pyridoxal phosphate in production of the nucleic acid precursor "active formaldehyde" via the serine-glycine interconversion. The mechanism of pyridoxine action in protein biosynthesis could, accordingly, be linked to its role in nucleic acid metabolism. Present experiments were designed to investigate this relationship in greater depth and entailed studies of the effect of pyridoxine deficiency on the content of polysomes in liver and spleen and the ability of these tissues to incorporate amino acids in vitro. Rate of synthesis of ribosomal RNA (r-RNA) and m-RNA in this deficiency was also investigated. A preliminary report of a portion of these studies has been presented.[2]

## EXPERIMENTAL PROCEDURE

*Animals and diets.* Male, weanling albino rats of the Holtzman strain were used. Care of the animals, composition of control and pyridoxine-deficient diets, and treatment of inanition controls have been described previously (7).

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[1] This study was aided by U.S. Public Health Service Research Grant no. A-727 from the National Institute of Arthritis and Metabolic Diseases, and by the Office of Naval Research under Contract 1833(00), NR 101-412.

[2] Trakatellis, A. C., A. E. Axelrod and M. Montjar 1964 Studies on nucleic acid metabolism in pyridoxine-deficient rats. Federation Proc., 23: 137 (abstract).

*Tracer compounds.* DL-Leucine-1-$C^{14}$ (specific activity = 22.3 mc/mmole), L-valine-1-$C^{14}$ (specific activity = 5.73 mc/mmole), and orotic-6-$C^{14}$ acid hydrate (specific activity = 3.45 mc/mmole) were obtained from the New England Nuclear Corporation, Boston.

*Preparation and fractionation of purified ribosomes (p-ribosomes).* Animals were decapitated after ether anesthesia. Livers and spleens were excised immediately after killing and homogenized in Hoagland's medium A (0.005 M Tris, 0.025 M KCl, 0.005 M $MgCl_2$ and 0.25 M sucrose) with a motor-driven Potter-Elvehjem tissue grinder fitted with a Teflon pestle. All isolation procedures were performed at 0° to 2°. Purified ribosomes (p-ribosomes) were obtained by centrifugation of the deoxycholate-treated postmitochondrial fraction through 2 layers of sucrose (9). Fractionation of the purified ribosomal fraction into polysomes of various aggregate sizes by zone centrifugation analysis in a linear sucrose gradient has been described previously (9).

*Sedimentation pattern of pulse-labeled RNA.* Rats were injected intraperitoneally with 20 μc of orotic-6-$C^{14}$ acid hydrate/100 g body weight and decapitated 4 hours later, after ether anesthesia. Liver p-ribosomes were prepared as described above and their RNA liberated by treatment with sodium dodecyl sulfate (SDS) according to the method of Kurland (10) with minor modifications. One milliliter of 0.5% SDS was added to a ribosomal pellet derived from approximately 1 g of liver. The suspension was stirred continuously with a Potter-Elvehjem all-glass homogenizer for 3 minutes at 37° and then layered over 30 ml of a cold linear sucrose gradient (0.5 to 1.0 M) containing 0.005 M Tris·HCl, pH 7.2. After centrifugation for 40 hours at 25,000 rpm and 1° in the SW 25 Spinco rotor, 1-ml fractions were collected from a needle inserted through the bottom of the centrifuge tube and their absorbancies at 254 mμ determined. Sedimentation constants of RNA components were determined by calibrating the linear sucrose gradient with the 18S and 29S ribosomal RNA (r-RNA) (3).

*Measurement of radioactivity.* After their absorbancies were determined, the collected 1-ml fractions of RNA were assayed for their radioactive RNA content by precipitation with $HClO_4$ according to the method of Staehelin et al. (3) with minor modifications. One milliliter of 0.6 N $HClO_4$ was added to each sample after addition of 20 mg of diatomaceous silica [3] and 0.5 ml of a bovine serum albumin solution (4 mg/ml) serving as a co-precipitant. Resulting precipitates were collected by suction on filter paper disks (Whatman no. 540) layered with 20 mg of diatomaceous silica [4] in a stainless steel filtration apparatus [5] and washed successively with 6 ml of 0.5 N $HClO_4$, twice with 5 ml of isopropanol-ethyl ether (1:2) and finally with 5 ml of isopropanol-ethyl ether-chloroform (2:2:1). Dried precipitates were transferred to glass-counting vials and 1 ml of 1 M *p*-(diisobutyl-cresoxy-ethoxy ethyl) dimethyl benzyl ammonium hydroxide [6] in methanol was added. After 1 hour at room temperature, 9 ml of a 0.5% solution of 2,5-diphenyl-oxazole [7] in toluene were added and radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer.

*Cell-free amino acid incorporating system.* p-Ribosomes of liver or spleen were suspended in 0.4 ml of Hoagland's salt buffer [8] and the following added: $MgCl_2$, 3.2 μmoles; Tris·HCl, pH 7.6, 30 μmoles; β-mercaptoethanol, 2.0 μmoles; potassium phosphoenol pyruvate, 10 μmoles; pyruvate kinase, 10 μg; ATP, 1 μmole; GTP, 0.4 μmole; DL-leucine-1-$C^{14}$, 0.1 μmole; or L-valine-1-$C^{14}$, 0.4 μmole in Hoagland's salt buffer, 0.1 ml of dialyzed post-microsomal supernatant and Hoagland's salt buffer to a final volume of 1 ml. The postmicrosomal supernatant was derived from a 25% rat liver homogenate in Hoagland's medium A after removal of the microsomal fraction by centrifuging at 105,000 × *g* for 80 minutes. This supernatant was dialyzed at 2° for 24 hours against a 50-fold volume of Hoagland's salt buffer containing 0.005 M β-mercapto-etha-

[3] Celite. Johns Manville and Company, Lontoc, California.
[4] See footnote 3.
[5] E-8B precipitation apparatus, Tracerlab, Waltham, Massachusetts.
[6] Hydroxide of Hyamine 10-X. Packard Instrument Company, La Grange, Illinois.
[7] Purchased from the Packard Instrument Company, La Grange, Illinois.
[8] Identical with Hoagland's medium A but with sucrose omitted.

nol. Optimal incorporation was obtained with this system containing p-ribosomes derived from 0.6 to 0.8 g of tissue and 0.1 ml of dialyzed liver supernatant. The dialized liver supernatant was also used in the incorporation system employing splenic ribosomes, since the corresponding preparation from spleen gave poor results. This observation has been made previously by others (1). In some experiments a mixture of 19 L-amino acids exclusive of the labeled amino acid was added. This mixture supplied 0.05 μmoles of each amino acid participating in the currently recognized coding system (12). After incubation at 37° for the desired time, incorporation was stopped by addition of 1 ml of 1 M NaOH. The mixture was kept at room temperature for 1 hour to remove radioactive leucine bound to transfer RNA and the protein was then precipitated with $HClO_4$, filtered, washed, solubilized with *p*-(diisobutyl-cresoxyethoxy ethyl) dimethyl benzyl ammonium hydroxide [9] and radioactivity measured in a Packard Tri-Carb scintillation spectrometer (2). Blank controls utilized in each experiment were kept at 0° and 1 M NaOH added at zero time. Counting efficiency was approximately 54%. Maximal deviation between duplicate samples above 100 counts/min was $< 10\%$ of the mean, whereas that above 1000 counts/min was $< 5\%$ of the mean.

## RESULTS

Experiments were conducted after the animals had been fed the experimental diets for 7 to 9 weeks. At this time, weights of pyridoxine-deficient, inanition controls, and ad libitum-fed controls were 95 to 115, 101 to 120, and 250 to 310 g, respectively. Each experiment was performed at least 4 times. The same pattern of results was observed in each case and only typical data comparing results obtained with pyridoxine-deficient rats and their inanition controls are presented in the figures. In all experiments, effects of the deficiency state were only slightly more pronounced if comparisons were made with ad libitum-fed controls. However, we have presented data only of inanition controls in order to minimize the factor of inanition present in pyridoxine-deficient rats.

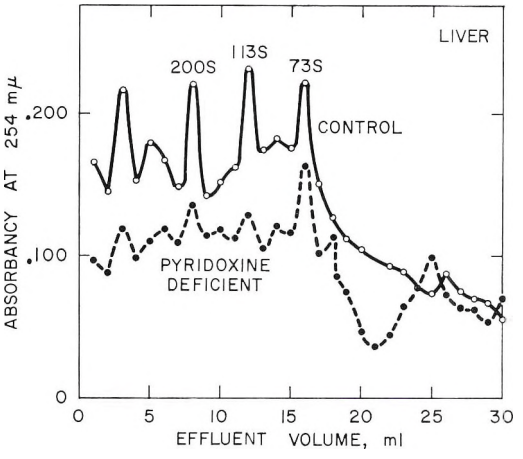


Fig. 1 Zone centrifugation analysis of purified liver ribosomes. Purified ribosomes (p-ribosomes) prepared from 0.5 g of liver obtained from a pyridoxine-deficient rat or its inanition control were layered as an inverted gradient (0–0.25 M sucrose) over 28 ml of a linear sucrose gradient (0.3–1.0 M). After centrifugation for 4 hours at 25,000 rpm and 1° in the SW 25 Spinco rotor, 1-ml fractions were collected from a needle inserted through the bottom of the centrifuge tube and their absorbancies at 254 mμ determined. Sedimentation constants are indicated at various peaks by *S* values.

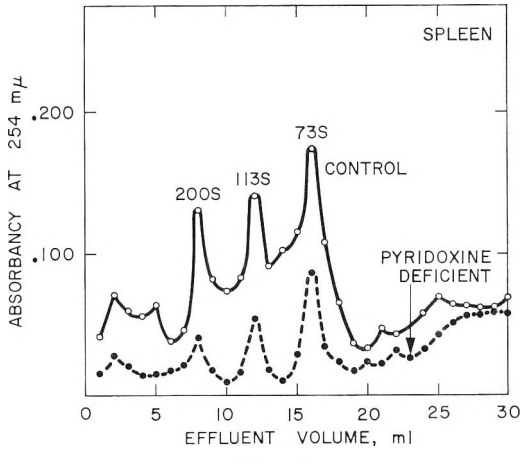


Fig. 2 Zone centrifugation analysis of purified splenic ribosomes. Purified ribosomes (p-ribosomes) were prepared from 0.3 g of spleen obtained from a pyridoxine-deficient rat or its inanition control. Ribosomes were fractionated with a linear sucrose gradient as described in figure 1.

[9] See footnote 6.

*Content of polysomes.* Zone centrifugation analysis of tissue polysomes is illustrated in figures 1 and 2. Number and state of aggregation of the polysomes are depicted by peaks in these diagrams. The polysomal population consists of a spectrum of aggregates of varying size reflecting their content of individual 73S ribosomes (monomers). Thus, the number of 73S ribosomes per polysomal aggregate increases with increase in sedimentation constant (S) of the observed peaks. Quantity of polysomes of each size is given by the height of the peak, namely, the degree of absorption at 254 mμ.

In comparison with its inanition control, the pyridoxine-deficient rat possessed fewer polysomes per unit weight of liver (fig. 1) and spleen (fig. 2) in each category of aggregate size. A more quantitative estimate of this decrease can be obtained from the in vitro incorporation studies discussed later in this paper.

*Incorporation of amino acids in vitro.* Abilities of purified ribosomes to incorporate labeled valine and leucine were studied in a cell-free system and results obtained are presented in figures 3–5. The ribosomal preparation derived from liver of pyridoxine-deficient rats possessed a reduced ability to incorporate DL-leucine-1-$C^{14}$ (fig. 3) and L-valine-1-$C^{14}$ (fig. 4) when compared to a ribosomal preparation isolated from liver of an inanition control. A similar effect of pyridoxine deficiency upon capacity of a splenic ribosomal preparation to incorporate DL-leucine-1-$C^{14}$ was also noted (fig. 5). Direct comparisons could be made since, in all cases, the ribosomal preparations were derived from the same weight of original tissue. In both tissues, the incorporating abilities of ribosomal preparations from deficient animals were 60 to 75% of those obtained from inanition controls.

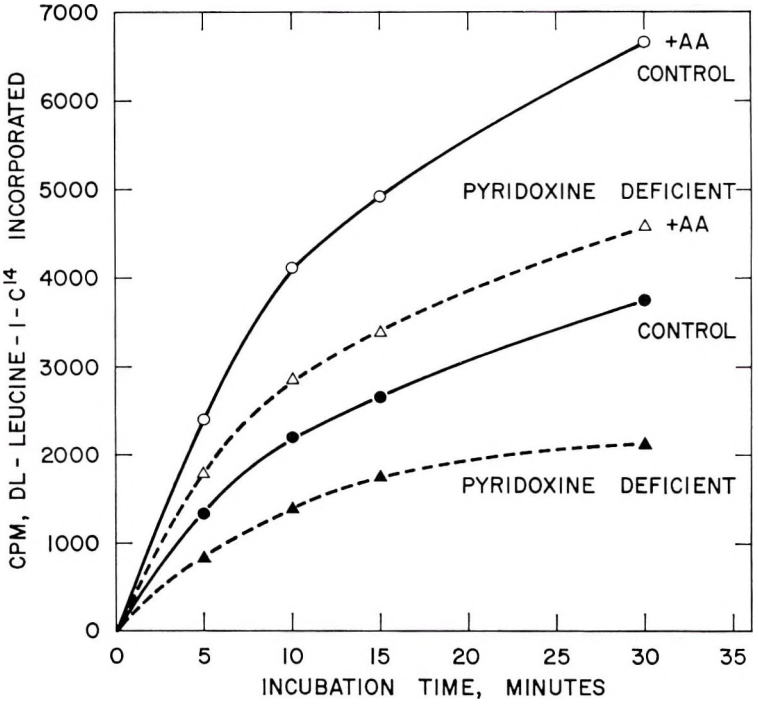


Fig. 3 In vitro incorporation of DL-leucine-1-$C^{14}$ by a cell-free system of liver. Purified ribosomal preparations were isolated from 0.8 g of liver obtained from a pyridoxine-deficient rat or its inanition control. Experimental details are given in the text. Addition of the mixture of 19 L-amino acids (see text) to the incorporating system is indicated by +AA.

The stimulatory effect of the mixture of 19 L-amino acids upon incorporation by ribosomal preparations from normal rats has also been observed by Wettstein et al. (2). In our experiments, this stimulation was evidenced in ribosomal preparations isolated both from normal and pyridoxine-deficient rat liver (fig. 3).

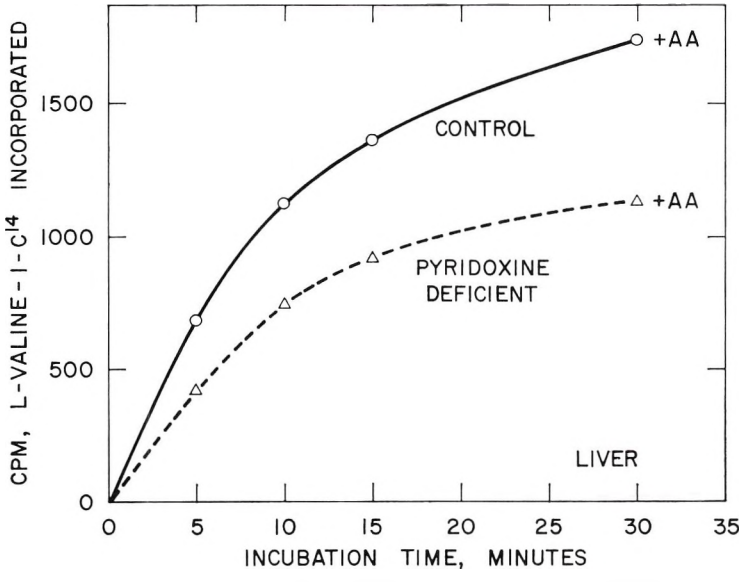


Fig. 4 In vitro incorporation of L-valine-1-$C^{14}$ by a cell-free system of liver. Experimental conditions were identical with those described in legend of figure 3.

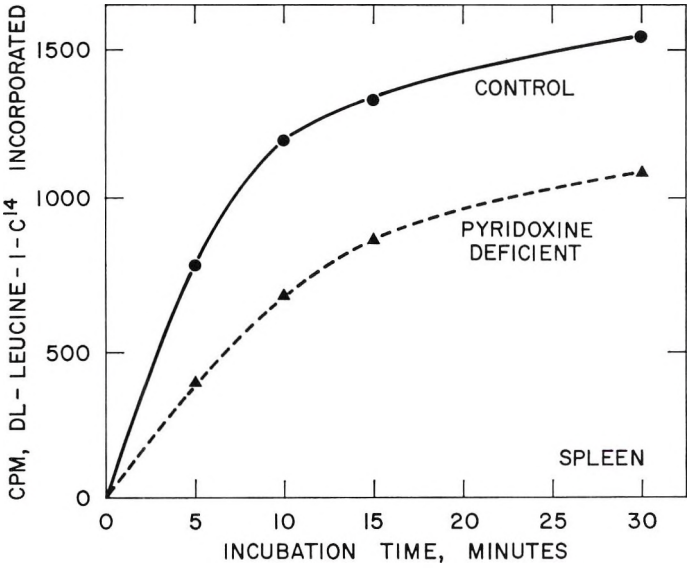


Fig. 5 In vitro incorporation of DL-leucine-1-$C^{14}$ by a cell-free system of spleen. Purified ribosomal preparations were isolated from 0.8 g of spleen obtained from pooled organs of pyridoxine-deficient or inanition control rats. Experimental conditions were identical with those described in legend of figure 3.

We utilized a dialyzed post-microsomal supernatant fraction prepared from liver of a control rat in all incorporation experiments reported in this paper. Similar results were obtained with a dialyzed post-microsomal supernatant fraction prepared from liver of pyridoxine-deficient rats.[10]

*Incorporation of labeled orotic acid into ribosomal RNA.* Effect of pyridoxine deficiency upon biosynthesis of the 18S and 29S components of ribosomal RNA (r-RNA) and of messenger RNA (m-RNA) was investigated in pulse-labeled experiments with orotic acid. Essentially, such experiments involved determination of absorbancy at 254 mμ (as measure of RNA) and radioactivity of fractions of RNA obtained from liver ribosomes 4 hours after injection of orotic-6-$C^{14}$ acid hydrate. RNA was fractionated on a linear sucrose gradient. A typical sedimentation pattern of pulse-labeled RNA extracted from purified liver ribosomes of a control rat is shown in figure 6. This pattern has been analyzed extensively in recent studies from other laboratories (3) as well as in our own.[11] The diagram clearly shows 3 radioactive peaks, *a*, *b*, and c and an area *b* to c with high radioactivity. The radioactive peaks, *a* and *b* coincide with the 29S and 18S r-RNA, respectively. Peak c which corresponds to an RNA component of approximately 5S consists of transfer RNA (3). The radioactive area, *b* to c, possesses a high specific activity and is obviously heterogeneous with components possessing sedimentation rates ranging from approximately 5S to 20S. This area overlaps with the 18S r-RNA and 5S components (3). These characteristics of the *b* to c area have been assigned to bacterial as well as rat liver m-RNA (3). A sedimentation pattern of pulse-labeled RNA extracted from purified liver ribosomes of a pyridoxine-deficient rat is shown in figure 7. Specific activities of the 29S and 18S components of r-RNA and of m-RNA are considerably less than the activities of corresponding RNA fractions of control rats (fig. 6).

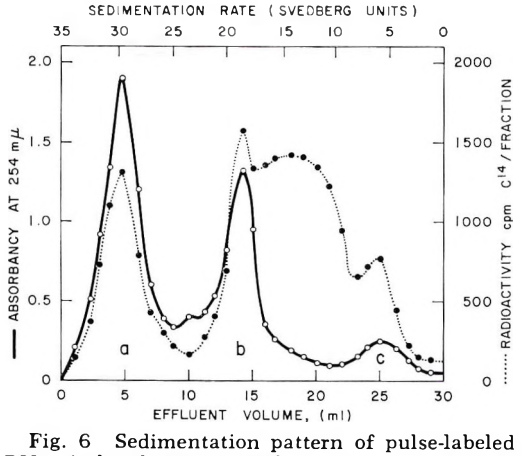


Fig. 6 Sedimentation pattern of pulse-labeled RNA isolated from purified ribosomes derived from 1.0 g of liver of a control rat. Experimental details are given in the text.

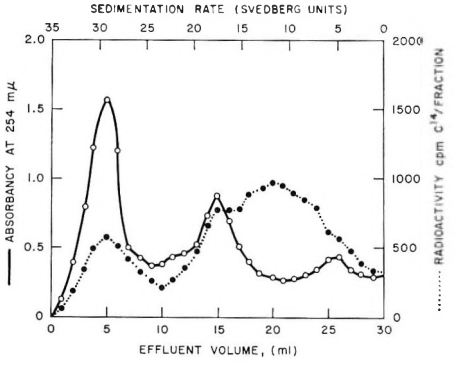


Fig. 7 Sedimentation pattern of pulse-labeled RNA isolated from purified ribosomes derived from 1.0 g of liver of a pyridoxine-deficient rat. Experimental procedures were identical with those utilized in experiment of figure 6.

## DISCUSSION

We reported previously that synthesis of DNA and RNA was impaired in pyridoxine deficiency. This is in agreement with our present experiments demonstrating a decreased incorporation of labeled orotic acid into ribosomal and messenger RNA. The deleterious effect of pyridoxine deficiency upon synthesis of these components of RNA, particularly messenger RNA, suggests a role for pyridoxine in protein synthesis, since messenger RNA is involved both in translation of genetic information to polypeptide synthesis and, as a binding substance, in formation of polysomal aggregates. Thus, the decreased number of polysomes per unit weight of

[10] Montjar, M., A. E. Axelrod and A. C. Trakatellis, unpublished observations.

[11] Trakatellis, A. C., A. E. Axelrod and M. Montjar, unpublished observations.

liver and spleen and the resultant decreased capacity of these tissues to incorporate labeled amino acids in vitro observed in our experiments can be referred to the inhibitory effect of a pyridoxine deficiency upon the synthesis of ribosomal and messenger RNA. This concept of the mechanism of action of pyridoxine also affords a rational explanation for the decreased incorporation of injected labeled valine into proteins of the pyridoxine-deficient rat previously noted (7).

This hypothesis developed to integrate the role of pyridoxine in nucleic acid metabolism with its effect on protein metabolism and cellular proliferation can be extended to explain the adverse effects of pyridoxine deficiency upon development of various immune phenomena (13). It is known that administration of an antigen stimulates intensive multiplication of host cells in certain organs concerned with immune responses, e.g., spleen and lymph nodes. Although the mechanism by which the antigen excites this proliferating process is not clear at present, there is no doubt that accelerated synthesis of DNA is required at this step in the immune process. By inhibiting DNA synthesis, a deficiency of pyridoxine could prevent the required cellular proliferation and, consequently, inhibit antibody production. Our previous observation that pyridoxine-deficient rats possessed fewer cells per unit weight of splenic tissue conforms with this concept. Furthermore, an accelerated production of specific messenger RNA possessing the information necessary for synthesis of antibody protein would be expected after antigenic stimulation under normal conditions. In pyridoxine deficiency, therefore, the impairment of synthesis of m-RNA, as well as ribosomal RNA, could also effectively reduce antibody synthesis. Thus, the deleterious effects of a pyridoxine deficiency upon immunological phenomena may be exerted at the site of cellular proliferation as well as on the synthetic capacity of the cell. These possibilities are being explored in studies with immunized animals.

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# The Lactation Value, a New Index of Protein Evaluation [1,2]

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*ABSTRACT* A new index is described for the evaluation of food proteins. It depends upon 3 simple measurements: body weight change occurring in the lactating mother, gain in body weight of litter during the first 14 days after birth and amount of protein consumed by the adult female during this period. The algebraic addition of the body weight changes of mother and litter expressed as per gram of protein consumed yielded an index which has been called the lactation value of the protein. Ten foods were investigated by this standardized technique and simultaneously by the conventional growth promoting value method.

The nutritive value of food proteins has been determined in the past by a variety of assay methods each of which uses a different parameter to attain the desired objective. The 2 assays most widely used have been the growth-promoting and the nitrogen balance methods. The parameter of the first is the gain in weight per gram of protein consumed, and that of the second is the nitrogen balance established with a specific protein ration. Several other methods have been described for the biological evaluation of food proteins, some having used reproduction and lactation performances as parameters.

The lactation performance in the rat has been quantitatively studied by indirect means using as criteria of measurement the number of young which survive weaning or the weight increment during specified periods. Using the first criterion, Clayton (1) studied the comparative value of milk, egg, and meats for reproduction and lactation in the rat and more recently Schultze (2–6), in a series of studies, investigated the adequacy of some food proteins and mixtures of pure amino acids for these same functions. Using weight increments as a criterion Cowie and Folley (7) studied the endocrine control of lactation; French et al. (8) investigated the influence of dietary fat and carbohydrate on reproduction and lactation; and Daggs (9), as part of a series of studies on lactation, described a method for its quantitative evaluation in the rat. This method depended on the logarithm of the weights of the young from the fourth to the tenth and from the eleventh to the seventeenth day of life plotted against age in days. The slope of these 2 lines which represented the growth rate constants were arbitrarily combined by simple addition and dropping of the decimal point into a figure which Daggs called lactation index. Later, he and Tomboulian (10) applied this method to study the effect of various dietary principles on lactation in rats. However, no one appeared to have considered the possibility of utilizing the gain in weight per gram of protein consumed by the mother rat-litter complex during lactation period as a parameter for protein evaluation.

It is the object of the present investigation to report the results obtained when food proteins are assayed by this standardized technique together with those obtained by the conventional growth-promoting method.

## EXPERIMENTAL

*Rations.* The experimental rations for both the lactation value and the growth-promoting techniques were all similar in composition except for the protein food which varied from ration to ration. The legumes were cooked by boiling in plain tap water until soft, then dried, including

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[1] This work was aided, in part, by grants from the Food and Nutrition Board, National Academy of Science — National Research Council of the United States and from the Nutrition Committee of Puerto Rico.

[2] A preliminary report of this work was presented at the 6th International Congress of Nutrition, Edinburgh, August, 1963.

any water left after boiling, at 65° to 70° in a hot-air oven and ground to 60 mesh. They were then analyzed for protein content. Enough of the analyzed protein food was added to make the protein content of the final ration 12% (N × 6.25) for the lactation value determination, and 10% for the growth-promoting value method. The other ingredients were as follows: (in per cent) corn oil,[3] 10; cellulose,[4] 4; salt mixture, USP XIV, 4; vitamin fortification mixture,[5] 2; and cornstarch to make 100. After compounded, all rations were analyzed for protein content by the official Kjeldahl method. Animals were fed ad libitum and food consumption was accurately recorded.

*Animals.* For the lactation value determination, litters from normal female rats, Wistar-School of Tropical Medicine strain, reared with laboratory chow,[6] were selected. Only first or second litters with at least 8 healthy young were used. Both mother and the whole litter were weighed the day of parturition. The mean body weight of the mothers varied from 263 to 320 g. Litter size was made constant by reducing the number of sucklings to six, three females and three males whenever possible. The mean body weight of the reduced litter at birth varied from 37.0 to 41.4 g. Any litter reduced by the death of one or more young was discarded. Litters were fed the experimental ration within the first 24 hours after parturition. For the final calculation those litters where the mother successfully fed and weaned to 21 days the 6 young alloted to her at birth were used. However, the weight used for the calculations was that attained at the end of he fourteenth day of life, that is, before the young started to consume any of the diet. Also, the change in body weight occurring in the mother during this period was used for the final calculation.

The nutritive index, called by us lactation value of a protein, is the algebraic sum of the weight changes of mother rat and standardized litter during the 14 days following parturition expressed as per gram of protein consumed.

Protein efficiency ratio determinations were simultaneously carried out using weanling male rats, Wistar-School of Tropical Medicine strain, 28 days of age, 12 for each ration. The assay period was 4 weeks. The animals were individually caged, and feeders, which reduced food spillage to a minimum, were provided for more accurate recording of consumption.

## RESULTS AND DISCUSSION

The results obtained for the 10 protein foods examined by this technique are presented in tables 1 and 2.

Table 1 summarizes the performance of the female rats and their litters during the lactation period (14 days following parturition). Each food protein was assayed using at least 6 mother-litter complexes although in some cases as many as 21 were used. All the mothers lost weight even when egg protein was fed. This was due to the low level of protein (12%) supplied by the experimental rations. We had to choose this level because the protein concentration in many of the legumes examined did not permit a higher percentage of protein in the ration without altering its uniform composition. This limitation subjected the mothers, in particular, to severe stress, thus probably increasing the sensitivity of the technique.

The results obtained indicate, in general, that there is a close inverse relationship between body weight loss by the adult females and both the mean gain in weight of the litters and the amount of protein consumed.

The relative size of the nursing young of mothers fed diets containing red kidney beans, chick peas, or whole dry egg is illustrated in figures 1–3.

The lactation values thus obtained are summarized in table 2 together with the growth-promoting values simultaneously determined.

The relative rank of the different food proteins investigated by the 2 methods agreed closely. The only 2 instances where there is not exact agreement are those of purified casein and great-northern beans whose indexes by the growth-promoting value method rank one step higher than by the lactation value technique.

[3] Mazola, Corn Products Company, Argo, Illinois.
[4] Cellu Flour, The Chicago Dietetic Supply House, Inc., Chicago.
[5] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland.
[6] Rockland Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois.

TABLE 1

*Performance of female rats and litters during lactation period (14 days)*

| Protein-rich food | No. of litters | Mean body wt loss of adult females | Mean body wt gain of litter at 14th day | Mean total protein consumed |
|---|---|---|---|---|
| | | *g* | *g* | *g* |
| Whole dry egg | 11 | 25.4 | 134.9 | 35.75 |
| Chick pea (*Cicer arietinum*), market | 10 | 40.0 | 95.1 | 25.15 |
| Purified casein | 21 | 52.2 | 102.1 | 28.63 |
| Pigeon pea (*Cajanus cajan*), green, frozen | 6 | 39.5 | 78.4 | 36.93 |
| Great northern bean (*Phaseolus vulgaris*), market | 10 | 69.1 | 71.4 | 24.76 |
| Lima bean, Calif. var. Ventura (*Phaseolus lunatus*) | 10 | 68.5 | 67.1 | 24.28 |
| Native white bean, var. Bonita (*Phaseolus vulgaris*) | 10 | 66.5 | 61.1 | 27.47 |
| Small white no. 38 bean, Calif. (*Phaseolus vulgaris*) | 11 | 73.5 | 61.7 | 20.59 |
| Red kidney bean, Calif. (*Phaseolus vulgaris*) | 10 | 80.0 | 60.9 | 15.73 |
| Native red bean, var. Bolito (*Phaseolus vulgaris*) | 11 | 96.5 | 57.4 | 20.38 |

TABLE 2

*Lactation and growth-promoting value of protein-rich foods*

| Protein-rich food | Lactation value | Growth-promoting value |
|---|---|---|
| Whole dry egg | $+3.06 \pm 0.12$ [1] | $4.20 \pm 0.12$ |
| Chick pea (*Cicer arietinum*), market | $+2.19 \pm 0.08$ | $2.23 \pm 0.15$ |
| Purified casein | $+1.74 \pm 0.21$ | $2.95 \pm 0.10$ |
| Pigeon pea (*Cajanus cajan*), green, frozen | $+1.05 \pm 0.20$ | $1.59 \pm 0.06$ |
| Great northern bean (*Phaseolus vulgaris*), market | $+0.09 \pm 0.08$ | $1.89 \pm 0.03$ |
| Lima bean, Calif. var. Ventura (*Phaseolus lunatus*) | $-0.06 \pm 0.14$ | $1.14 \pm 0.05$ |
| Native white bean, var. Bonita (*Phaseolus vulgaris*) | $-0.19 \pm 0.17$ | $1.40 \pm 0.10$ |
| Small white no. 38 bean, Calif. (*Phaseolus vulgaris*) | $-0.57 \pm 0.32$ | $1.14 \pm 0.10$ |
| Red kidney bean, Calif. (*Phaseolus vulgaris*) | $-1.21 \pm 0.32$ | $0.88 \pm 0.06$ |
| Native red bean, var. Bolito (*Phaseolus vulgaris*) | $-1.92 \pm 0.29$ | $0.22 \pm 0.07$ |

[1] SE of mean.

The determination of the lactation value index of food proteins could be relatively rapid and easily performed in a nutrition laboratory which maintains a good breeding colony. This standardized technique could be of particular importance in providing specific information as to the value of protein foods for the very important function of milk production and mother-litter maintenance and development during the preweaning period.

The results presented here, in which the lactation value technique is described, are part of a more comprehensive investigation in which legume proteins have been evaluated by a variety of biological and chemical procedures. These studies should prove to be of considerable practical importance for those areas where legume proteins constitute a significant item of the daily diet, and a selection of the proper type of legume may contribute signifi-

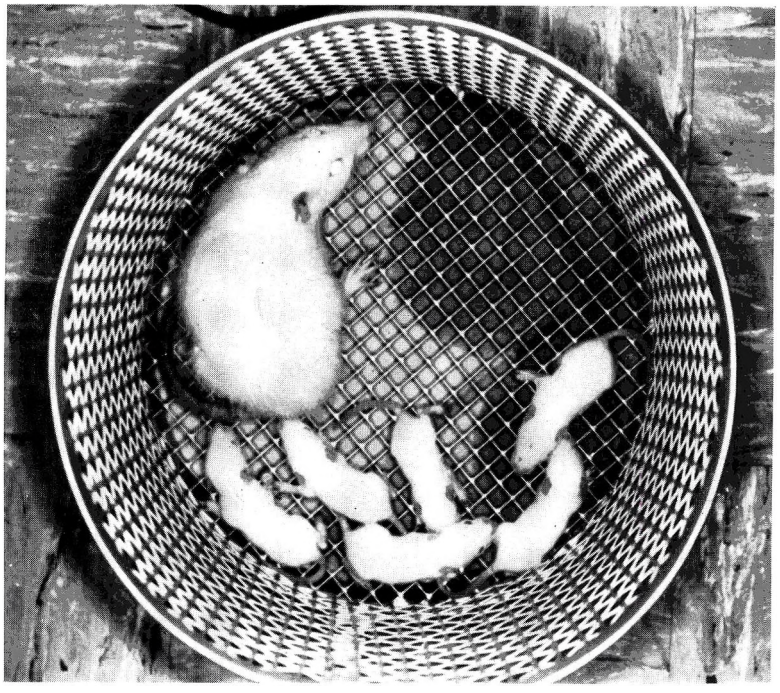

Fig. 1 Influence of dietary protein source on lactation performance — lactating female rat fed red kidney beans (litter 14 days old).

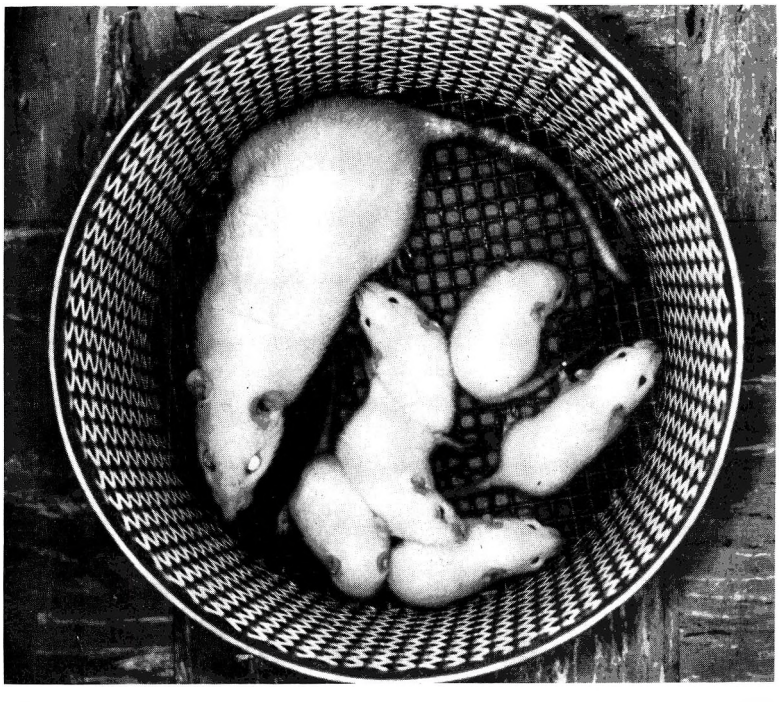

Fig. 2 Influence of dietary protein source on lactation performance — lactating female rat fed chick peas (litter 14 days old).

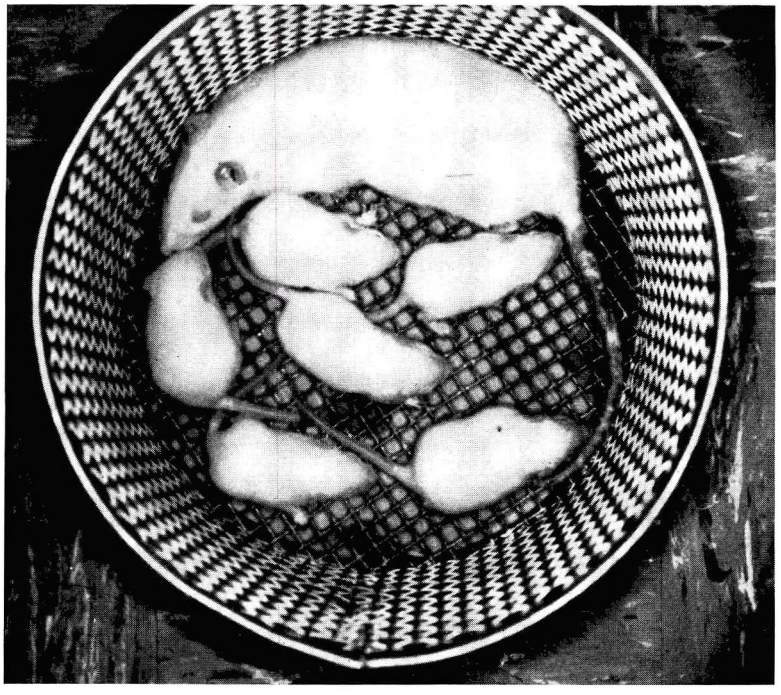

Fig. 3 Influence of dietary protein source on lactation performance — lactating female rat fed whole dry egg (litter 14 days old).

cantly toward improving the diet of expectant and nursing mothers and also of infants and school children.

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# Effect of Dietary Energy Intake on Protein Deficiency Symptoms and Body Composition of Baby Pigs Fed Equalized but Suboptimal Amounts of Protein [1]

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*ABSTRACT* Yorkshire pigs weaned at 3 to 5 weeks of age, weighing an average of 7.2 kg initially in experiment 1 and 4.9 kg in experiment 2, were pair-fed 3% protein purified diets containing 5 or 25% corn oil. The daily feed intake of the member of each pair fed the low fat diet was equalized with the ad libitum consumption of its mate fed the high fat diet. The total calorie consumption per pig was approximately 29% greater in the high fat group than in the low fat group. A similar diet containing 24% protein was fed as a positive control diet at a level of 4.5% of body weight daily in experiment 1 and 4.0% in experiment 2. Total serum protein level and percentage of albumin decreased significantly ($P < 0.01$) after feeding the low protein diets for 8 weeks. Pigs fed the low protein-high fat diet showed a more severe depression in serum proteins than those fed the low protein-low fat diet even though protein intake was equal for the 2 groups. Liver lipid concentration was 34.1% in the pigs fed low protein-high fat as compared with 9.7% in those fed low protein-low fat and 12.3% in positive control pigs. Pigs in the former group developed typical kwashiorkor-like symptoms, including severe edema in the umbilical and genital regions and general feebleness and apathy. In contrast, pair-fed mates fed the low protein-low fat diet failed to develop these symptoms and remained thin in appearance and active, indicating 2 different manifestations of protein deficiency, one resembling kwashiorkor, the other marasmus. Gross energy concentration of whole empty bodies of pigs fed the 2 low protein diets was similar (6.5 vs. 6.2 kcal/g of dry sample) but that of positive control pigs was significantly lower ($P < 0.01$) (5.6 kcal /g). This similarity in energy storage in the 2 low protein groups, despite the 29% difference in calorie intake, indicates not only an increased specific dynamic effect with low protein diets, but an additional increase when greater calorie intake accompanies protein deficiency.

The biochemical and anatomical changes observed in the protein-deficient pig (1–3) are similar to those reported in human infants suffering from severe protein deficiency (4–9). In the work of Lowrey et al. (1) no attempt was made to equalize protein intake among groups, and hence the severity of the symptoms was associated with the amount of protein consumed as well as with the protein quality and energy intake.

The present work was designed to study the effect of energy intake of baby pigs fed equalized but suboptimal amounts of protein on: 1) the development of signs of protein deficiency, and 2) the chemical composition of the whole body.

## EXPERIMENTAL

*General.* Yorkshire pigs weaned at 3 to 5 weeks of age were used in 2 experiments. They were assigned at random by trios to dietary treatments according to litter and body weight and kept individually in concrete-floor pens with plywood partitions. Water was supplied ad libitum and feed was given as a gruel in metal troughs in 2 equal morning and evening portions. The composition of the diets used in experiments 1 and 2 is shown in table 1. Pigs fed the 2 low protein diets

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TABLE 1

*Composition of diets (exps. 1 and 2)*

| | Exp. 1 diets | | | Exp. 2 diets | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| | Low protein-low fat | Low protein-high fat | High protein | Low protein-low fat | Low protein-high fat | High protein |
| | % | % | % | % | % | % |
| Casein [1] | 3.0 | 3.0 | 24.0 | — | — | — |
| Soybean meal [2] | — | — | — | 6.00 | 6.0 | 48.0 |
| Glucose [3] | 59.4 | 35.4 | 38.4 | 60.66 | 38.66 | 18.66 |
| Dextrin [4] | 25.0 | 25.0 | 25.0 | 25.00 | 25.00 | 25.00 |
| Corn oil [5] | 5.0 | 5.0 | 5.0 | 3.00 | 25.00 | 3.00 |
| Hydrogenated vegetable oil [6] | — | 24.0 | — | — | — | — |
| Salt mixture [7] | 4.0 | 4.0 | 4.0 | 5.34 | 5.34 | 5.34 |
| Dicalcium phosphate | 0.6 | 0.6 | 0.6 | — | — | — |
| Vitamin mixture [8] | 3.0 | 3.0 | 3.0 | + | + | + |
| Metabolizable kcal/g | 4.1 | 5.3 | 4.0 | 3.9 | 5.0 | 3.6 |

[1] Crude, 30-mesh, National Casein Company, New York.
[2] Solvent extracted, dehulled, 50% crude protein.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] General Biochemicals, Inc., Chagrin Falls, Ohio.
[5] Mazola, Corn Products Company, Argo, Illinois.
[6] Primex, Procter and Gamble, Cincinnati.
[7] Jones, J. H., and C. Foster, J. Nutrition, 24: 245, 1942, (obtained from Nutritional Biochemicals Corporation, Cleveland). Fortified with $ZnCO_3$ and $CuSO_4 \cdot 7H_2O$ to supply 100 ppm Zn and 15 ppm Cu in the diet, respectively, in experiment 1. Composition as follows in experiment 2: (g/kg of diet) $CaHPO_4 \cdot 2H_2O$, 15.4; $CaCO_3$, 12.32; $KH_2PO_4$, 17.16; NaCl, 6.16; $CuSO_4 \cdot 7H_2O$, 0.57; $FeSO_4 \cdot XH_2O$, 0.66; $MnSO_4$, 0.185; $ZnCO_3$, 0.66; MgO, 0.818; $CoCl_2$, 0.011; KI, 0.0004.
[8] Vitamin mixture for experiment 1 supplied per kilogram of diet: (in milligrams) $\alpha$-tocopheryl acetate, 110; menadione, 49.5; thiamine·HCl, 22; riboflavin, 22; niacin, 99; Ca pantothenate, 66; pyridoxine·HCl, 22; inositol, 110; *p*-aminobenzoic acid, 110; folic acid, 0.2; biotin, 0.44; ascorbic acid, 990; and choline chloride, 1.65 g; vitamin $B_{12}$, 30.0 $\mu$g; and vitamin A, 1980 IU; vitamin $D_3$, 220 IU (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation, Cleveland). Vitamin mixture for experiment 2 supplied per kilogram of diet: (in milligrams) $\alpha$-tocopheryl acetate, 45; menadione, 2.0; thiamine·HCl, 1.2; riboflavin, 3.0; niacin, 20.0; Ca pantothenate, 12.0; pyridoxine, 1.0; *p*-aminobenzoic acid, 100; folic acid, 1.0; biotin, 1.0; and (in grams) inositol, 1.0; choline chloride, 1.0; and vitamin $B_{12}$, 20.0 $\mu$g; vitamin A, 1600 IU; Vitamin $D_3$, 200 IU.

in each experiment were pair-fed, based on the voluntary diet consumption the previous day by the mate receiving the high fat diet. Pigs fed the high protein diet were given feed equal to 4.5% of their body weight daily in experiment 1 and 4.0% in experiment 2. Body weights were recorded twice weekly and feed intake of pigs fed high protein diets was adjusted accordingly.

Room temperature was maintained near 21° with a thermostatically controlled electric space heater. Wood shavings were used as bedding.

*Experiment 1.* Seventeen pigs, averaging 7.2 kg body weight, taken from 3 litters, were divided into 6 trios (5 trios plus 1 pair) and assigned to casein diets as shown in table 1. The protein-deficient diets contained 3.0% casein (2.55% protein). Lowrey et al. (1) obtained slow but constant body weight gains with a similar diet containing 5% protein from casein, and only minor protein deficiency symptoms were observed. The level of protein was reduced in the present experiment in an attempt to maintain body weight gains at or near zero and produce more severe protein deficiency symptoms. A blood sample was obtained from the anterior vena cava of each pig at the beginning of the experiment for determination of total serum proteins (10) and serum protein fractions using the Spinco Model R paper electrophoresis system. After 51 days, 2 pairs of pigs fed low protein that showed symptoms of advanced protein deficiency were changed from the depletion diets along with their high protein mates. Blood was obtained from these pigs for serum protein determination. The remaining 12 pigs (4 trios) were continued on experiment until day 87 when blood samples were obtained as above. All depleted pigs were refed the high protein diet for repletion. During the first 3 weeks of repletion

periodic blood samples were taken for blood ammonia determination. A preliminary report of results has been given.[2]

*Experiment* 2. Twenty-four pigs, averaging 4.9 kg body weight, taken from 6 litters, were divided into 8 trios and assigned to solvent-extracted, dehulled soybean meal diets as shown in table 1. Preliminary unpublished data had indicated that pigs fed low protein diets containing soybean protein developed more severe protein deficiency symptoms, including edema, than those fed casein at the same level of protein. Four of the 8 trios were selected for slaughter after 58 or 60 days on experiment to obtain body composition data. The survivors from the remaining 4 trios were refed the high protein diet to obtain information on the permanency of biochemical, pathological and behavioral changes associated with protein deprivation. A preliminary summary of these results has been reported.[3]

Blood samples were obtained as in experiment 1, from 4 trios at the beginning of the experiment and after 58 or 60 days for serum protein determinations. The pigs to be used for body composition measurements were fasted approximately 18 hours before being killed by ether. The liver of each pig was removed for ether extraction and the right femur was removed and its length measured with calipers. The contents of the gastrointestinal tract were removed and the urinary bladder emptied. The weight of the whole body minus the gastrointestinal contents and urine was recorded as the "empty body weight." Care was taken to minimize loss of blood from the carcass. There was some unmeasured loss of subcutaneous fluids during dissection, from pigs with extensive edema, representing mainly the low protein-high fat dietary group. These losses were minimized insofar as possible. Tissue samples from several organs were saved for histopathology. The results will be reported elsewhere. The whole empty body of each pig was frozen, then ground 7 times through a large auger-type meat grinder. A representative sample of approximately 500 g was saved for analysis. This sample was freeze-dried[4] then ground to a fine powder through a Wiley mill. Dry ice was mixed with the sample to maintain a low temperature of the carcass lipids during grinding. The finely ground sample was then exposed at room temperature overnight to allow release of all carbon dioxide and was stored at 5° in an air-tight bottle until analysis was performed. Gross energy was determined by bomb calorimetry and protein, ether extract and ash by proximate analysis.

## RESULTS AND DISCUSSION

*Experiment 1.* The body weight, calorie intake and feed intake curves are shown in figures 1, 2 and 3, respectively, and the serum protein patterns are in table 2.

Pigs fed the diets containing 2.55% protein from casein essentially maintained their original body weight at either level of energy intake (fig. 1). This was true despite the fact that those fed the high fat diet consumed approximately 29% more calories over the entire period than their mates fed the low fat diet (fig. 2). The success of the paired feeding technique in terms of achieving equal feed intake is illustrated by the shape of the curves in figure 3. No long-term difficulties were encountered with digestive upsets, although occasional intermittent diarrhea was observed in the late stages of the experiment in pigs fed the low protein-high fat diet (diet 2). Pigs fed diet 2 developed edema in the umbilical and genital areas during the final week before refeeding and appeared more feeble and in a more severe state of protein deficiency than their mates fed the low protein-low fat diet (diet 1). The comparative condition of the 2 groups of pigs corresponds roughly to the description of human infants suffering from marasmus as compared with kwashiorkor, as outlined by Graham and Morales (9).

Both groups of pigs fed low protein diets (diets 1 and 2) showed lower total serum protein values at the end of the experiment ($P < 0.01$) as compared with pigs fed the high protein diet (diet 3). This reduction

[2] Barnes, R. H., W. G. Pond, E. Kwong and G. Fiala 1963 Blood ammonia changes in experimental protein malnutrition. Proc. 6th International Congress on Nutrition, Edinburgh, Scotland, p. 10.

[3] Moore, A. U., R. H. Barnes, W. G. Pond, R. B. MacLeod, H. N. Ricciuti and L. Krook 1964 Behavioral abnormality associated with a kwashiorkor-like syndrome in pigs. Federation Proc., 23: 397 (abstract).

[4] Desivac, F. J. Stokes Corporation, Philadelphia.

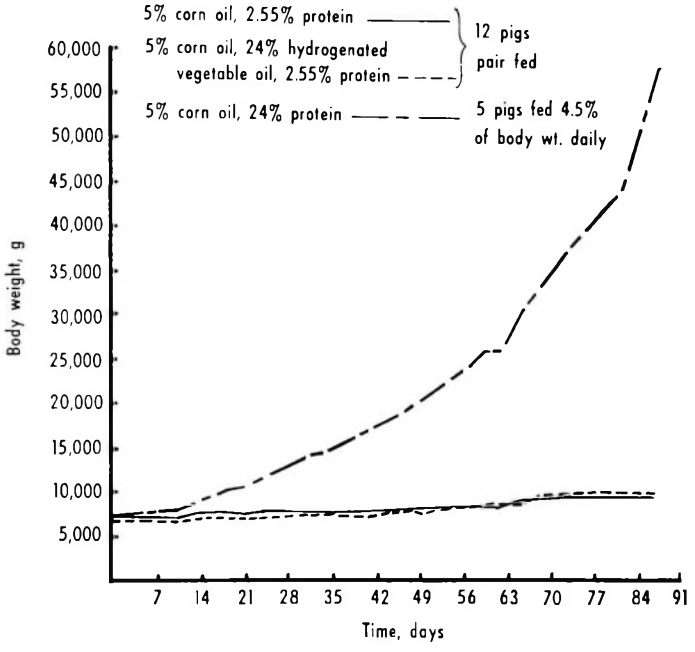


Fig. 1 Effect of diet on body weight (exp. 1).

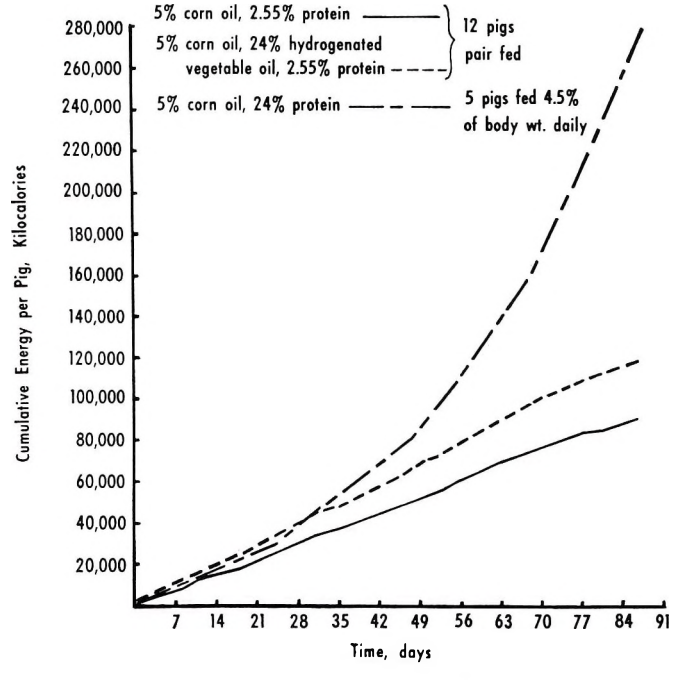


Fig. 2 Cumulative energy consumption (exp. 1).

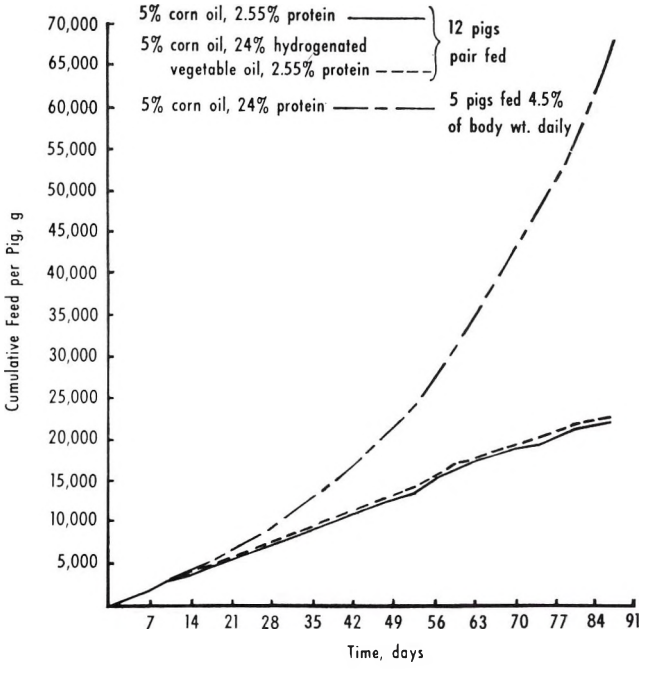


Fig. 3 Cumulative feed consumption (exp. 1).

TABLE 2

*Effect of dietary protein level and of energy level of low protein diets on serum proteins of baby pigs (exp. 1)*

| Diet no. | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| Diet designation | 3% casein + 5% corn oil | | 3% casein + 5% corn oil + 24% hydrogenated vegetable oil | | 24% casein + 5% corn oil | |
| No. of pigs [1] | 6 | | 6 | | 6 | |
| Serum proteins [2] | Initial | Final | Initial | Final | Initial | Final |
| Total, g/100 ml | 4.8 | 5.0 | 5.0 | 4.8 | 6.2 | 7.0** |
| Albumin, % of total | 39.0 | 32.1 | 40.6 | 30.0 | 49.8 | 50.2** |
| $\alpha_1$-Globulin, % of total | 7.3 | 5.3 | 5.3 | 4.9 | 4.3 | 2.7* |
| $\alpha_2$-Globulin, % of total | 25.7 | 27.8 | 24.2 | 31.6 | 23.7 | 17.3** |
| $\beta$-Globulin, % of total | 14.1 | 16.9 | 13.5 | 10.5 | 11.9 | 13.2 |
| $\gamma$-Globulin, % of total | 13.8 | 17.9 | 16.3 | 23.1 | 10.5 | 16.6 |

** Significantly different from low protein groups ($P < 0.01$).
* Significantly lower than low protein groups ($P < 0.05$).
[1] Pigs fed diet 1 pair-fed the amount of diet voluntarily consumed by pair mates fed diet 2. Pigs fed diet 3 given 4.5% of their body weight daily. See figures 1, 2, 3 for body weight, calorie intake and feed intake curves.
[2] Two pigs from each diet were sampled at 51 days, the remainder at 87 days. The final means represent the combined 51- and 87-day values.

in total serum proteins was associated with a decrease in the albumin fraction ($P < 0.01$) and an increase in the $\alpha_1$-globulin ($P < 0.05$) and $\alpha_2$-globulin ($P < 0.01$) fractions expressed as a percentage of the total. The decrease in albumin in the 2 protein-deficient groups was evident but not marked. There is slight evidence that the decrease was greater in the pigs receiving the low protein-high fat diet.

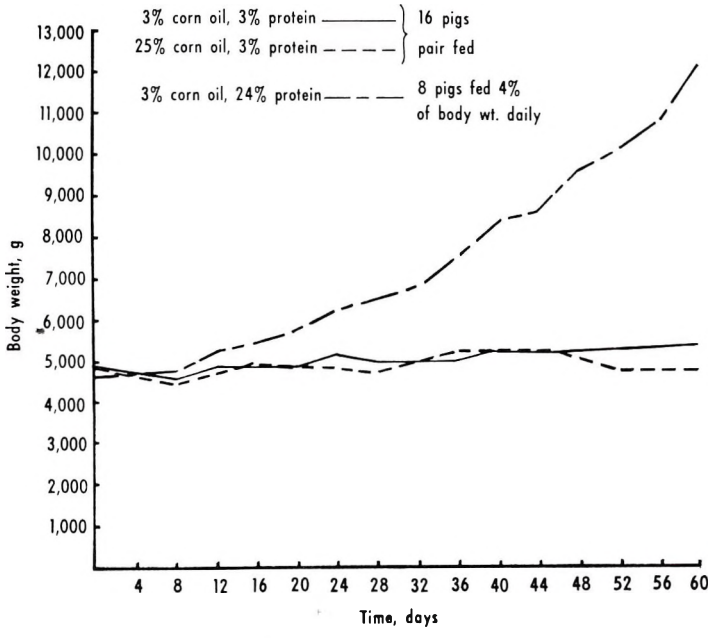


Fig. 4 Effect of diet on body weight (exp. 2).

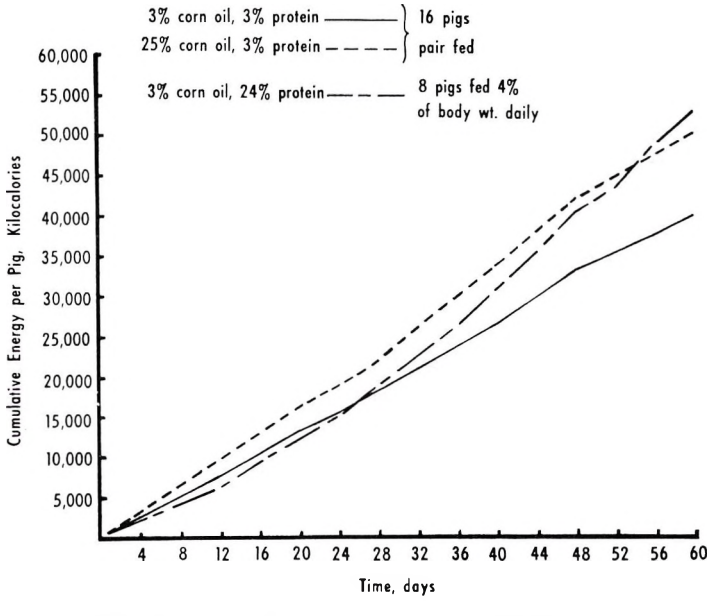


Fig. 5 Cumulative energy consumption (exp. 2).

*Experiment* 2. The body weight, calorie intake and feed intake curves are shown in figures 4, 5 and 6, respectively. Serum protein patterns are summarized in table 3 and body composition and liver fat data in table 4. As in experiment 1, pigs fed the low protein (6% soybean meal) diets failed to gain body weight during the ex-

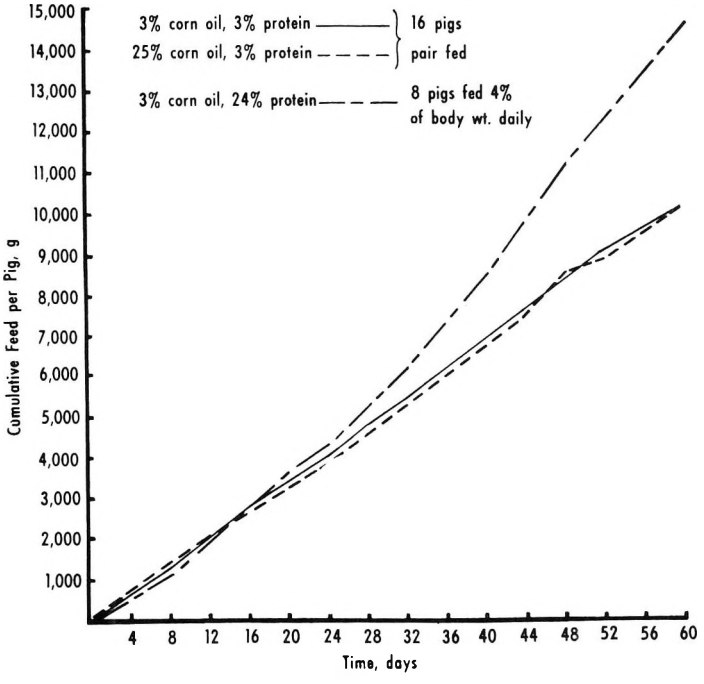


Fig. 6 Cumulative feed consumption (exp. 2).

periment (fig. 4). Feed consumption of pigs fed the low protein diets containing high or low fat was effectively equalized by the paired-feeding technique (fig. 6), but total calorie intake of those fed the high fat diets was approximately 29% greater (fig. 5). No problems were encountered with diarrhea in any treatment group. By day 50, pigs fed the low protein-high fat diet showed typical kwashiorkor-like symptoms, including severe edema in the umbilical and genital regions and general feebleness and apathy. In contrast, pair-fed mates receiving the low protein-low fat diet failed to develop these symptoms and remained thin in appearance and active, indicating 2 different manifestations of protein deficiency, one resembling kwashiorkor, the other marasmus. Terminal blood serum protein showed a significant reduction ($P < 0.01$) in total concentration and in the albumin fraction in pigs fed low protein as compared with pigs fed high protein. Furthermore, the decline in total serum protein and in albumin was greater in pigs fed the low protein-high fat diet (average final total protein for low and high fat groups, 5.7 and 4.4 g/100 ml and % albumin, 29.8 and 15.9, respectively, as compared with 7.2 and 47.2 for the high protein group) than in pair-fed pigs fed the low protein-low fat diet. The serum protein changes were much greater than in experiment 1, in which casein was fed. The initial value for total serum protein in experiments 1 and 2 was lower than the value recorded in control pigs by Lowrey et al. (1) after 6 weeks. This difference is probably related to the increase in total serum protein level that occurs with increasing age in baby pigs (10). Even after the pigs had been supplied with the low protein diet for 12 weeks, serum albumin was not as low as for pigs fed the soybean diet for 8 weeks; and with the soybean diets, high energy intake caused a much greater decrease in albumin than was obtained with the low protein-low fat diet. This difference in serum protein response due to energy intake is notably similar to the rela-

TABLE 3

*Effect of dietary protein level and of energy level of low protein diets on serum proteins of baby pigs (exp. 2)*

| Diet no. | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| Diet designation | 6% soybean meal + 3% corn oil | | 6% soybean meal + 25% corn oil | | 48% soybean meal + 3% corn oil | |
| No. of pigs [1] | 4 | | 3 | | 3 | |
| Serum proteins | Initial | Final | Initial | Final | Initial | Final |
| Total, g/100 ml | 5.7 | 5.7 | 5.9 | 4.4 | 5.9 | 7.2** |
| Albumin, % of total | 56.1 | 29.8 | 54.2 | 15.9 | 55.9 | 47.2** |

** Significantly higher than low protein groups ($P < 0.01$).
[1] Pigs fed diet 1 pair-fed the amount of diet voluntarily consumed by pair mates fed diet 2. Pigs fed diet 3 given 4% of their body weight daily. See figures 4, 5 and 6 for body weight, feed intake and calorie intake curves.

TABLE 4

*Effect of dietary protein level and of energy level of low protein diets on body composition of baby pigs (exp. 2)*

| Diet no. | 1 | 2 | 3 |
|---|---|---|---|
| Diet designation | 6% soybean meal + 3% corn oil | 6% soybean meal + 25% corn oil | 48% soybean meal + 3% corn oil |
| No. of pigs [1] | 4 | 4 | 4 |
| Avg initial wt, g | 5188 | 5100 | 4625 |
| Avg final wt, g | 5513 | 5188 | 10,788 |
| Avg empty body wt at slaughter, g [2] | 5040 | 4643 | 9863 |
| Avg gross energy/g (dry basis), kcal | 6.5 | 6.2 | 5.6** |
| Avg protein (dry basis), % | 39.5 | 39.5 | 58.1** |
| Avg ether extract (dry basis), % | 46.2 | 45.5 | 26.8** |
| Avg ash (dry basis), % | 11.7 | 13.1 | 12.9 |
| Avg femur length, % | 8.5 | 7.8 | 10.5** |
| Nitrogen-free extract (dry basis), % | 2.6 | 1.9 | 2.2 |
| Liver dry matter, % | 27.0 | 30.3 | 26.4 |
| Liver fat (dry basis), % | 9.7 | 34.1** | 12.3 |

** Significantly different from other groups ($P < 0.01$).
[1] Pigs fed diet 1 were pair-fed the amount of diet voluntarily consumed by pair mates fed diet 2. Pigs fed diet 3 were given 4% of their body weight daily.
[2] Whole body minus gastrointestinal and urinary bladder contents.

tively normal serum proteins in the marasmic infant as compared with the low total protein and albumin in the kwashiorkor infant.

The liver lipid concentration was far greater in pigs fed low protein-high fat than in those fed low protein-low fat diets or in control pigs fed high protein (34.1 vs. 9.7 and 12.3%, respectively). These differences in liver fat further support the clinical observations and the serum protein differences indicating 2 distinctly different manifestations of protein deficiency in the pig related to dietary energy intake. The increased lipid content in livers from pigs fed high fat may be associated with a greater degree of necrosis and cirrhosis, but a complete description of pathology awaits further study.

The gross energy concentration of the whole empty bodies of pigs fed low protein-low fat diets was not significantly different from that of bodies from pair-fed mates fed low protein-high fat diets, despite the greater total calorie intake of the latter pigs. Pigs fed high protein had a significantly lower ($P < 0.01$) body energy concentration than either of the other groups of pigs (5.6 vs. 6.5 and 6.2 kcal/g of dry sample). This can be explained on the basis of the higher protein content and lower ether extract content of the pigs fed high protein as compared with values for the pigs in the other groups (58.1 vs. 39.5 and 39.5% protein and 26.8 vs. 46.2 and 45.5% ether extract, respectively). The identical amount of protein and very similar amount of ether extract in the bodies of

the 2 groups of pigs fed low protein strongly indicates that the approximately 11,000 (50,000 vs. 39,000) extra kilocalories consumed, on the average, by the pigs fed high fat were used for heat production. In 1946 Kleiber (12) reviewed the subject of dietary deficiencies and energy metabolism and provided many examples of increased conversion of food energy to heat in the presence of certain dietary deficiencies, including protein. Presumably metabolizable energy is available in excess of the body's ability to use the energy for body substance or useful work. In protein deficiency, others have provided substantiation of this phenomenon. For example, Miller and Payne (13), have shown that weight maintenance in rats and pigs can be achieved over a wide range of caloric intakes and conclude that food energy may be converted directly into heat. Cabak et al. (14) reported recently that rats receiving a high protein diet required fewer calories to maintain body weight than rats receiving a low protein diet. In the present studies a somewhat different situation exists. Two groups of pigs received a diet severely deficient in protein. Both groups consumed exactly the same daily intake of protein, but one group consumed more calories due to the fact that fat increased the caloric density of the diet. The total retention of protein, as measured by carcass analysis, was the same for the the 2 groups. There was a marked difference in the nutritional status of the group receiving the high calorie intake as evidenced by the lower total serum proteins and serum albumin, the increased severity of liver damage, the gross evidence of edema as well as the 29% increase in calorie intake without a corresponding increase in body substance or physical activity. In other words, there was not only an increased specific dynamic effect with low protein diets, as described by Kleiber (12), but an additional increase when increased total calorie intake accompanied protein deficiency.

Other possible fates of the extra calories consumed by the pigs fed high fat would include fecal or urinary excretion, or both. No fecal or urinary collections were made in the present experiments and therefore a final conclusion cannot be drawn, but it appears highly unlikely that losses of the magnitude necessary to account for the differences in calorie intake could have occurred. The absorbability of protein in similar low protein diets by pigs of this age is not influenced by dietary fat level (15) and in older pigs dry matter and protein absorbability were not altered by dietary fat level (16).

The average length of the femurs from pigs fed high protein was significantly greater ($P < 0.05$) than that of femurs from pigs fed low protein, as would be expected from the differences in body weight. The slightly longer femurs from the pigs fed low fat-low protein than from their pair-fed mates receiving high fat-low protein indicates that some bone growth might have occurred in the former group in the absence of an increase in body weight. This has been shown to occur in underfed cockerels (17).

The over-all appearance of pigs fed the low fat-low protein diet in the present study resembled that described by McCance (18) and Heard et al. (2) for growing pigs subjected to severe undernutrition by means of restricted intake of a nutritionally balanced diet and by Graham and Morales (9) in marasmic infants. The low liver lipid levels in undernourished pigs described by Widdowson et al. (19) also resemble the case observed in the pigs fed low fat-low protein in the present study. The pigs receiving the low protein diets with relatively higher energy intake, resembled more nearly the syndrome described as kwashiorkor in human infants. Comparison of the physical signs and the serum protein patterns in experiments 1 and 2 suggests that soybean meal protein elicits a more severe kwashiorkor-like syndrome than casein when fed at a similar protein level in the diet. The nutritional basis for this apparent difference is under study in this laboratory.

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# Studies in Chickens Fed a Copper-deficient Diet Supplemented with Ascorbic Acid, Reserpine and Diethylstilbestrol [1,2,3]

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*ABSTRACT* Ascorbic acid, 5 g/kg of feed, reduced growth, lowered hemoglobin and hematocrit levels and increased mortality in chickens when added to a semi-purified diet containing 8 ppm copper. A small percentage of birds died of arterial rupture. Increasing the copper content of the diet 3- and 5-fold counteracted somewhat the growth depression by ascorbic acid, more completely protected against depression of hematopoiesis and completely prevented arterial rupture. In most experiments ascorbic acid intensified the effects in chickens of a copper-deficient diet. Although incidence of arterial rupture was not always increased, average age to rupture was consistently reduced by ascorbic acid supplementation. Reserpine and estrogen did not significantly alter the incidence of arterial rupture in chickens fed a copper-deficient diet.

Young growing chickens fed a diet deficient in copper present a number of deficiency signs. These include poor growth, loss of feather pigmentation, and anemia (1, 2). Sudden deaths occur due to rupture of the aorta and other arteries manifested grossly by hemopericardium and hemoperitoneum (3. 4). Deficient chickens develop a leg weakness which ultimately interferes with their ability to walk upright. Although gross deformity of the long bones does not occur, marked alterations were observed in the epiphyseal plate areas when bone sections were examined microscopically (5).

During studies on the pathology of copper deficiency in the chick (3, 5), it was observed that groups of chickens which were fed a copper-deficient diet supplemented with ascorbic acid often presented an increased incidence of arterial rupture and the average age to rupture was consistently lower in these groups. These data suggested that ascorbic acid may have interfered in some manner with copper utilization by the chick. Studies were undertaken to investigate further this interaction and the results of these studies form the basis of the present report. Additional studies were undertaken on the supplementation of the copper-deficient diet with reserpine and diethylstilbestrol. The results of these studies are also included.

## MATERIALS AND METHODS

Six experiments were completed with one-day-old cross-bred male broiler chickens obtained from commercial hatcheries. The birds were distributed at random into lots of 15 to 20 birds each. They were housed in electrically heated battery brooders coated with a plastic spray paint [5] which was also used to coat the feeding troughs. Glass-distilled water was provided in flint glass jars equipped with plastic fountain bases. Feed and water were supplied ad libitum.

The semi-purified diet consisted of dried whole milk and sucrose adequately supplemented with vitamins, minerals and amino acids. It was mixed in stainless steel equipment and stored under refrigeration in plastic bags. Diets were mixed at medium speed in a Hobart mixer for about 15 minutes. Storage time of diets varied but never exceeded 2 weeks in duration. Copper was added as reagent grade

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copper sulfate ($CuSO_4 \cdot 5H_2O$) as a premix to provide a control diet with a copper content of 8 ppm. Copper sulfate was ground to a fine powder in a mortar and pestle and mixed with sucrose. Additional amounts of copper sulfate were added to certain diets in experiments 3 and 4 in amounts shown in the tables. L-Ascorbic acid [6] was incorporated into the diets at the time of mixing. L-Ascorbic acid was purchased as a fine powder and added as such to the diet without any additional handling. Small quantities were purchased and hence storage was of short duration. The levels used are shown in the tables.

The basal diet had the following composition: (in per cent) dried whole milk, 57; sucrose, 34.5; soybean oil, 2.0; glycine, 1.5; L-arginine·HCl, 1.0; DL-methionine, 0.5; choline chloride, 0.2; reagent grade dicalcium phosphate, 1.0; and sodium chloride, 0.5.

The vitamin mixtures contained the following vitamins: (mg/kg of diet) menadione, 22; α-tocopherol, 22; thiamine, 8.8; riboflavin, 8.8; pyridoxine, 8.8; Ca pantothenate, 27.0; niacin, 44.0; inositol, 440.0; folacin, 17.6; cobalamin, 0.044; biotin, 0.44; and vitamin A, 4499 USP units; and vitamin $D_3$, 374 ICU units. The mineral mixtures contained: (mg/kg of diet) manganese, 100; iron, 50; and iodine, 24.

The birds were observed daily and weighed weekly. Hemoglobin and hematocrit determinations were made every 2 weeks. Hemoglobin was determined by a modification of the method described by Bankowski (6). The packed-cell volume was measured with heparinized microhematocrit capillary tubes. Necropsy was carried out on all birds dying during the experimental period. Tissues were taken at necropsy, fixed in formalin and prepared for paraffin sections. Selected tissues were cut at 5 to 7 μ on a rotary microtome and stained with hematoxylin and eosin for microscopic examination.

## RESULTS

In experiment 1, ascorbic acid, 5 g/kg of feed, added to the copper-deficient diet significantly reduced ($P < 0.01$) growth and hemoglobin levels when compared with either the copper-deficient or control groups. There was slightly over 100 g difference in average weights between the copper-deficient group and the copper-deficient ascorbic acid group (table 1). The vitamin appeared not to have a direct toxic effect as degenerative lesions or necrosis were not observed in sections of the liver, spleen, kidney, adrenal glands, or intestinal tract.

Ascorbic acid supplementation at the levels of 1 and 2 g/kg of feed was studied in experiment 2. It was added to both the

[6] Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

*Average weights, hematocrit and hemoglobin levels (exps. 1 and 2)*

| | No. of birds | Avg wt [1] | Avg hemoglobin [1] | Avg hematocrit [1] |
|---|---|---|---|---|
| | | *g* | *g/100 ml blood* | *%* |
| Experiment 1 | | | | |
| Control | 24 | 293.7 | 9.2 | 30.6 |
| Copper-deficient | 20 | 285.2 | 8.7 | 27.0 |
| Copper-deficient + ascorbic acid [2] | 22 | 170.5 | 6.3 | 21.6 |
| Experiment 2 | | | | |
| Control | 11 | 355.1 | 10.9 | 31.6 |
| Control + ascorbic acid [3] | 18 [4] | 265.3 | 8.7 | 30.2 |
| Control + ascorbic acid [5] | 19 [6] | 294.4 | 9.4 | 29.9 |
| Copper-deficient | 18 | 156.2 | 7.9 | 27.6 |
| Copper-deficient + ascorbic acid [3] | 19 | 142.3 | 8.5 | 29.4 |
| Copper-deficient + ascorbic acid [5] | 18 | — [7] | — [7] | — [7] |

[1] Sixth week.
[2] Five g/kg of feed.
[3] One g/kg of feed.
[4] Four birds died of arterial rupture.
[5] Two g/kg of feed.
[6] Three birds died of arterial rupture.
[7] All birds dead.

control and copper-deficient diets. The weight gains of the 2 control groups receiving ascorbic acid did not differ significantly. However, the addition of the vitamin had a growth-depressing effect as weight gains of these groups were significantly lower ($P < 0.05$) than those of the control birds. Some of these birds became deficient in copper, as evidenced by arterial rupture and presence of microscopic lesions of copper deficiency (3). Ascorbic acid-supplemented deficient birds grew nearly as well as unsupplemented deficient birds in this experiment, but there was greater mortality in the group which consumed the higher level of ascorbic acid. All were dead at the sixth week (table 1).

In experiment 3, copper supplementation at 2 levels was made to the basal diet containing 5 g/kg of ascorbic acid so that the total concentration of copper was 24 and 40 ppm. These 2 levels were used to determine whether increasing the copper concentration of the diet might counteract the apparent interference of ascorbic acid with copper utilization. The addition of ascorbic acid to the control diet resulted in a reduction of growth. Increasing the copper level of this diet by three- and fivefold counteracted in part the growth-depressing effect of ascorbic acid (table 2). The combined weight averages of the 2 groups receiving extra amounts of copper were significantly greater ($P < 0.01$) when compared with those made by the control + ascorbic acid group. The copper-deficient + ascorbic acid group grew poorly and all birds were dead by the sixth week (table 2).

Ascorbic acid supplementation of the control diet caused a significant reduction in hemoglobin and hematocrit levels ($P < 0.01$). Increasing the copper levels of this diet by three- and fivefold protected against the decrease in hemoglobin caused apparently by the addition of ascorbic acid (table 2).

Experiment 4 was essentially a replication of experiment 3 with very similar results: ascorbic acid supplementation, 5 g/kg of feed, of the control diet containing 8 ppm of copper resulted in a significant reduction ($P < 0.01$) in growth and hemoglobin levels. These effects were overcome in part by increasing by threefold the copper content of the diet (table 3).

In experiment 5, dietary reserpine failed to prevent vascular rupture in copper-deficient chicks (table 4). The highest death rate from internal hemorrhage (90% of total birds) so far achieved was obtained in the copper-deficient + reserpine group. The average age of rupture was very low in this group, (21.7 days). Some birds in this group died of hemopericardium as early as 14 days of age. Average age to rupture was extremely high in the copper-deficient group of this study (61.8 days). A marked deficiency was produced later in this group than in previous studies (3). A "conditioned" copper deficiency was apparently produced in some of the birds of the control + ascorbic acid group; 3 birds died of arterial rupture.

Diethylstilbestrol did not appear to significantly affect the incidence of vascular rupture when added to the copper-deficient diet (table 5). Atheromatous lesions were

TABLE 2

*Average weights, hematocrit and hemoglobin levels (exp. 3)*

| | No. of birds | Avg wt [1] | Avg hemoglobin [1] | Avg hematocrit [1] |
|---|---|---|---|---|
| | | *g* | *g/100 ml blood* | *%* |
| Experiment 3 | | | | |
| Control | 9 | 314.9 | 10.1 | 31.3 |
| Control + ascorbic acid [2] | 18 [3] | 204.1 | 6.7 | 19.5 |
| 3 × Copper [4] + ascorbic acid [2] | 18 | 234.1 | 10.3 | 27.6 |
| 5 × Copper [5] + ascorbic acid [2] | 17 | 239.4 | 10.1 | 30.3 |
| Copper-deficient | 17 | 290.0 | 10.0 | 30.5 |
| Copper-deficient + ascorbic acid [2] | 17 | — [6] | — [6] | — [6] |

[1] Sixth week.
[2] Five g/kg of feed.
[3] One bird died of arterial rupture.
[4] 24 ppm copper.
[5] 40 ppm copper.
[6] All birds dead.

TABLE 3

*Average weights, hematocrit and hemoglobin levels (exp. 4)*

| | No. of birds | Avg wt [1] | Avg hemoglobin [1] | Avg hematocrit [1] |
|---|---|---|---|---|
| | | *g* | *g/100 ml blood* | *%* |
| Experiment 4 | | | | |
| Control | 20 | 287.2 | 9.1 | 30.4 |
| Control + ascorbic acid [2] | 20 [3] | 195.3 | 7.2 | 26.7 |
| Control + 3 × copper [4] | 20 | 291.7 | 9.3 | 30.9 |
| Control + 3 × copper [4] + ascorbic acid [2] | 20 | 216.6 | 8.1 | 27.3 |
| Copper-deficient | 20 | — [5] | — [5] | — [5] |
| Copper-deficient + ascorbic acid [2] | 20 | — [5] | — [5] | — [5] |

[1] Sixth week.
[2] Five g/kg of feed.
[3] Three birds died of arterial rupture.
[4] 24 ppm copper.
[5] All birds were dead.

TABLE 4

*Incidence of vascular rupture (exp. 5)*

| | No. of birds | Mortality | Arterial rupture | Rupture/ total birds | Rupture/ mortality | Avg age to rupture | Range of ages |
|---|---|---|---|---|---|---|---|
| | | | | *%* | *%* | *days* | |
| Experiment 5 | | | | | | | |
| Control | 20 | 4 | 0 | — | — | — | |
| Control + ascorbic acid [1] | 20 | 5 | 3 | 15.0 | 60.0 | 36.3 | |
| Copper-deficient | 20 | 18 | 12 | 60.0 | 66.7 | 61.8 | (44–75) |
| Copper-deficient + ascorbic acid [1] | 20 | 20 | 12 | 60.0 | 60.0 | 26.5 | (17–44) |
| Copper-deficient + reserpine [2] | 20 | 20 | 18 | 90.0 | 90.0 | 21.7 | (14–27) |

[1] Five g/kg of feed.
[2] 0.23 mg/kg of feed.

TABLE 5

*Incidence of vascular rupture (exp. 6)*

| | No. of birds | Mortality | Arterial rupture | Rupture/ total birds | Rupture/ mortality | Avg age to rupture |
|---|---|---|---|---|---|---|
| | | | | *%* | *%* | *days* |
| Experiment 6 | | | | | | |
| Control | 15 | 0 | — | — | — | — |
| Control + estrogen [1] | 15 | 3 | 0 | — | — | — |
| Copper-deficient | 15 | 11 | 9 | 60.0 | 81.8 | 34.3 |
| Copper-deficient + estrogen [1] | 15 | 11 | 10 | 66.7 | 90.0 | 39.5 |

[1] 110 mg/kg of feed.

not observed in sections of arteries from estrogen-supplemented birds.

## DISCUSSION

In several experiments of this study ascorbic acid added to the copper-deficient diet at levels of 5 g/kg of feed caused a greater reduction in growth than the copper-deficient diet alone (tables 1 and 2). Several possible mechanisms can be postulated to account for this action of dietary ascorbic acid: 1) the vitamin could have reduced feed consumption with resultant growth retardation; 2) ascorbic acid may have a direct toxic action; or 3) it may have interfered with copper

assimilation. This latter hypothesis could include the formation of a copper-ascorbic acid complex which might have an antagonistic action. Our studies do not include an investigation of this mechanism of action.

Our feed consumption studies [7] suggest that the feed differential (1 g/bird/day) between the groups was not so great as to account for the weight depression and the high mortality observed. There is no evidence in the literature to suggest that ascorbic acid is toxic at the levels of intake in this study. No toxic effects in chickens were observed by Satterfield and coworkers (7) when 200 mg of ascorbic acid were injected within a week. They (8) did not observe toxic effects after subcutaneous injections of ascorbic acid into young birds over a period of 21 weeks; an average of 2,150 mg were injected. Heywang and Kemmerer (9) observed that 0.1% ascorbic acid produced no toxic effects when included in the diet of laying hens. This level of diet supplementation resulted in an increased vitamin level of serum but without toxic effects (10). Stimulation of growth by dietary ascorbic acid has been reported (11). March and Biely (12) observed that ascorbic acid at levels of 360 mg to 0.5 g/454 g of feed stimulated growth in chicks fed a natural diet deficient in folic acid. No toxic effects were reported. A direct toxic effect was discounted on the basis of absence of lesions in the parenchymatous organs and intestines suggestive either of toxemia or of a caustic action by the vitamin.

In experiments 2, 3, and 4, even in the presence of adequate copper, growth was reduced by the addition of ascorbic acid. The reduction in weight in the control + ascorbic acid groups might be explained on the basis of an interference with copper assimilation and the production of a "conditioned" copper deficiency. In support of this interference, using arterial rupture as the criterion, copper deficiency was produced in some of the birds consuming the control diets supplemented with ascorbic acid. A low percentage of birds from these groups died of arterial rupture (tables 1–4). A few deaths not attributable to arterial rupture were recorded in all groups. Some of the birds died of starvation early in the experimental period as they failed to consume adequate amounts of the diets.

If ascorbic acid interfered with copper assimilation, the addition of extra copper to the diet might correct the "conditioned" deficiency. In experiments 3 and 4, three- and fivefold levels of copper counteracted in part the growth-suppressive effect of ascorbic acid. These levels protected more completely against the suppressive action on hematopoiesis and completely protected the chickens against arterial rupture.

Although Barnett (13) observed that reserpine is effective in preventing aortic rupture in turkeys under field conditions, it did not at the same dosage level reduce the incidence of arterial rupture in our studies.

A high incidence of fatal dissecting aneurysms in male turkeys was observed in a commercial flock that had been implanted with pellets of stilbestrol (14). Beall and associates (15) observed that weekly implantation of turkeys with diethylstilbestrol increased the incidence of aortic ruptures. Diethylstilbestrol added to our diets failed to increase the incidence of arterial rupture and no lesions attributable to feeding of the drug were observed in sections of the arteries. These differences might be explained by the shorter interval of exposure in our studies and the different dosage regimen.

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# Response of the Liver to Prolonged Protein Depletion

## V. NEUTRAL GLYCERIDES AND CHOLESTEROL; PRODUCTION OF FATTY LIVERS BY CERTAIN AMINO ACIDS FED IN A PROTEIN-FREE RATION [1]

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*ABSTRACT* The influence of prolonged protein deprivation followed by protein repletion in the adult male rat upon liver neutral glycerides and cholesterol was studied. Neutral glyceride and cholesterol concentrations increased for 8 weeks. Thereafter neutral glycerides decreased to normal and cholesterol to below normal after 14 weeks of deficiency. Attempts to alleviate the slightly fatty liver produced in the simple protein deficiency by including 0.3% DL-methionine in the protein-free ration produced a marked increase in these liver lipids. These excessive accumulations also reached a maximum after 8 weeks, followed by a decline towards normal. Reintroduction of protein into the rations at the terminal stages of protein deficiency caused a rebound of neutral glycerides and cholesterol to far above normal levels. The exact pattern of this response, however, was influenced markedly by whether methionine had been included in the ration during development of the protein deficiency. Cystine and methionine produced very fatty livers when added individually to the protein-free ration, whereas valine increased neutral glycerides and cholesterol to some extent. Leucine was quite lipotropic, however, and maintained the concentrations of these liver lipids normal or below. Phenylalanine was also somewhat lipotropic. Other essential amino acids had no effect. Omission of the dietary source of lipids (corn oil) had no effect on the excessive accumulation of neutral glycerides or cholesterol produced by methionine in the protein-free ration.

During the early phases of development of a dietary protein deficiency, lipid accumulates in the liver. Although the adult male albino rat survives for approximately 100 days with a protein-free ration, liver lipid changes have not been studied beyond 30 days (1). It was therefore decided to follow the lipid changes throughout the entire course of protein depletion followed by repletion.

The present study is part of a continuing investigation of changes in intracellular components closely related to the maintenance of energy metabolism throughout the development of a protein deficiency. Previous studies of this series have been reported (2–5). Because neutral lipids are perhaps not involved as functional parts of those cellular components necessary for the utilization of substrates for energy, they undoubtedly serve as substrates themselves in this process. Although the functions of cholesterol in the liver are not known with certainty, its presumed importance in the animal as a whole encouraged its study also.

In the present studies we have also investigated the possibility of preventing the production of the slightly fatty liver produced in simple protein deficiency by including a low level of methionine in a protein-free ration, since under certain conditions methionine is lipotropic. An investigation of the effect of methionine and other individual amino acids on liver neutral glycerides and cholesterol is also presented. Current studies of changes in total phospholipids, plasmalogens, and fatty acids in protein deficiency will be reported separately.

### EXPERIMENTAL METHODS

The study was divided into 2 phases. In the first, the changes in neutral glycerides

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[1] A preliminary report of these studies was presented at the VIth International Congress of Nutrition, Edinburgh, Scotland, 1963; and in Nature, 200: 472, 1963.

and cholesterol associated with prolonged protein depletion were followed during progressive protein depletion and the subsequent period of repletion. In this phase the effect of including methionine in the protein-free ration was also studied. In the second phase the effects of adding individually all other essential amino acids as well as cystine, glutamic acid, and arginine at equimolar levels in a protein-free ration were studied.

Liver lipids were extracted and washed by the procedure of Folch et al. (6). Total lipid was assayed by the method of Bragdon (7); phospholipid, by the method of Fiske and Subbarow (8); total cholesterol, by the method of Pearson et al. (9); and liver DNA, by the extraction procedure of Schneider (10) followed by color development with diphenylamine (11). Neutral glycerides were calculated by taking the difference between total lipid and phospholipid plus cholesterol and calculating this difference as micromoles of tripalmitin.

*Phase 1.* Three hundred seventy-two male rats of the Sprague-Dawley strain were fed a complete purified diet (diet R 1) (2) for a 3-week adjustment period. The complete diet consisted of: (in per cent) casein, 20; DL-methionine, 0.3; corn oil, 5; salts N plus molybdate (12), 6.5; glucose monohydrate, 63.5; choline chloride, 0.2; *i*-inositol, 0.02; and water-soluble vitamin mix in sucrose (13),[2] 4.5. Fat-soluble vitamins (2)[3] were given weekly to each rat in 2 drops of corn oil. The rats, which weighed 290 g after the initial adjustment period, were separated at random into 4 groups. Group 1 received diet R1 ad libitum. Group 2 received diet R1 but was pair-fed with group 4 (average daily food consumption). Group 3 received ad libitum diet R1 from which the casein was omitted. Group 4 received ad libitum diet R1 from which both casein and methionine were omitted. The food consumption of groups 3 and 4 was almost identical.

At intervals after feeding the various rations, rats from each group were killed and the livers removed, rapidly weighed, and portions frozen and stored in the freezer until analyzed for lipids. The number of animals killed at each point in each group was as follows: zero time, 8; 24 days, 8; 56 days, 10; 102 days, 8; 3 days post-repletion, 4–8 of groups 2–4; 6 days post-repletion, 4 of groups 2–4; 15 days post-repletion, 4 of groups 2–4; 25 days post-repletion, 8 of group 1; 56 and 86 days post-repletion, 6–8.

*Phase 2.* When it had been observed from phase 1 of the study that methionine produced a markedly anti-lipotropic effect, another group of 112 rats was fed for a 3-week adjustment period with the complete diet to study the effects of other individual amino acids. After the adjustment period, the rats were separated into 17 groups of 8 rats each (groups 1A–17A) and fed the individual diets for 8 weeks. The diet fed to each group was as follows: a normal control diet (1A); a protein and amino acid-free diet (2A); protein-free diets containing various amino acids equimolar with 0.3% L-methionine (0.42 L-arginine·HCl 3A; 0.24% L-cystine, 4A; 0.30% L-glutamic acid, 5A; 0.42% L-histidine·HCl·$H_2O$, 6A; 0.20% L-isoleucine, 7A; 0.26% L-leucine, 8A; 0.37% L-lysine·HCl, 9A; 0.30% L-methionine, 10A; 0.33% L-phenylalanine, 11A; 0.24% L-threonine, 12A; 0.41% L-tryptophan, 13A; 0.24% L-valine, 14A); a normal control diet less corn oil, 15A; a protein and amino acid-free diet less corn oil, 16A; and a protein-free diet containing 0.30% L-methionine, less corn oil, 17A. In groups 15A–17A corn oil was omitted from the rations to examine whether the accumulation of liver lipids is entirely dependent upon the presence of lipids in the diet or whether the lipids may arise from other dietary components or from redistribution of lipids in the body of the animal. In all cases, L-amino acids equimolar with 0.3% methionine were included in the protein-free diet.

## RESULTS

The results are presented as concentration of lipid component per milligram of DNA. Since the concentration of DNA

[2] The concentrations of the water-soluble vitamins in the ration were as follows: (mg vitamin/100 g diet) thiamine·HCl, 0.8; riboflavin, 0.8; Ca pantothenate, 2.0; choline chloride, 200; niacin, 10; pyridoxine·HCl, 0.8; D-biotin, 0.03; folic acid, 0.3; vitamin $B_{12}$, 0.002; and inositol, 20.

[3] The composition of the fat-soluble vitamin mixture prepared in corn oil was as follows: (mg/100 ml corn oil) vitamin A acetate, 966; vitamin $D_3$, 0.7; DL-α-tocopheryl acetate, 4900, and menadione, 420.

per average liver cell nucleus has been found to be constant during protein deprivation in adult rats (14), concentration of liver cellular components expressed per unit weight of DNA should be directly proportional to concentration of a component per average liver cell. The results in phase 1 were analyzed using Student's *t* test to determine whether the differences between the means of groups 2–4 versus 1, groups 3 and 4 versus 2, and group 4 versus 3 at any one time are significant. The method of noting the significant differences is shown in the legend of the figures. The results in phase 2 are presented as bar graphs for the 17 different groups. The results were also analyzed statistically and the range of 2 standard errors is drawn for each bar to denote significance of difference among the means.

*Phase 1.* In figure 1 are presented the results for the response of neutral glycerides to protein depletion, with and without 0.3% DL-methionine. The results for the ad libitum-fed group (group 1) and pair-fed controls (group 2) have been separated from the 2 protein-deficient groups (groups 3 and 4) for clarity. The values for the ad libitum-fed controls are presented as micromoles of neutral glycerides (calculated as tripalmitin) per milligram of DNA. The results for the other 3 groups are presented as a percentage of the ad libitum-fed control values to negate the influence of the fluctuations in the points for group 1 upon the other groups. Thus a clearer picture of the effects of the diets per se on the variables is obtained.

The simple protein deficiency (group 4) produced an increase in neutral glycerides which reached a maximum of 6 times normal after 8 weeks and then returned to nearly normal at the final stages of protein deficiency. The presence of 0.3% DL-methionine in the protein-free ration markedly increased neutral glycerides per average liver cell even after 3 weeks; and after 8 weeks, their concentration per cell reached 14..5 times normal. They then began to return towards normal. Only a relatively slight change in neutral lipids in the pair-fed controls occurred throughout the entire study.

When protein was replaced in the ration of group 3, the neutral glycerides returned rapidly to a high level after 3 days and then rapidly decreased towards normal. In group 4, the neutral glycerides returned quickly to an extremely high level after 3 days (about 16 times normal), remained there for about 2 weeks and then returned to normal. This difference in response of groups 3 and 4 after addition of protein to the rations points to a real dissimilarity in the physiology of the 2 groups of animals at the point of maximal depletion (100 days). What this difference is and why the presence of only 0.3% methionine in otherwise identical rations during protein depletion produces this effect is a subject for future study. It should be emphasized that the rations of groups 3 and 4 were identical during repletion.

In figure 2 are presented the results for liver total cholesterol. In general, the results are quite similar to those for neutral glycerides, except with respect to magnitude of the changes. Again in the simple protein deficiency (group 4), cholesterol reached a maximum after 8 weeks, which, however, was only 1.3 times normal. The presence of 0.3% methionine in the ration caused an increase in cholesterol of 2.1 times normal after 8 weeks, which, although a significant increase, was much less than found with the neutral glycerides. Upon repletion with protein, essentially the same picture was observed for group 3 compared with group 4 as for the neutral glycerides, although again the magnitude of the rebound of cholesterol concentrations was much less in both cases than for the neutral glycerides. In the pair-fed controls (group 2), cholesterol decreased gradually during the depletion period. When normal food intake was again supplied to this group, a slow return of cholesterol to slightly above normal levels occurred.

*Phase 2.* In figures 3 and 4 are presented the results of studies with groups of rats fed the protein-free ration with the addition of individual amino acids. Also, 3 extra groups are included (15A–17A), which were fed diets identical to those of groups 1A, 2A and 10A, respectively, except that corn oil was omitted. These

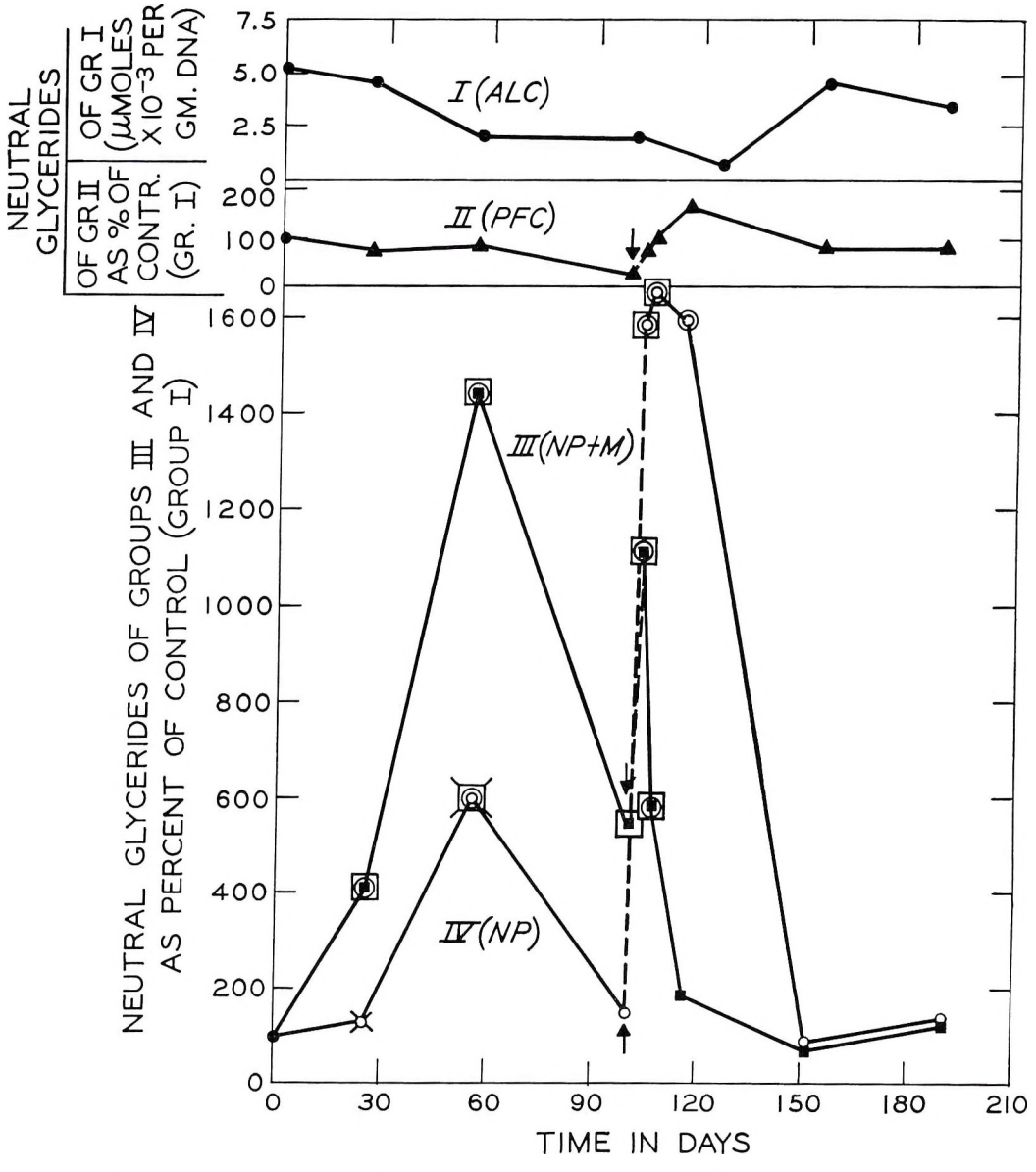


Fig. 1 The response of liver neutral glycerides to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. I (ALC) = • = ad libitum-fed controls; II (PFC) = ▲ = pair-fed controls; III (NP + M) = ■ = protein-deficient rats fed diet supplemented with 0.30% DL-methionine; IV (NP) = o (small open circles) = protein-deficient rats. Repletion was begun at the arrows. The results for groups 2–4 are expressed as a percentage of the ad libitum-fed controls (I).

Tests for significance of difference between means

| Group | versus | Group | Notation for $P < 0.01$ |
|---|---|---|---|
| II, III, IV | | I | ○ (large circles) |
| III, IV | | II | □ |
| IV | | III | × |

The number of rats at each point is shown in the text.

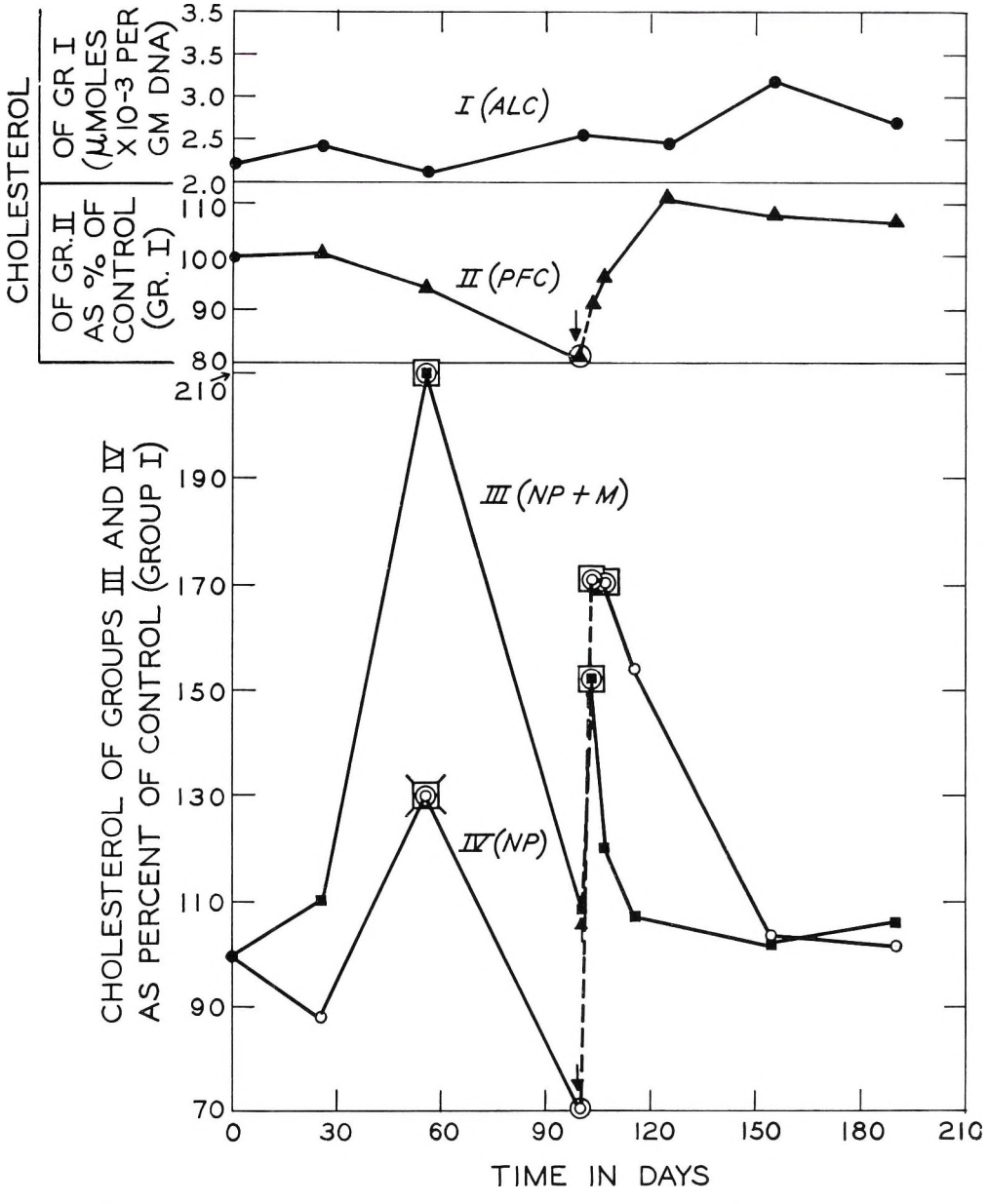


Fig. 2 The response of liver total cholesterol to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. I (ALC) = • = ad libitum-fed controls; II (PFC) = ▲ = pair-fed controls; III (NP + M) = ■ = protein-deficient rats fed diet supplemented with 0.30% DL-methionine; IV (NP) = o (small open circles) = protein-deficient rats. Repletion was begun at the arrows. The results for groups 2–4 are expressed as a percentage of the ad libitum-fed controls (I).

Tests for significance of difference between means

| Group versus | Group | Notation for $P < 0.01$ |
|---|---|---|
| II, III, IV | I | ○ (large circles) |
| III, IV | II | □ |
| IV | III | × |

diets were fed for 8 weeks, which was the time required for peak accumulation of liver lipids as shown in figures 1 and 2. In figure 3 are the results for neutral glycerides; and in figure 4, for cholesterol.

Cystine and methionine were markedly anti-lipotropic under these conditions (fig. 3). Valine (group 14A) was somewhat anti-lipotropic. On the other hand, leucine (group 8A) quite markedly depressed the level of neutral glycerides. Phenylalanine (group 11A) also tended to depress the neutral glyceride level, but not as markedly as leucine. When corn oil was omitted from the diets (groups 15A–17A), almost identical results were obtained as with oil in the diet. The simple protein deficiency (group 16A) still caused some neutral glyceride accumulation, and methionine again produced a very marked accumulation of neutral glycerides.

The results for liver cholesterol (fig. 4) were almost identical to those for neutral glycerides, except that the magnitude of the changes were less for cholesterol. Methionine and cystine increased cholesterol markedly, each to about the same extent. Leucine, phenylalanine, and glutamic acid lowered cholesterol to some extent. The absence of corn oil from the diets again had no influence on the large accumulation of cholesterol produced by methionine.

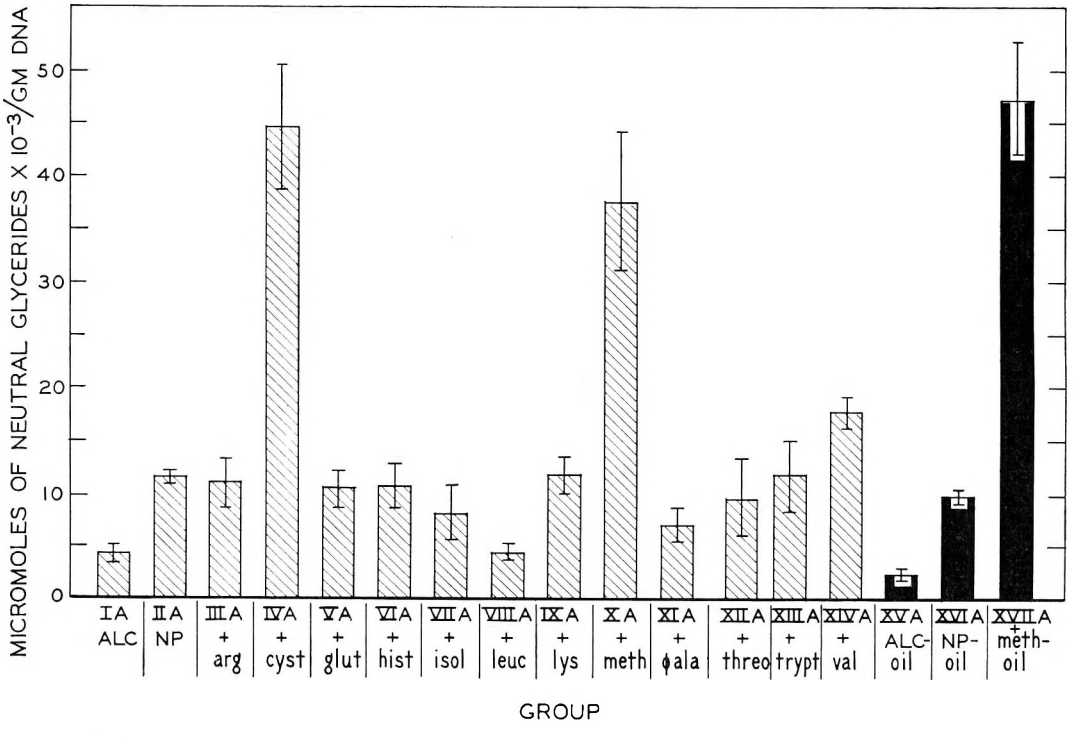


Fig. 3 Liver neutral glyceride concentration per milligram of DNA in rats fed a normal control diet (IA), a protein and amino acid-free diet (IIA), protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA–XIVA), and diets similar to IA, IIA, and XA but without corn oil (XVA–XVIIA) for 56 days. The meanings of the abbreviations in the figure are as follows: arg, 0.42% L-arginine·HCl; cyst, 0.24% L-cystine; glut, 0.30% L-glutamic acid; hist, 0.42% L-histidine·HCl·$H_2O$; isol, 0.20% L-isoleucine; leuc, 0.26% L-leucine; lys, 0.37% L-lysine·HCl; meth, 0.30% L-methionine; ϕala, 0.33% L-phenylalanine; threo, 0.24% L-threonine; trypt, 0.41% L-tryptophan; val, 0.24% L-valine; ALC = group IA = ad libitum-fed controls; NP = group IIA = rats fed protein and amino acid-free diet. Twice the standard error of each group is represented by the vertical line through the mean. Each mean was obtained from 8 rats.

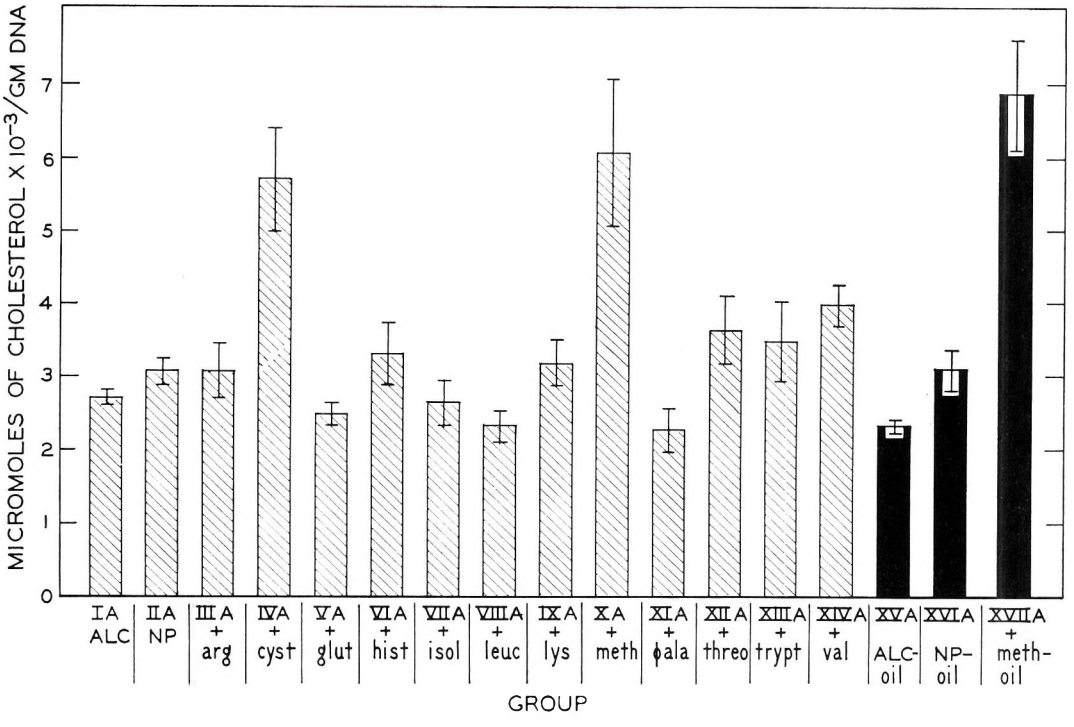


Fig. 4 Liver total cholesterol concentration per milligram of DNA in rats fed a normal control diet (IA), a protein and amino acid-free diet (IIA), protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA–XIVA), and diets similar to IA, IIA, and XA but without corn oil (XVA–XVIIA) for 56 days. The meanings of the abbreviations are shown in the legend of figure 3. Twice the standard error of each group is represented by the vertical line through the mean. Each mean was obtained from 8 rats.

## DISCUSSION

Fatty livers have been shown to be produced in a variety of ways. Some of the more important of these are as follows: the administration of certain foreign substances such as 4-aminopyrazolopyrimidine (15), chloroform, carbon tetrachloride, and yellow phosphorus; deficiencies of insulin (16), essential fatty acids (17), protein (1), choline (18), pyridoxine (17), pantothenic acid (19), thyroid hormone (cholesterol fatty liver) (20), or threonine (partial deficiency) (21); excesses of dietary fat (low carbohydrate diet) (22), cystine (in an otherwise adequate ration) (23), orotic acid (24), alcohol (25), guanidoacetic acid (23), thiamine (26, 27), biotin (28), niacin (29), anterior pituitary hormone (30), adrenal cortical hormones (31), and estrone and estradiol (32); and administration of certain agents which block protein synthesis such as ethionine (33) and puromycin (34).

In the present studies the methods of producing fatty livers (methionine, cystine, or valine in a protein-free ration, and protein-repletion of protein deficient animals) appear to be quite different from any in the foregoing list. The production of the fatty livers by methionine, cystine, or valine is contingent upon their being fed in a protein-free ration. It has been known for a long time that cystine increases liver lipids if fed at a high level in an otherwise normal ration (23), whereas methionine under the same conditions produces only a slight increase in liver cholesterol with no concomitant accumulation of neutral lipids (35). The one condition in which methionine has been shown to produce a fatty liver is when young rats are fed a 9% casein ration supplemented with 0.1% methio-

nine. However, this effect has actually been shown to result from the production of an amino acid imbalance in which the addition of methionine produces a partial threonine deficiency (21).

The relatively small but significant ability of valine to increase liver neutral glycerides and of leucine and phenylalanine to decrease these components under the conditions of these experiments cannot as yet be explained. The picture appears to be complicated and further work will be necessary to explain these effects.

ACKNOWLEDGMENTS

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

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# Response of the Liver to Prolonged Protein Depletion

## VI. TOTAL PHOSPHOLIPIDS AND PLASMALOGENS, AND PROTECTION OF PHOSPHOLIPIDS BY METHIONINE AND CYSTINE

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*ABSTRACT* Adult male rats maintained with a protein-free ration for 100 days progressively lost phospholipids from the liver cells. The presence of 0.3% DL-methionine in the protein-free ration prevented maximal loss of cellular phospholipids. Plasmalogens were also lost during depletion but not to as great an extent as total phospholipids. Inclusion of methionine in the protein-free ration had no effect on the loss of plasmalogens, in contrast with the effect on total phospholipids. It was shown that the loss of plasmalogens was due entirely to the lowered food intake in protein deficiency, again in contrast with the total phospholipids. Study of other amino acids added individually to the protein-free ration for 8 weeks indicated that only methionine and cystine protected against loss of phospholipids.

In the present series of studies (1–6) concerning the changes in liver cells associated with prolonged protein deprivation and subsequent repletion, emphasis has been placed upon those components closely related to the maintenance of cellular energy metabolism. The succinic oxidase system was chosen for detailed study as a model mitochondrial system which is closely related to the maintenance of high energy phosphate. Recently several reports (7–10) have appeared which positively implicate the phospholipids as necessary for the activity of at least 3 segments of the electron-transport chain in the succinic oxidase system. Biezinski and Spaet (11) have reported that liver mitochondria contain at least 10 times as much phospholipid as any other cell fraction. Fleischer et al. (12) have shown that 90% of the total heart mitochondrial lipid is phospholipid and that 40% of this lipid is plasmalogen. Whether these concentrations are the same in liver mitochondria is not yet known, although much functional parallelism exists between liver and heart mitochondria.

Kosterlitz (13) followed the changes in liver phospholipid concentration during protein depletion for 4 weeks and noted that 35% of the total phospholipids was lost. In our preivous studies (1–6) it was observed that the maximal changes associated with protein depletion did not occur in many variables studied until after 8 to 14 weeks. Thus, because of the direct implication of phospholipids in mitochondrial enzymic function and because of the dearth of information concerning changes in total phospholipids and plasmalogens during protein deprivation, we have studied liver phospholipids and plasmalogens during progressive protein depletion followed by repletion.

In the preceding paper (6) it was shown that methionine and cystine, when included in a protein-free ration, are markedly anti-lipotropic with respect to neutral lipid and cholesterol. Therefore, the influence of individual amino acids upon liver total phospholipids and plasmalogens was also studied in the present investigation.

### EXPERIMENTAL METHODS

The plan of the study and feeding and management of the rats had been presented in detail previously (6). The livers used for analysis in the present study were the same as those used in the preceding study for neutral lipids and cholesterol. Briefly, the investigation consisted of 2

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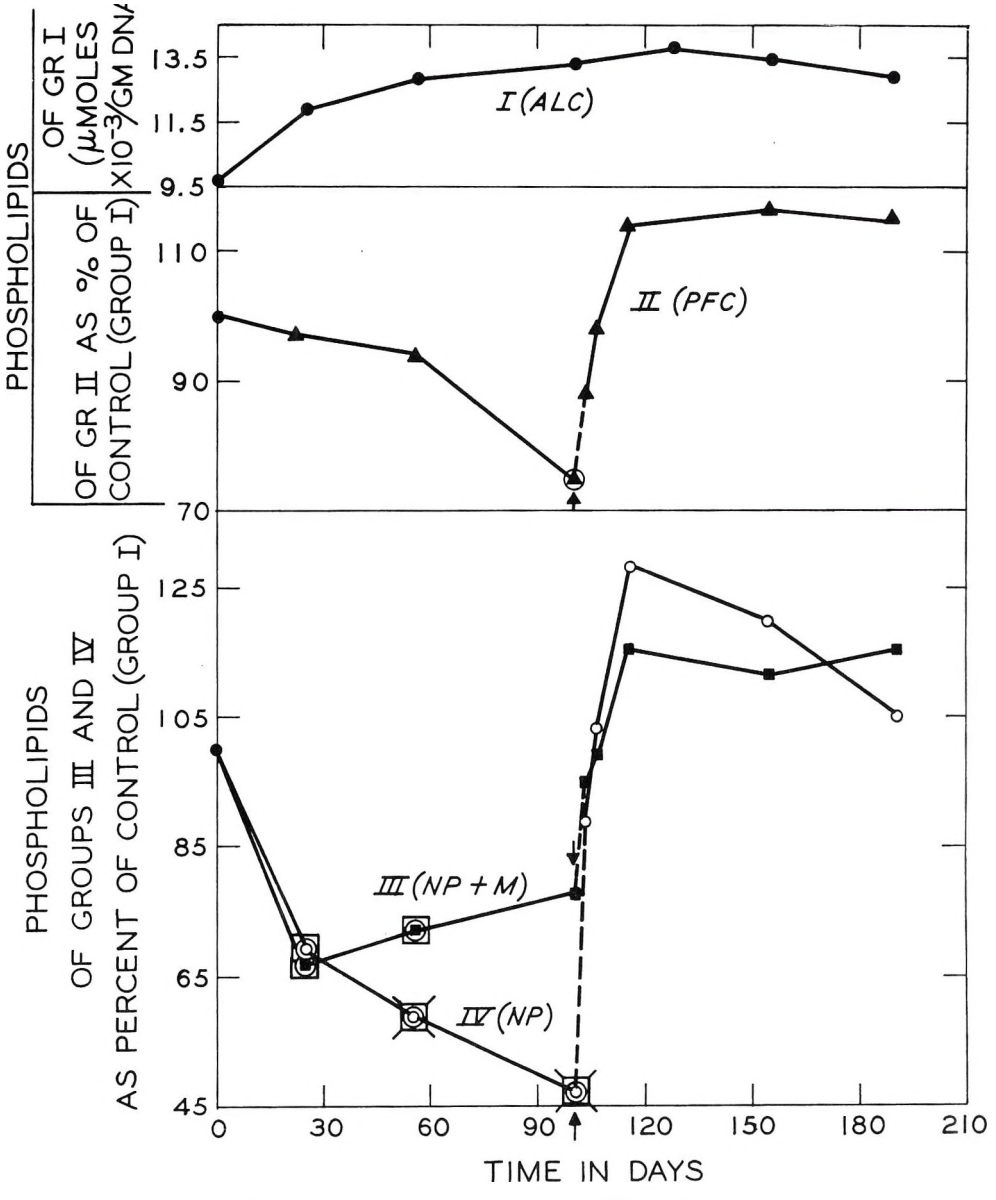


Fig. 1 The response of liver phospholipids to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion.

I (ALC) = ● = ad libitum-fed controls; II (PFC) = ▲ = pair-fed controls; III (NP + M) = ■ = protein-deficient rats fed diet supplemented with 0.30% DL-methionine; IV (NP) = o (small open circles) = protein-deficient rats. Repletion was begun at the arrows. The results for groups II–IV are expressed as a percentage of the ad libitum-fed controls (I).

Tests for significance of difference between means

| Group versus | Group | Notation for $P < 0.01$ |
|---|---|---|
| II, III, IV | I | ○ (large circles) |
| III, IV | II | □ |
| IV | III | × |

The numbers of rats at each point are as follows: zero time, 8; 24 days, 8/group; 56 days, 10/group; 102 days, 8/group; 3 days post-repletion, 4–8 of groups II–IV; 6 days post-repletion, 4 of groups II–IV; 15 days post-repletion, 4 of groups II–IV; 21 days post-repletion, 8 of group I; 56 and 86 days post-repletion, 6–8 of each group.

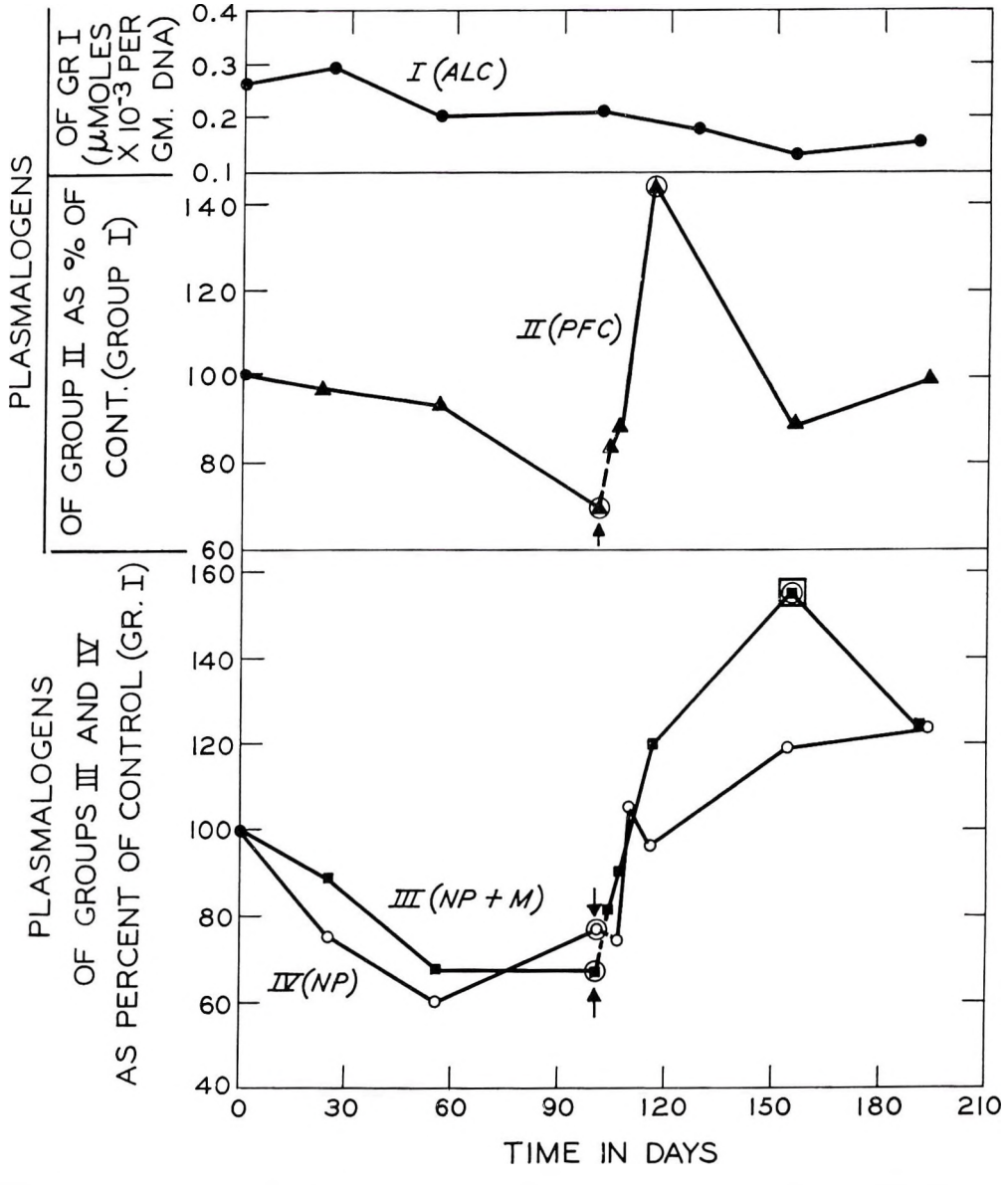


Fig. 2 The response of liver plasmalogens to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion.

I (ALC) = ● = ad libitum-fed controls; II (PFC) = ▲ = pair-fed controls; III (NP + M) = ■ = protein-deficient rats fed diet supplemented with 0.30% DL-methionine; IV (NP) = o (small open circles) = protein-deficient rats. Repletion was begun at the arrows. The results for groups II–IV are expressed as a percentage of the ad libitum-fed controls (I).

Tests for significance of differences between means

| Group | versus | Group | Notation for $P < 0.01$ |
|---|---|---|---|
| II, III, IV | | I | ○ (large circles) |
| III, IV | | II | □ |
| IV | | III | × |

The number of rats at each point is shown in the legend of figure 1.

phases. In phase 1 the influence of progressive protein depletion followed by repletion upon liver total phospholipds and plasmalogens was followed. Four groups of rats were included in this phase: group 1, ad libitum-fed controls; group 2, pair-fed controls (fed the average daily food consumption of group 4); group 3, protein-deficient group with 0.3% DL-methionine included in the ration; and group 4, protein-deficient group. In phase 2 the effect of cystine, glutamic acid, arginine, and the essential amino acids added individually to the protein-free ration upon total phospholipids and plasmalogens was studied. In addition, the effect of methionine in the absence of dietary fat was studied. The diets in this phase were fed for 8 weeks.

Liver DNA was extracted by the procedure of Schneider (14), followed by color development with diphenylamine (15). Lipid phosphorus was determined by the method of Fiske and Subbarow (16); and plasmalogens, by the method of Williams et al. (17).

## RESULTS

*Phase 1.* The results for the total phospholipid changes are presented in figure 1; and for the plasmalogens, in figure 2. The values for the ad libitum-fed controls are presented as micromoles of phospholipids or plasmalogens per milligram of DNA. The results for the other 3 groups are presented as a percentage of the ad libitum-fed control values to negate the influence of the fluctuations in the points for group 1 upon the other groups. Thus, a clearer picture of the effects of the diets per se on the variables is obtained. The simple protein deficiency

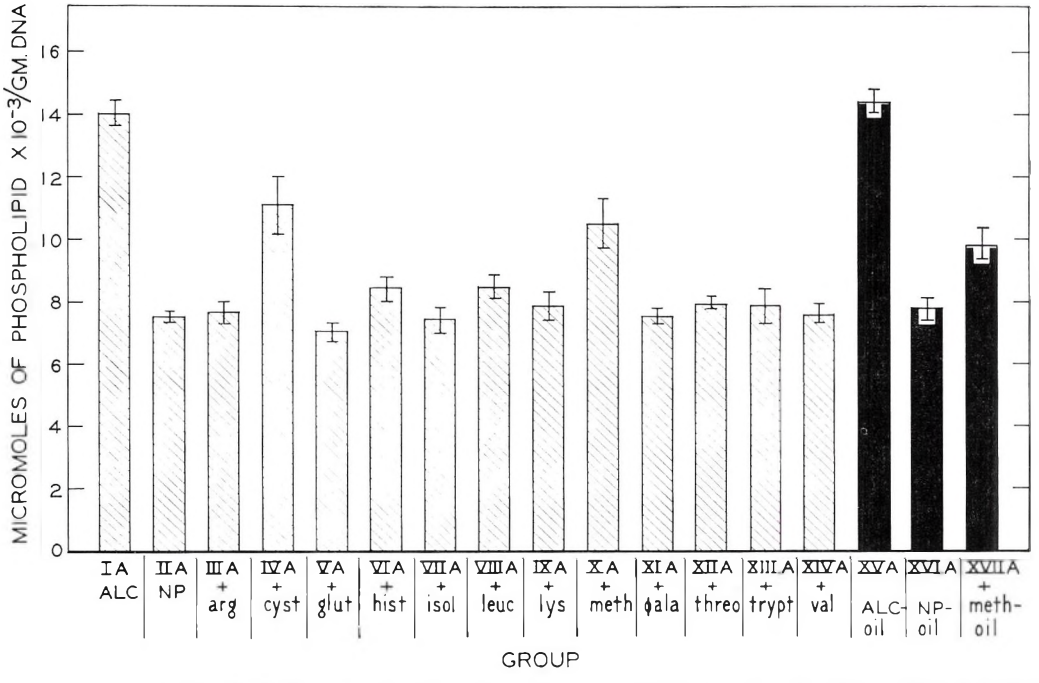


Fig. 3 Liver phospholipid concentration per milligram of DNA in rats fed for 56 days a normal control diet (IA), a protein and amino acid-free diet (IIA), protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA–XIVA), and diets similar to IA, IIA, and XA but without corn oil (XVA–XVIIA). The meaning of the abbreviations in the figures are as follows: arg, 0.42% L-arginine·HCl; cyst, 0.24% L-cystine; glut, 0.30% L-glutamic acid; hist, 0.42% L-histidine·HCl·$H_2O$; isol, 0.20% L-isoleucine; leuc, 0.26% L-leucine; lys, 0.37% L-lysine·HCl; meth, 0.30% L-methionine; ϕala, 0.33% L-phenylalanine; threo, 0.24% L-threonine; trypt, 0.41% L-tryptophan; val, 0.24% L-valine; ALC = group IA = ad libitum-fed controls; NP = group IIA = rats fed protein and amino acid-free diet. Twice the standard error of each group is represented by the vertical line through the mean. Each mean was obtained from 8 rats.

(group 4) produced a progressive decline in total phospholipids for 100 days (fig. 1). The animals could not be continued beyond this point since they began to die from the deficiency. When 0.3% DL-methionine was included in the protein-free ration (group 3), considerable protection of phospholipids occurred after the initial decrease at 21 days. The pair-fed controls (group 2) showed a slow decline in phospholipids, which was less than that of the protein-deficient group (group 4), however. Upon supplementation of the diet of groups 2 and 4 with protein, a rapid return towards normal occurred; and after 2 weeks, liver phospholipids returned to normal. When food was returned to the pair-fed controls, liver phospholipid concentrations also returned to normal.

Although the plasmalogens make up one of the classes of phospholipids, the effect of protein deficiency on plasmalogen concentration was different from that on total phospholipid concentration. The plasmalogen concentration in group 4 ceased to decline further after 56 days of depletion. Also, the presence of methionine in the ration (group 3) had no effect on the plasmalogen concentration. The pair-fed controls lost as much cellular plasmalogen as either of the 2 protein-deficient groups. Upon supplementation of protein to the diet of groups 3 and 4, a return towards normal occurred but at a considerably slower rate than for the total phospholipids. In fact, the plasmalogen concentration of group 3 reached a higher than normal value at 156 days and then returned to normal after 190 days. The plasmalogen concentration of the pair-fed controls rebounded rapidly to above normal after 2 weeks of repletion and then decreased to normal after 156 days.

*Phase* 2. In figures 3 and 4 are presented the results of studies with groups of rats fed the protein-free ration with the addition of individual amino acids.

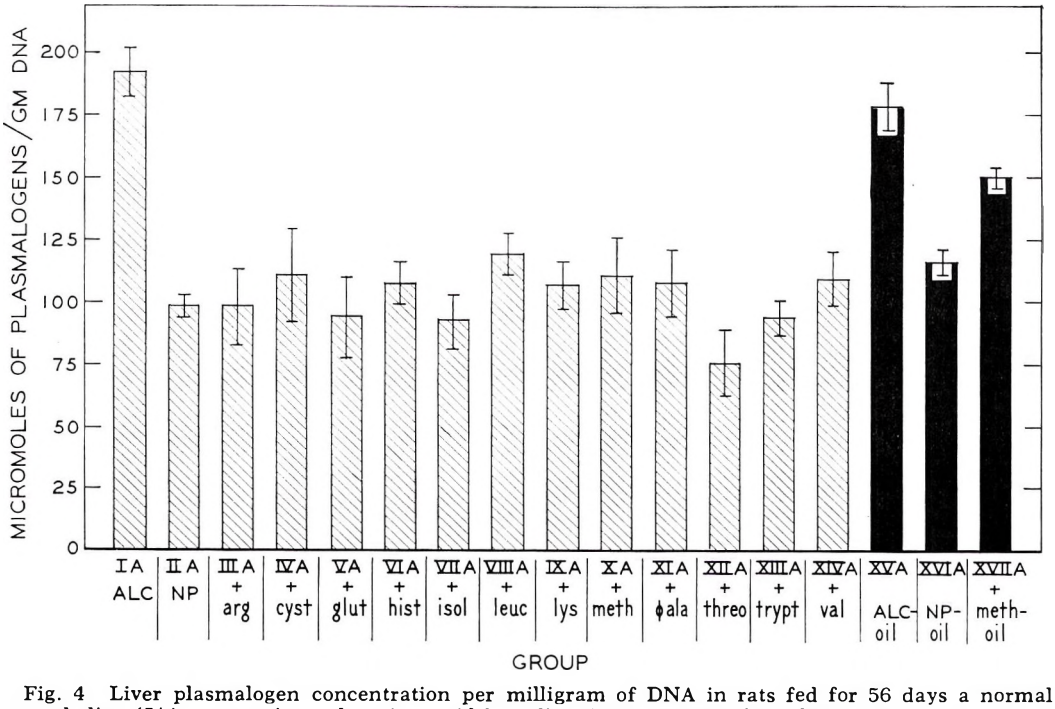


Fig. 4 Liver plasmalogen concentration per milligram of DNA in rats fed for 56 days a normal control diet (IA), a protein and amino acid-free diet (IIA), protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA–XIVA), and diets similar to IA, IIA, and XA but without corn oil (XVA–XVIIA). The meanings of the abbreviations are shown in the legend of figure 3. Twice the standard error of each group is represented by the vertical line through the mean. Each mean was obtained from 8 rats.

Three additional groups were included (15A–17A) which were fed diets identical to those of groups 1A, 2A and 10A, respectively, except that the dietary fat (corn oil) was omitted. The total phospholipds of all groups, except for those supplemented with cystine (4A) and methionine (10A), decreased to about 50% of normal after 8 weeks (fig. 3). Methionine and cystine protected against the loss of phospholipids. Omission of fat from the diet had no effect either on the loss of phospholipids in group 16A or on the protection against loss by methionine. The results in figure 4 indicate that no protection against loss of plasmalogens by any of the amino acids tested occurred when dietary fat was present (groups 1A–14A). When fat was omitted from the diet, however, methionine protected the plasmalogens to some extent (groups 15A–17A). Further work will be required to clarify this result.

## DISCUSSION

In marked contrast with the results for neutral lipids and cholesterol (6) under the same conditions as in the present study, phospholipids were decreased by simple protein deficiency; and methionine and cystine protected against this loss. Also, whereas the neutral lipids and cholesterol reach a peak after 8 weeks and then decline almost to normal as the simple deficiency progresses, total phospholipids declined throughout the course of the deficiency. Thus it appears that the mechanisms controlling the concentrations of neutral lipid and cholesterol in the liver cell during protein depletion are different from those which control cellular phospholipid concentration. During protein repletion the patterns of response of neutral lipids and cholesterol are different from those of the phospholipids. Concentrations of the former return rapidly to extremely high levels within 3 days after repletion, whereas the phospholipids simply return to normal after 2 weeks.

It appears that the protein deficiency per se had no effect on the plasmalogens but rather that the loss observed in groups 3 and 4 was simply due to lowered food intake. If the plasmalogens are refractory to protein deficiency and if methionine exhibits its effect only in conjunction with protein deficiency, the lack of effect of methionine (or cystine) on plasmalogen concentration would be explained.

The results for the total phospholipids were very similar to those for succinic oxidase and succinic dehydrogenase reported earlier (4). Simple protein depletion caused a progressive loss of these cellular enzymes to about the same extent as of the phospholipids. Methionine protected against the loss of the enzymes to almost exactly the same extent as for the phospholipids. It has been shown earlier (2, 4), under conditions in which the activity of the overall system is influenced by protein deficiency, that the rate-limiting enzyme of the succinic oxidase system is the initial succinic dehydrogenase. Also, in particulate heart preparations, succinic dehydrogenase has been shown to possess 2 reaction sites; if the enzyme is detached from the particulate structure, its activity is decreased because of loss of one of the reactive sites (18, 19). That phospholipid is required for the transfer to electrons from succinate to coenzyme Q reductase has been demonstrated by Brierley et al. (10). Thus, although a phospholipid requirement for activity of the initial isolated dehydrogenase has not yet been demonstrated, it is possible that phospholipid is required for the normal functioning of mitochondrial succinic dehydrogenase by enabling the proper attachment of the flavoprotein to the particulate structure of the mitochondrion.

In earlier work (2) it was observed that approximately 85% of cytochrome oxidase activity is lost from liver cells after about 12 weeks of protein depletion. Recently Brierley and Merola (9) observed that purified cytochrome oxidase loses almost all its activity after phospholipids are removed by solvent extraction and that the activity is partially restored by addition of phospholipid. Neutral lipids had no effect in this respect. Thus it is possible that the large loss of cytochrome oxidase activity after prolonged protein depletion (2) may be due in part to the loss of phospholipids observed in the present studies. It will be of interest in future studies to observe to what extent cytochrome oxidase, succinic oxidase, or other enzyme activities lowered by protein deficiency can be restored by

in vitro addition of phospholipid. In this way an indication of the extent of actual loss of the enzymes themselves or of activity due to loss of phospholipid might be obtained.

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

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# Influence of Hemicellulose A and B on Cellulose Digestion, Volatile Fatty Acid Production and Forage Nutritive Evaluation [1,2]

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*ABSTRACT* Forages were delignified with chlorous acid and holocellulose, α-cellulose and hemicellulose A and B fractions were isolated and studied in an artificial rumen system to determine the influence of individual components on the microbial digestion of cellulose and the nutritive value of forages. Forage delignification, resulting in the holocellulose fraction, always increased the amount of cellulose digested. Further removal of hemicellulose A and B increased the amount of cellulose digested in the resulting α-cellulose in most instances as much as 100%. The increased digestibility of cellulose in α-cellulose over holocellulose was attributed to the removal of hemicellulose A and B. Addition of hemicellulose B reduced cellulose digestion in the forage, holocellulose, or α-cellulose depending on the level added. Digestibility of cellulose in forages, holocellulose or α-cellulose was reduced to zero by an equal weight of hemicellulose B. Hemicellulose B addition was always accompanied by an increased VFA production and an increased molar ratio of propionate with corresponding reduction in acetate, the magnitude depending upon the level added. Hemicellulose A and B produced similar effects. Comparison of cellulose digestibility of forage, holocellulose and α-cellulose with equal cellulose level in all treatments, resulted in 41, 55 and 75% digested, respectively, with alfalfa and 25, 44 and 71% digested, respectively, with the reed canarygrass. The hemicellulose B content of reed canarygrass was approximately 3 times higher than alfalfa. Other constituents were similar.

Polysaccharides constitute approximately 75% of the dry weight of plants with the abundance of cellulose far transcending all other carbohydrates (1). Cellulose and hemicellulose A and B are the predominant roughage constituents of plant material, with the latter group ranging from 10 to 30% (2). Cellulose can be utilized by the mammalian system only through symbiotic microbial action in the gastrointestinal tract. Under such conditions certain chemical linkages may be more resistant than others to microbial digestion as in the case of plant hemicellulose glycuronosidic linkages which resist soil microbial attack (1, 3). The preferential breakdown of chemical linkages of forage polysaccharides by the bacterial and protazoan population of the rumen is not presently defined.

Efforts have been made to establish a measure of the nutritive value of a forage by correlation of in vitro rumen microbial digestion of cellulose or volatile fatty acid production with in vivo digestible energy measures (4–6). An in vitro method of predicting forage nutritive value would eliminate expensive and time-consuming digestion trials and in addition be a useful tool in evaluating the nutritive value of different genotypes in forage breeding programs. The present paper establishes the effect of isolated hemicellulose A and B on in vitro cellulose digestion of forages and isolated α-cellulose.

## MATERIALS AND METHODS

*Plant materials.* The forages used in this study were reed canarygrass (*Phalaris arundicacea* L.) and Vernal alfalfa. They had previously been observed to have highly similar digestibility of protein, dry matter and cellulose in sheep.[7] Their chemical composition was highly similar with the exception of hemicellulose B. The forages were grown on adjacent plots,

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[2] This study was supported in part from NC-64 Regional Project, Laboratory Methods for Estimating Forage Quality, and State Chemist Funds.
[3,4,5,6] Departments of Biochemistry, Animal Sciences, USDA-Agricultural Research Service, and Agronomy, respectively.
[7] Barnes, R. F. 1963 The *in vivo* and *in vitro* evaluation of selected reed canarygrass clones differing in palatability. A Ph.D. thesis, Purdue University.

dried at 60° in a forced-draft oven and ground to pass through a 40-mesh screen prior to their delignification and chemical separation into holocellulose, α-cellulose, hemicellulose A and hemicellulose B. The soluble carbohydrate content of the whole plant was 5.84 and 5.15% for alfalfa and reed canarygrass, respectively.[8] The polysaccharide content of the forages shown in table 1 indicates little difference in their chemical composition with the exception of the higher hemicellulose composition and lower α-cellulose of reed canarygrass. The significant difference in polysaccharide composition is that reed canarygrass contains more than twice the level of the hemicellulose B fraction than alfalfa. This difference in hemicellulose content in these experimental forages is also evident in the pentosan content of the reed canarygrass which is more than double that of alfalfa.[9]

*Chemical analysis.* The holocellulose fraction of the plant was isolated by the chlorous acid procedure of Whistler (7, 8) except that the reaction period was increased by 1.5 hours. This was necessary because of the higher protein content than that of the materials analyzed by Whistler. Whistler and Smart (1) cite numerous references of evidence indicating that the polysaccharide structures are left practically in their original state following delignification as long as the procedure is not continued to reduce the lignin value to 2% or less. The lignin content of the holocellulose, as determined by the method of Crampton and Maynard (10), averaged 4% and the ash averaged 5% of the dry weight.

The isolation and purification of α-cellulose, hemicellulose A and hemicellulose B fractions were carried out by a combination of procedures described previously (1, 8). Four hundred grams of holocellulose were extracted 15 hours with 3.5 liters of 10% KOH at 25° under nitrogen to remove hemicellulose. The α-cellulose residue was washed with dilute alkali and then with water until no trace of color remained. It was then treated with 10% acetic acid, washed until free of acid and air-dried at room temperature. The filtrate was neutralized with 50% acetic acid in the presence of ice, allowed to stand in the cooler for 15 hours and the hemicellulose A was separated in a Sharples supercentrifuge and purified. The hemicellulose B fraction remained in the brown opalescent solution and was precipitated by adding 3 times its volume of 95% ethanol with rapid stirring. The hemicellulose B was filtered, avoiding exposure to air, and washed with 95% ethanol several times and then with ether to facilitate drying in a vacuum desiccator.

The α-cellulose fraction contained, in addition to cellulose, some ash, alkali-resistant pentosans and nitrogen. No methods are available for extracting pure cellulose from forages without extensive degradation.

The compositional analysis shown in table 1 was determined on 30 g of the fat-extracted forages using the micro-quantitative procedure (9) and a Servall

[8] O'Donovan, P. B. 1964 Ad libitum intake and digestibility of forages by lambs as related to soluble and structural components. A Ph.D. thesis, Purdue University.

[9] See footnote 8.

TABLE 1

*Polysaccharide composition of experimental forages, dry matter basis* [1]

| Forage | Experiment used | Cellulose | Holocellulose | α-Cellulose | Hemicellulose A | Hemicellulose B |
|---|---|---|---|---|---|---|
| | | % | % | % | % | % |
| Alfalfa (1962) | 1,4,5 | 31.59 | 67.03 | 36.34 | 10.32 | 5.81 |
| Reed canarygrass (1962) | 3,4,5,6 | 28.46 | 66.84 | 27.10 | 8.06 | 14.00 |
| Alfalfa (1963) | 7 | 26.11 | 64.99 | 38.31 | 9.25 | 4.28 |
| Reed canarygrass (1963) | 7 | 22.65 | 65.20 | 28.17 | 8.55 | 13.01 |

[1] Cellulose was determined by the method of Crampton and Maynard (10). The holocellulose was an isolated forage fraction containing the α-cellulose and hemicellulose fractions. The α-cellulose fraction was the remaining product of 10% KOH extraction of holocellulose. The hemicellulose fractions were soluble in 10% KOH and hemicellulose A was recrystallized at neutral pH and removed. Hemicellulose B was then recrystallized by ethanol addition.

superspeed centrifuge (20,000 × *g*) to quantitatively recover the hemicelluloses.

Cellulose digestibility was determined (10) on the flask contents and if there was no more than 1% loss in the 24-hour incubation, digestion was considered zero. Total volatile fatty acid (VFA) concentration was determined by steam distillation of 20 ml of the rumen fluid sample to which 2 ml concentrated $H_2SO_4$ and 10 g of $MgSO_4$ were added to increase the rate of distillation. The individual VFA's were determined by gas chromatography on the artificial rumen fluid after the protein had been precipitated by *m*-phosphoric acid. The conditions for the determination were as follows: injector temperature, 200°; furnace temperature, 130°; hydrogen flow rate, 20 ml/minute; nitrogen flow rate, 16 ml/minute. The column was 0.3-cm copper tubing, 183 cm in length with a column packing made up of 20% LAC-296 + 2% $H_3PO_4$ on Chromosorb W (60–80). The instrument used was an Aerograph Model A600B equipped with a hydrogen generator, flame ionization detector and a Honeywell-Brown integrated recorder.

*In vitro analysis.* Fermentation was carried out in 50-ml Erlenmeyer flasks continuously shaken in a Dobnoff metabolic shaking incubator under an atmosphere of carbon dioxide gas. Each incubation flask contained 25 ml of fluid consisting of 6.25 ml of strained rumen inoculant, 15.5 ml of mineral solution (11), urea to make contents 0.02 M and deionized water up to volume. Each treatment was run in duplicate. Duncan's multiple range test was used for making significance tests among individual treatment means of replicated experiments (12). The period of incubation was 24 hours. The inoculum was obtained from a number of mature fistulated sheep. The results of this study were obtainable on inoculum from animals supplied with forage as well as with concentrate diets.

Experiment 1 was conducted with alfalfa harvested in the pod formation stage of the initial spring growth. Reed canarygrass harvested at the same time, but in the seed stage of the initial spring growth, was used in experiment 3. Reed canarygrass harvested in the late jointing stage of the first regrowth was used in experiments 2a and 2b. The forage, holocellulose and α-cellulose substrates were added on an equal weight basis in experiments 1, 2a, 2b and 3 resulting in variable cellulose content in the fermentation flask. Experiments 4 and 5 were replicates using the same forages as in experiments 1 and 3 but the substrates were added to give equal cellulose content in all treatments. In experiment 6, the isolated constituents of the reed canarygrass used in experiments 3, 4 and 5 were added at different levels and combinations to determine the relative influence of one constituent on the other. Experiment 7 was designed to determine the influence of hemicellulose A or B on cellulose digestion in α-cellulose and alfalfa and reed canarygrass. These forages were harvested during the first regrowth from the same plots used in earlier experiments, but one year later. Four replicate experiments were run with single flask for each treatment. The in vivo dry matter and cellulose digestibility in the alfalfa and reed canarygrass samples were the same with ad libitum feeding but the intake with the alfalfa was 30% greater.[7,8]

## RESULTS

Table 2 shows the influence of graded levels of hemicellulose B on the in vitro cellulose digestibility of its respective alfalfa forage and α-cellulose in experiment 1. In treatments 1, 2 and 6, with holocellulose, forage and α-cellulose, respectively, cellulose digestion was considerably increased by removal of other forage constituents in the isolation process. The addition of hemicellulose to the forage resulted in a large reduction of the amount of cellulose digested, a higher VFA production, and a change in the molar ratio of acetate and propionate. As less hemicellulose was added, more cellulose was digested and less VFA produced. Essentially the same effect was obtained when graded levels of hemicellulose B were added to the isolated α-cellulose of alfalfa (treatments 7, 8 and 9) as observed when added to the forage.

When similar comparisons of hemicellulose B addition were made with reed canarygrass in experiments 2a and b (table 3), hemicellulose B reduced cellu-

TABLE 2

*Influence of level of isolated hemicellulose B of alfalfa on cellulose digestibility and volatile fatty acid (VFA) production*

| Treatment no.[1] | Substrate | Cellulose digested | | VFA produced | Molar ratio of VFA produced | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Acetate | Propionate | Butyrate | Isovalerate | Valerate |
| | | *mg* | % | *mEq/100 ml* | | | | | |
| 1 | Holocellulose (750) [2] | 196 | 52.8 | 14.21 | 70.3 | 23.4 | 4.1 | 0.6 | 1.7 |
| 2 | Forage (750) | 106 | 44.7 | 14.14 | 68.5 | 21.2 | 7.3 | 1.2 | 1.8 |
| 3 | Forage (750) + hemicellulose B (250) | 70 | 29.7 | 16.27 | 65.0 | 27.0 | 6.3 | 0.4 | 1.1 |
| 4 | Forage (750) + hemicellulose B (100) | 96 | 40.7 | 15.74 | 66.0 | 24.7 | 7.0 | 0.7 | 1.6 |
| 5 | Forage (750) + hemicellulose B (50) | 95 | 40.1 | 14.43 | 66.2 | 24.1 | 7.2 | 0.9 | 1.7 |
| 6 | α-Cellulose (750) | 278 | 47.6 | 13.31 | 72.1 | 22.5 | 3.7 | — | 1.8 |
| 7 | α-Cellulose (750) + hemicellulose B(250) | 163 | 27.9 | 14.50 | 68.5 | 26.2 | 4.6 | — | 1.2 |
| 8 | α-Cellulose (750) + hemicellulose B (100) | 213 | 36.6 | 13.59 | 72.1 | 22.6 | 4.1 | — | 1.2 |
| 9 | α-Cellulose (750) + hemicellulose B (50) | 252 | 43.3 | 13.51 | 73.0 | 21.9 | 4.1 | — | 1.1 |

[1] All treatments run in duplicate.

[2] Numbers in parentheses indicate milligrams of substrate added. The cellulose content of the forage was 31.6%, holocellulose 49.5% and α-cellulose 77.7% to supply 236, 371, and 583 mg of cellulose, respectively, in 750 mg of sample.

TABLE 3

*Influence of level of reed canarygrass hemicellulose B on volatile fatty acid (VFA) production and digestibility of cellulose*

| Treatment no.[1] | Substrate | Cellulose digested [2] | | Total VFA [2] | Molar ratio of VFA produced [2] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Acetate | Propionate | Butyrate | Isovalerate | Valerate |
| | | *mg* | *%* | *mEq/100 ml* | | | | | |
| 1 | Holocellulose (750) [3] | 119.9 [b] | 36.1 | 12.53 [b] | 60.9 [b] | 30.6 [b] | 6.4 [d] | 0.9 [b] | 1.3 [b] |
| 2 | Forage (750) | 92.5 [c] | 46.1 | 13.27 [b] | 65.5 [a] | 23.2 [c] | 8.2 [b] | 1.6 [a] | 1.6 [a] |
| 3 | Forage (750) + hemicellulose B (750) | 0 | 0 | 20.44 [a] | 56.3 [b] | 31.7 [b] | 9.7 [a] | 0.5 [c] | 1.9 [a] |
| 4 | Forage (750) + hemicellulose B (250) | 49.2 [d] | 24.5 | 16.75 [b] | 61.0 [b] | 29.6 [b] | 7.6 [c] | 0.6 [c] | 1.5 [a] |
| 5 | Forage (750) + hemicellulose B (100) | 84.7 [c] | 42.1 | 15.14 [b] | 62.0 [a] | 27.3 [c] | 8.1 [b] | 1.2 [b] | 2.0 [a] |
| 6 | Forage (750) + hemicellulose B (50) | 92.0 [c] | 45.9 | 13.92 [b] | 63.7 [a] | 25.1 [c] | 8.5 [b] | 1.3 [b] | 1.6 [a] |
| 7 | Hemicellulose B (750) | 0 | 0 | 15.87 [b] | 56.8 [b] | 36.5 [a] | 6.1 [d] | 0.4 [c] | 1.1 [b] |
| 8 | α-Cellulose (750) | 268.3 [d] | 41.7 | 11.63 [b] | 62.6 [a] | 29.1 [b] | 7.1 [c] | 0.5 [c] | 0.7 [b] |
| 9 | α-Cellulose (750) + hemicellulose B (750) | 0 | 0 | 12.66 [b] | 55.6 [b] | 37.2 [a] | 5.8 [a] | 0.3 [c] | 1.0 [b] |
| 10 | α-Cellulose (750) + hemicellulose (250) | 131.5 [b] | 20.4 | 13.44 [b] | 60.7 [b] | 31.4 [b] | 6.9 [c] | 0.2 [c] | 0.7 [b] |

[1] All treatments run in duplicate, 2 replicate experiments.

[2] Those means within a column followed by a different letter superscript differ significantly ($P < 0.01$).

[3] Numbers in parentheses indicate air-dried weight of substrate added in milligrams. Cellulose content in forage is 26.75%; holocellulose, 44.21%; α-cellulose, 85.82%.

lose digestibility in a manner similar to that of alfalfa. The amount of cellulose digested in the α-cellulose was more than double that of holocellulose.

Addition of an equal quantity of hemicellulose B to forage prevented cellulose digestion, significantly increased VFA production, increased the molar ratio of propionate and decreased that of acetate ($P < 0.01$). A nearly 1:1 ratio of α-cellulose to hemicellulose B (treatment 9) resulted in zero cellulose digestibility, and a molar ratio of VFA similar to hemicellulose B substrate only (treatment 7). The reed canarygrass holocellulose produced a molar ratio of VFA's similar to that for hemicellulose B even though considerable cellulose was digested. This is in contrast with alfalfa holocellulose which closely resembled the forage.

The data in experiment 3 (table 4) with reed canarygrass cut at the same time as the alfalfa of experiment 1 substantiate results of previous experiments in that hemicellulose produced a marked inhibition on cellulose digestibility, in both the forage and the α-cellulose fraction. Isolated α-cellulose produced 69.6% of acetate whereas isolated hemicellulose B produced 59.2. When the hemicellulose was added in equal amounts to a flask with cellulose, cellulose digestibility was again reduced to zero and the molar ratio and total quantity of VFA's produced were very similar to that of hemicellulose B alone. Again the amount of cellulose digested in the holocellulose was less than that with α-cellulose but considerably greater than the forage.

Table 5 shows results and statistical analysis of experiments 4 and 5. Substrates of experiments 1 and 3 were added to give equal cellulose content in all treatments. In treatments 1 through 3 with equal cellulose substrates available, the cellulose digestibility of α-cellulose was greater than in holocellulose, which in turn was greater than the alfalfa forage. The same was true in treatments 4, 5 and 6 with reed canarygrass.

The same general trends in cellulose digestion and VFA production observed in alfalfa can be observed in the reed canarygrass; however, the canarygrass forage samples have a lower cellulose digestibility and correspondingly lower amounts of VFA's were produced. Considerable difference in the molar ratio of acetate and propionate was observed between the holocellulose fraction of alfalfa and reed canarygrass. This difference also occurred in previous experiments. The addition of hemicellulose B to α-cellulose or forage again resulted in a decrease in cellulose digested and an increase in VFA production corresponding to the level added.

Experiment 6, comparing relative quantities and ratios of isolated α-cellulose, hemicellulose B, and holocellulose of reed canarygrass from the previous experiment is shown in table 6. Hemicellulose B added alone to the in vitro system increased VFA production at the 750-mg level, but did not influence the molar ratio. In comparison with α-cellulose, there was a reduction in the acetate fraction and a one-third increase in propionate with the addition of hemicellulose.

An equal quantity of hemicellulose B added to α-cellulose reduced cellulose digestibility to zero and the VFA production and molar ratio of the VFA were almost identical to hemicellulose B substrate. Hemicellulose B was utilized in preference to the cellulose.

Decreasing the hemicellulose B content in the in vitro system, again caused a comparable increase in cellulose digestion. Under these conditions, the molar ratio of acetate increased and that of propionate decreased with decreased hemicellulose levels.

With treatments 8, 9 and 10 the hemicellulose B substrate was held constant but α-cellulose level was varied. This produced a decrease in the amount of cellulose digested only when α-cellulose was reduced to the 250-mg level and indicates the dependence of cellulose digestibility upon cellulose substrate concentration. The presence of 100 mg of hemicellulose B reduced the amount of cellulose digested from 259 mg (treatment 3) to 197 mg (treatment 8). We had previously determined that the amount of cellulose digested in this in vitro system is not reduced until α-cellulose levels were reduced to below 500 mg and that levels as high as 1000 mg do not increase the amount of cellulose digested in the 24-hour incubation.

TABLE 4

*Influence of hemicellulose B of reed canarygrass on volatile fatty acid (VFA) production and the digestibility of cellulose*

| Treatment no.[1] | Substrate | Cellulose digested | | Total VFA | Molar ratio of VFA produced | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Acetate | Propionate | Butyrate | Isovalerate | Valerate |
| | | *mg* | *%* | *mEq/100 ml* | | | | | |
| 1 | Holocellulose (750) [2] | 156 | 45.2 | 13.03 | 61.1 | 30.9 | 6.5 | 0.4 | 1.0 |
| 2 | Forage (750) | 72 | 33.6 | 11.76 | 64.3 | 24.3 | 8.6 | 1.1 | 1.7 |
| 3 | Forage (750) + hemicellulose B (750) | 0 | 0 | 19.80 | 60.8 | 31.9 | 5.9 | 0 | 1.4 |
| 4 | Forage (750) + hemicellulose B (250) | 45 | 21.0 | 14.94 | 61.6 | 29.3 | 7.6 | 0.4 | 1.3 |
| 5 | Forage (750) + hemicellulose B (100) | 70 | 33.0 | 13.72 | 62.6 | 27.4 | 8.1 | 0.6 | 1.4 |
| 6 | Forage (750) + hemicellulose B (50) | 78 | 36.4 | 12.79 | 61.5 | 28.1 | 8.3 | 0.7 | 1.0 |
| 7 | Hemicellulose B (750) | 0 | 0 | 16.01 | 59.2 | 33.2 | 6.7 | 0 | 1.0 |
| 8 | α-Cellulose (750) | 269 | 42.6 | 12.19 | 69.6 | 22.7 | 7.3 | 0 | 0.6 |
| 9 | α-Cellulose (750) + hemicellulose B (750) | 0 | 0 | 14.02 | 61.8 | 30.8 | 6.4 | 0 | 1.0 |

[1] All treatments run in duplicate.

[2] Numbers in parentheses indicate milligrams of dry weight of substrate added. The forage holocellulose and α-cellulose contained 213, 344 and 632 mg of cellulose, respectively/750-mg sample.

TABLE 5

*Cellulose digestibility and volatile fatty acid (VFA) production of forage and constituents when added at equal cellulose levels* [1,2,3]

| Treatment no. | Substrate | Cellulose digested | VFA produced | Molar ratio of VFA produced | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Acetate | Propionate | *n*-Butyrate | Iso-valerate | *n*-Valerate |
| | | % | *mEq/100 ml* | | | | | |
| | | | | Vernal alfalfa | | | | |
| 1 | Forage | 41.0 $^{d}$ | 15.55 $^{b}$ | 66.3 $^{ab}$ | 27.1 $^{d}$ | 5.1 $^{b}$ | 0.6 $^{bc}$ | 1.1 $^{d}$ |
| 2 | Holocellulose | 55.3 $^{b}$ | 11.57 $^{d}$ | 68.0 $^{ab}$ | 26.8 $^{d}$ | 3.6 $^{c}$ | 0.4 $^{cd}$ | 1.0 $^{d}$ |
| 3 | α-Cellulose | 73.8 $^{a}$ | 10.39 $^{ef}$ | 69.1 $^{a}$ | 25.8 $^{d}$ | 4.2 $^{bc}$ | 0 $^{e}$ | 0.9 $^{de}$ |
| | | | | Reed canarygrass, genotype 6017 | | | | |
| 4 | Forage | 24.6 $^{g}$ | 12.90 $^{c}$ | 62.9 $^{bc}$ | 27.8 $^{cd}$ | 6.2 $^{a}$ | 1.2 $^{a}$ | 1.7 $^{c}$ |
| 5 | Holocellulose | 44.3 $^{c}$ | 11.0 $^{de}$ | 58.3 $^{cd}$ | 36.2 $^{a}$ | 4.3 $^{bc}$ | 0.5 $^{bc}$ | 1.1 $^{d}$ |
| 6 | α-Cellulose | 71.1 $^{a}$ | 9.60 $^{f}$ | 66.4 $^{ab}$ | 29.2 $^{bc}$ | 4.2 $^{bc}$ | 0.1 $^{de}$ | 1.1 $^{e}$ |
| 7 | α-Cellulose + hemicellulose B (250) [4] | 33.3 $^{e}$ | 10.87 $^{de}$ | 63.1 $^{bc}$ | 31.0 $^{ab}$ | 5.1 $^{b}$ | 0 $^{e}$ | 0.9 $^{de}$ |
| 8 | Forage + hemicellulose B (500) | 0 $^{i}$ | 17.41 $^{a}$ | 56.3 $^{d}$ | 34.8 $^{ab}$ | 6.2 $^{a}$ | 0.1 $^{de}$ | 2.5 $^{a}$ |
| 9 | Forage + hemicellulose B (250) | 12.4 $^{h}$ | 16.72 $^{a}$ | 57.3 $^{d}$ | 34.3 $^{ab}$ | 5.9 $^{a}$ | 0.5 $^{bc}$ | 2.1 $^{b}$ |
| 10 | Forage + hemicellulose B (100) | 28.0 $^{f}$ | 15.42 $^{b}$ | 58.2 $^{cd}$ | 33.2 $^{abc}$ | 6.2 $^{a}$ | 0.8 $^{b}$ | 1.7 $^{c}$ |
| 11 | Hemicellulose B (500) | 0 $^{i}$ | 11.62 $^{d}$ | 58.5 $^{cd}$ | 35.3 $^{ab}$ | 5.2 $^{b}$ | 0.1 $^{de}$ | 1.1 $^{d}$ |

[1] Enough substrate added to supply equal weights (350 mg) of cellulose per flask.
[2] All treatments were run with duplicate flasks and 2 replicate experiments.
[3] Those means within a column followed by the same letter superscript do not differ significantly. All others differ significantly ($P < 0.05$).
[4] Numbers in parentheses indicate milligrams of hemicellulose B added.

TABLE 6

*Comparative influence of relative quantity of substrate on cellulose digestibility in an in vitro system*

| Treatment no.[1] | Substrate added [2] | | | Cellulose digested | | Total VFA [3] | Molar ratio of VFA produced | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | α-Cellulose | Hemicellulose B | Holocellulose | | | | $C_2$ | $C_3$ | $C_4$ | $C_5$ iso- [4] | $C_5$ |
| | *mg* | *mg* | *mg* | *mg* | % | *mEq/100 ml* | | | | | |
| 1 | 0 | 750 | 0 | 0 | 0 | 16.80 | 63.8 | 31.0 | 5.2 | 0 | 0 |
| 2 | 0 | 500 | 0 | 0 | 0 | 13.13 | 66.1 | 30.3 | 3.0 | 0 | 0.5 |
| 3 [1] | 750 | 0 | 0 | 259 | 41.0 | 11.52 | 74.0 | 20.0 | 6.0 | 0 | 0 |
| 4 | 750 | 750 | 0 | 0 | 0 | 16.26 | 64.9 | 29.4 | 5.0 | 0 | 0.8 |
| 5 | 750 | 500 | 0 | 0 | 0 | 14.18 | 64.7 | 29.6 | 5.7 | 0 | 0 |
| 6 [1] | 750 | 250 | 0 | 99 | 15.8 | 11.78 | 68.0 | 26.4 | 5.62 | 0 | 0 |
| 7 [1] | 750 | 150 | 0 | 162 | 25.7 | 12.27 | 69.5 | 24.8 | 5.8 | 0 | 0 |
| 8 [1] | 750 | 100 | 0 | 197 | 31.2 | 12.00 | 69.7 | 23.4 | 7.0 | 0 | 0 |
| 9 | 500 | 100 | 0 | 190 | 45.0 | 11.62 | 68.7 | 23.1 | 8.2 | 0 | 0 |
| 10 | 250 | 100 | 0 | 158 | 75.1 | 10.34 | 67.1 | 25.3 | 7.6 | 0 | 0 |
| 11 | 500 | 500 | 0 | 0 | 0 | 14.14 | 63.6 | 29.2 | 7.1 | 0 | 0 |
| 12 [1] | 250 | 250 | 0 | 98 | 46.6 | 12.21 | 66.6 | 26.6 | 6.9 | 0 | 0 |
| 13 | 0 | 0 | 1000 | 145 | 31.5 | 13.45 | 58.9 | 30.6 | 6.3 | 1.1 | 2.5 |
| 14 | 0 | 0 | 750 | 163 | 47.4 | 12.65 | 64.7 | 30.7 | 3.7 | 0.2 | 0.8 |
| 15 | 0 | 0 | 500 | 166 | 72.0 | 9.21 | 68.8 | 24.7 | 4.8 | 0.5 | 1.3 |
| 16 | 0 | 0 | 250 | 113 | 98.4 | 4.00 | 71.0 | 20.9 | 5.9 | 1.0 | 1.2 |
| 17 | 0 | 100 | 750 | 116 | 33.7 | 12.04 | 67.5 | 27.0 | 4.8 | 0.3 | 0.5 |
| 18 | 0 | 250 | 750 | 48 | 13.9 | 13.74 | 66.5 | 26.8 | 5.6 | 0 | 1.2 |

[1] All treatments run simultaneously with common inoculum. Treatments run in duplicate are marked with superscript 1, other means are single flask.
[2] All constituents were isolated from same sample of reed canarygrass.
[3] VFA indicates volatile fatty acid.
[4] Values below 0.2 for molar ratios are indicated as zero because these levels were supplied by the inoculum alone.

Numerous workers have erroneously used the percentage of cellulose digestibility in comparing substances of different cellulose content. Decreasing the α-cellulose in the in vitro system progressively reduced the amount of cellulose digested (treatments 8, 9 and 10); however; the percentage of cellulose digested was being increased from about 31 to 75%. The percentage cellulose digestion is often used as a sole criterion for evaluating forage nutritive quality and microbial activity in vitro. It thus appears to be important to give adequate attention to comparing substances with equal cellulose content.

Reducing the holocellulose substrate level from 1000 mg (treatment 13) to 750 mg (treatment 14) resulted in an increase in cellulose digestion. This was likely due to the corresponding higher quantity of hemicellulose A and B in the 1000 mg of holocellulose since the concentration of cellulose in both treatments was well above the amount necessary for maximal utilization in the system. In treatments 17 and 18, the addition of small amounts of hemicellulose B to the holocellulose produced a similar reduction in cellulose digestibility. A further reduction of holocellulose to 500 mg did not reduce the amount of cellulose digested, but the corresponding reduction of hemicellulose A and B available for utilization can be noted in the decrease in VFA produced as well as the shift in molar ratio of VFA characteristic of predominantly cellulose digestion. Note in treatment 16 that VFA production was reduced due to a substrate deficiency but the cellulose of holocellulose was 98% utilized.

Further results of added hemicellulose A or B to alfalfa and reed canarygrass forages and α-cellulose (exp. 7) are shown in table 6. The reduction in cellulose digestion in the forages is evident with either hemicellulose A or B. A similar reduction was produced when hemicellulose A and B were added to α-cellulose. That microbial digestion was occurring is evident in the increased amount of VFA produced over that of the forage or α-cellulose alone. The molar ratio of VFA's was similar to that in previous experiments and hence the results were not included in the tabular data.

## DISCUSSION

The complexity of microbial fermentation products and the heterogeneous rumen microflora makes the following of taxonomy and metabolic changes in population as influenced by forage components almost an impossibility. The molar ratio of VFA produced was indicative of the major forage components being utilized. Lack of adequate methods for the quantitative analysis of individual plant components presently makes impossible the assessment of the importance of individual plant components on forage digestibility, intake, growth and general nutritive value. A clear picture is not available from previous studies to explain the systematic processes of forage digestion in the rumen and factors controlling its passage through the alimentary tract.

The marked inhibition by hemicellulose of enzymic cellulose hydrolysis may indicate that a highly competitive substrate system exists between microbial species in the rumen or existing species alter their metabolism with different substrates. The increase in molar ratio of propionate and decrease in acetate in cultures utilizing predominantly hemicellulose does not indicate which of the above changes are taking place. Alteration in the metabolism of existing species appears likely from observations in this laboratory that a pure culture of rumen cellulolytic *Bacteroides succinogenes* utilized hemicellulose in preference to α-cellulose and increasing levels of hemicellulose lowered the amount of cellulose digested.[10] The data in the in vitro studies indicate further that rumen microflora preferentially utilize non-cellulose polysaccharides (pentosans) and utilize cellulose when little else is available. Cellulose digestion did not occur in forages, holocellulose or α-cellulose when quantities of hemicellulose A and B to support a superior VFA production were added. Previous studies in this laboratory have shown that essentially the same reduction of cellulose digestibility can be obtained by increasing the amount of forage or holocellulose substrate in the artificial rumen.[11] Whether the inhibition of

[10] Ragheb, H. S., and L. V. Packett, unpublished experiments.
[11] See footnote 10.

cellulose digestion is a result of preferential hemicellulose utilization or a change in microflora is not settled, but its action both in forage as well as delignified holocellulose and α-cellulose is evident.

Siu (13) presented referenced evidence that inhibition of cellulolytic activity in pure cellulolytic cultures by added pentoses and hexoses (major hydrolysis products of hemicelluloses) are probably due to the perferential utilization and breakdown of these substances. Breakdown of cellulose began only after growth on these substances ceased. Hemicellulose added at low levels in our study to α-cellulose or forages permitted some cellulose digestion, but always resulted in an increase in VFA production over that of either alone. The hemicellulose was probably utilized first followed by cellulose utilization. Observations of the microbial hydrolysis of gelatin being permanently inhibited by glucose levels exceeding 0.3% have been reported, whereas lower levels only inhibited hydrolysis until the glucose was utilized (13).

Monod (14) has termed the phenomenon in which a second compound is not utilized until the first has been completely exhausted as "diauxie." Instances of "triauxie" were observed in *Escherichia coli* on a mixture of glucose, sorbitol and glycerol. Forage components may present a "polyauxie" phenomenon for rumen microflora. The ideal composition for maximal digestibility, intake and productivity remains to be defined.

Current studies on soluble cellulose derivatives (carboxy methyl cellulose) indicate that microbial "cellulase" may include at least 2 distinct types of enzymes that are dependent on the action of each other for cellulose digestion. The first makes the cellulose molecule available by severing covalent, nonglucosidic linkages supposedly producing linear cellulose thus exposing β-D-(1 → 4) glucopyranose linkage for a second type of hydrolyzing enzyme (15,16). The ability to utilize β-D-(1 → 4) glucosidic linkages found in linear cellulose is not limited to cellulose decomposing microorganisms. It thus appears that the activity of the first type of enzymes may be a rate-limiting factor in cellulose utilization in forages.

The nutritional relationship between hemicellulose and cellulose undoubtedly is greatly influenced by chemical linkages to other cell wall constituents. Uronic acid-containing hemicelluloses cannot be extensively removed chemically prior to delignification nor can lignin be removed prior to some hemicellulose decomposition. Pure cultures of fungi are reported to digest exclusively forage hemicelluloses during the first 5 days with little removal of cellulose until later (17). This does not answer the question of whether chemical linkages resistant to enzymic hydrolysis are preventing the digestion of cellulose. The negative correlation between percentage of lignin in feeds and digestibility of the organic matter (10, 18) has stimulated a widespread concept of lignin as an "encrusting substance" preventing microbial enzymic activity on cellulose. Burdick and Sullivan (19) reported that greater lignification also hindered the acid hydrolysis of hemicellulose. This agrees with the increase in VFA production and cellulose digestion in the holocellulose fraction over that of the lignified forage. The removal of soluble proteins in the delignification may be responsible for some of the increase in cellulose digestibility. The delignification of the forage increased the digestibility of the cellulose in the holocellulose fraction (up to 98% of the cellulose was digestible). Removal of hemicellulose A and B from holocellulose further increased the digestibility (α-cellulose fraction), thus indicating that both the lignin and hemicellulose components affect cellulose digestion. Kamstra et al. (20) have previously concluded that lignin, but not hemicellulose, has an inhibitory effect upon cellulose digestion on the basis of similarities in the percentage of digestibility of α-cellulose and hollocellulose fractions. This conclusion resulted from a comparison of α-cellulose treatments containing 0.3 g of cellulose with holocellulose treatments containing only 0.2 g of cellulose even though their preliminary experiments showed cellulose substrate level affected digestibility. If the weight of cellulose digested had been used by Kamstra et al. (20) as a comparison, in every instance less cellulose was digested in flasks containing holocellulose. In addi-

tion it can not be assumed that the only difference in the holocellulose and α-cellulose fractions was the hemicelluloses, because the holocellulose obtained from forages, unlike that obtained from wood and straw, contains a considerable amount of protein.

The alfalfa used in this study had a higher content of lignin than the reed canarygrass (7.84 vs. 5.12% in 1962 and 6.45 vs. 4.35% in 1963), yet the in vivo digestibility of dry matter and cellulose was not different.[12] The cellulose digestibility in these in vitro studies indicates that alfalfa forage has a higher cellulose digestibility. This would not be expected on the basis of lignin content. The higher cellulose digestibility also occurred in the holocellulose of alfalfa (tables 2, 4, 5). Since most of the forage protein is removed from this fraction, the difference can be more specifically attributed to lower hemicellulose content of the alfalfa holocellulose. Further evidence of the influence of hemicellulose on cellulose digestibility is that the addition of hemicellulose to forage holocellulose, or α-cellulose substrate decreases digestibility. The pentosan level of forages increases with maturity just as lignin.

The use of cellulose digestion as an in vitro measure of the nutritive value of a forage is affected by a number of forage constituents. Whether it represents the total digestive process remains to be determined. The best results have been obtained with comparisons of the same forage species (21, 22). It appears that knowledge of the chemical interrelation of forage constituents may be essential to the understanding of microbial utilization of a given constituent.

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# Effect of Dietary Level of Thiamine on Reproduction in the Rat

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*ABSTRACT* Reproductive performance and certain aspects of maternal biochemical response were studied in female rats of the Wistar strain maintained with a basal diet containing either an adequate or a low level of thiamine from weaning until mating. During pregnancy the rats were fed an adequate basal diet, or the same basal diet with all vitamin levels doubled. Fewer young were produced by animals fed at the low level of thiamine prior to pregnancy. Albumin and γ-globulin were the only serum proteins that appeared to be influenced by thiamine intake. In other respects reproductive performance and maternal biochemical response were normal even when food intake and thiamine level were low prior to pregnancy. The young were of normal weight. Total serum protein, hemoglobin and hematocrit were similar. Doubling the intake of thiamine and other vitamins during pregnancy had no effect on reproductive performance. Thiamine content in the young and in livers of mothers was related to thiamine intake during pregnancy and was not influenced by low thiamine level prior to pregnancy.

Although the effect of the total absence of dietary thiamine upon reproductive performance of the rat has been investigated (1, 2), the effect of a prolonged partial deficiency has not been reported. The present study is concerned with the effect of a chronic thiamine deficiency from weaning until mating, and the reproductive performance and maternal biochemical response when thiamine levels during pregnancy meet or exceed those generally considered adequate.

## EXPERIMENTAL

Female rats of the Wistar strain were fed ad libitum from weaning a basal diet containing initially either 2.5 or 0.5 mg thiamine/kg diet. The basal diet consisted of the following: (in per cent) vitamin-free casein, 25; sucrose, 40.75; cornstarch, 18; beef suet, 8; corn oil, 2; salts (3), 4; cellulose,[2] 2; L-cystine, 0.15; and choline chloride, 0.1. Other vitamins were added in the following amounts per kilogram of diet: (in milligrams) riboflavin, 5; Ca pantothenate, 25; niacin, 10; pyridoxine·HCl, 2.5; folic acid, 2; biotin, 0.2; vitamin $B_{12}$ (0.1% mannitol trituration), 0.2; inositol, 200; *p*-aminobenzoic acid, 100; and menadione, 2.5. Fat-soluble vitamins were administered once weekly in 0.05 ml of corn oil and supplied 5 mg *dl*-α-tocopheryl acetate, 1250 IU vitamin A acetate and 120 IU vitamin $D_3$ per rat per week.

To permit normal estrous cycles, the thiamine level of the diet containing originally 0.5 mg/kg was raised progressively to 0.75 mg/kg after 4 weeks and to 1.0 mg/kg at the beginning of the sixth week of experiment.

At approximately 70 days of age, animals were mated with male Wistar rats that had been reared with commercial laboratory chow,[3] and if sperm were observed in the vaginal smear, the rats were considered pregnant. Following mating, some of the animals of each initial group were fed the basal diet containing 2.5 mg thiamine/kg of diet. Others were fed the basal diet in which levels of all vitamins except choline were doubled. This diet hereafter will be designated as the 5.0 mg/kg diet. Animals were weighed and vaginal smears examined daily to detect any deviation from the normal course of pregnancy.

On the 22nd day of gestation, the animals were anesthetized with sodium amy-

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[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

tal. Blood was removed by tail bleeding for determination of hematocrit [4] and hemoglobin [5] and from the heart for determination of total serum protein (4) and serum protein fractions by paper electrophoresis.[6] Maternal livers and entire litters were removed and frozen immediately and were analyzed later for thiamine using the thiochrome method (5).

## RESULTS

*Maternal weight gain and reproductive performance.* Animals fed 2.5 mg thiamine/kg prior to pregnancy were approximately 65 g heavier at the time of mating than those fed 0.5 to 1.0 mg/kg. Total weight gain during pregnancy was similar in all groups (table 1). Rate of gain, however, differed significantly with level of thiamine fed prior to pregnancy. Animals fed at the lower level gained more during the first week of pregnancy ($P < 0.01$); animals fed at the higher level gained more during the last week ($P < 0.05$).

Animals reared with the basal ration produced larger litters than those fed at the lower level of thiamine prior to mating (table 1). Doubling the level of vitamins fed during pregnancy had no effect on litter size. Weight of young and number of resorptions were not significantly different among the diet groups.

*Serum protein, hematocrit and hemoglobin.* Total serum protein was similar in all groups (table 2). There was a consistent tendency for the percentage of albumin in the serum proteins to parallel the level of thiamine intake whether comparisons were based on prepregnancy or pregnancy levels, although the differences were not statistically significant. In contrast, γ-globulin was high when thiamine intake was low. The γ-globulin level of 7.1% observed in the sera of animals fed 0.5 mg/kg prior to pregnancy and 2.5 mg/kg during pregnancy was significantly higher than that of either group of animals fed 2.5 mg/kg prior to pregnancy ($P < 0.01$). An increase in thiamine intake from 0.5 mg/kg prior to pregnancy to 5.0 mg/kg during pregnancy resulted in a restriction in γ-globulin to a level similar to the higher prepregnancy levels of thiamine and differed significantly from the pregnancy level of 2.5 mg/kg ($P < 0.05$).

No differences in hemoglobin or hematocrit due to diet prior to or during pregnancy were observed.

*Thiamine content of maternal livers and fetuses.* The thiamine content of maternal livers and of the fetuses is shown in table 3. Significantly higher levels of thiamine ($P < 0.01$) were noted in livers and young of mothers fed 5.0 mg/kg during pregnancy than in those fed the diet containing 2.5 mg/kg. No differences due to diet prior to pregnancy were observed.

## DISCUSSION

Thiamine deficiency is known to reduce fertility in the rat (1, 2). In the present study, the average number of resorptions was similar for all groups and was of the same order observed in animals fed stock diet and maintained under the same experimental conditions.[7] The small size of

[4] 75-mm capillary tubes centrifuged for 3 minutes at 12,300 rpm.

[5] Hycel, Inc., Houston, Texas 1959 Cyanmethemoglobin Determination.

[6] Beckman Instrument Company, Spinco Division, Model R Paper Electrophoresis Instruction Manual RIM-5, Palo Alto, California.

[7] Unpublished data.

TABLE 1

*Influence of level of dietary thiamine prior to and during pregnancy on weight gain and reproductive performance of the rat*

| Dietary thiamine | | No. of litters | Avg maternal wt gain | | | | Avg no. of young/litter | Avg fetal wt | Avg no. resorptions |
|---|---|---|---|---|---|---|---|---|---|
| Prepregnancy | Pregnancy | | Week 1 | Week 2 | Week 3 | Total | | | |
| *mg/kg* | *mg/kg* | | | | | | | *g* | |
| 2.5 | 2.5 | 10 | 32 | 31 | 69 | 130 | 10.1 | 4.8 | 1.3 |
| 2.5 | 5.0 | 8 | 29 | 30 | 74 | 132 | 10.1 | 4.9 | 0.9 |
| 0.5–1.0 | 2.5 | 6 | 40 | 28 | 57 | 125 | 6.8 | 4.9 | 1.3 |
| 0.5–1.0 | 5.0 | 13 | 41 | 31 | 55 | 127 | 7.2 | 5.3 | 2.5 |

TABLE 2

*Serum proteins, hematocrit, and hemoglobin of pregnant rats fed at different levels of thiamine prior to and during pregnancy*

| Dietary thiamine | | No. of sera analyzed | Serum proteins | | | | | Hematocrit | Hemoglobin |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Globulins | | | | | |
| Pre-pregnancy | Pregnancy | | Total | Albumin | $\alpha_1$- | $\alpha_2$- | $\beta$- | $\gamma$- | |
| *mg/kg* | *mg/kg* | | *g/100 ml* | % | % | % | % | % | *g/100 ml* |
| 2.5 | 2.5 | 9 | 5.7 | 39.5 | 29.1 | 11.2 | 15.4 | 5.1 | 35.5 | 12.2 |
| 2.5 | 5.0 | 8 | 5.4 | 43.5 | 26.6 | 11.3 | 14.0 | 4.6 | 35.8 | 12.6 |
| 0.5 | 2.5 | 6 | 6.0 | 36.0 | 27.3 | 12.0 | 17.6 | 7.1 | 36.0 | 11.8 |
| 0.5 | 5.0 | 11 | 5.7 | 40.0 | 27.7 | 10.8 | 15.9 | 5.6 | 36.3 | 12.2 |

litters in animals with a limited intake of thiamine prior to pregnancy therefore appears to reflect lowered fertility rather than a high resorption rate. The failure to affect litter size by increasing level of thiamine during pregnancy was further indication that litter size was the result of nutritional status at the time of conception rather than of diet eaten during pregnancy. The rapid gain in weight during the first week of pregnancy following a low level of thiamine was the result of the increase in food intake which occurred immediately when the level of dietary thiamine was increased. The weight of the young was similar with all of the diets investigated, but differences in litter size were sufficient to account for differences in weight gain during the final week of pregnancy.

Chow [8] has recently reported permanent stunting of the progeny when dietary restriction was imposed on the pregnant rat, although such restriction failed to affect the reproductive system of the mother during subsequent pregnancies.[9] According to Hafez (6), fetal weight depends upon the plane of nutrition in the last part of pregnancy rather than diet in early pregnancy. The ability of the rat to produce young of normal size in spite of a low intake of a thiamine-deficient diet prior to pregnancy is further evidence that diet during pregnancy is more important for the development of young of normal size than diet prior to pregnancy.

Although protein depletion may result in a depression in the concentration of albumin in the sera of rats (7, 8), the consistent tendency for serum albumin to parallel dietary thiamine suggests that thiamine intake contributed to the results obtained in the present study.

Differences in $\gamma$-globulin level in the sera of rats fed deficient diets prior to pregnancy also appear to be the result of vitamin intake during pregnancy. Significant differences in serum $\gamma$-globulin have been reported by other investigators when vitamin-deficient diets were fed. LaCommare (9) observed a large increase in serum

[8] Chow, B. F., and C. J. Lee 1964 The effect of dietary restriction during pregnancy and lactation on food utilization. Federation Proc., 23: 291 (abstract).

[9] Chow, B. F. 1964 Further observations on maternal dietary restriction and subsequent pregnancy. Federation Proc., 23: 291 (abstract).

TABLE 3

*Thiamine content of maternal liver and of fetuses*

| Dietary thiamine | | Maternal livers | | | Fetuses | | |
|---|---|---|---|---|---|---|---|
| Pre-pregnancy | Pregnancy | No. analyzed | Avg wt | Thiamine | No. of litters analyzed | Avg litter wt | Thiamine |
| *mg/kg* | *mg/kg* | | *g* | *μg/g* | | *g* | *μg/g* |
| 2.5 | 2.5 | 10 | 12.5 | 4.61 ± 1.01 [1] | 10 | 47.5 | 1.70 ± 0.24 |
| 2.5 | 5.0 | 7 | 12.6 | 8.42 ± 1.21 | 8 | 49.4 | 2.19 ± 0.13 |
| 0.5 | 2.5 | 5 | 11.5 | 4.61 ± 0.67 | 5 | 31.5 | 1.75 ± 0.18 |
| 0.5 | 5.0 | 11 | 10.4 | 8.46 ± 1.03 | 13 | 37.0 | 2.14 ± 0.12 |

[1] SE of mean.

γ-globulin of pigeons subjected to experimental beriberi. Increased γ-globulin in the sera of riboflavin-deficient rats was reported by Jacquot-Armand (10).

The thiamine content of maternal livers and fetuses reflects the diet fed during pregnancy rather than that fed prior to pregnancy. Liver storage and possibly fetal transfer of the vitamin were maximum with the diet containing 5 mg thiamine/kg. Morrison and Sarett (11) reported liver thiamine levels of 8.7 μg/g and 8.4 μg/g in female rats postpartum when the animals were fed a diet containing 75 mg thiamine/kg during pregnancy. These levels of liver thiamine are comparable with that of 8.4 μg/g observed in animals receiving 5 mg/kg in the present study. Everson et al. (12) reported the thiamine content of the fetus at term to be 2.4 μg/g when mothers were maintained with a diet containing 140 to 145 μg thiamine/day. In the present study, the values for fetuses averaged 2.2 μg/g. Average thiamine intakes of animals fed 5 mg/kg ranged from 76 to 93 μg/day from the first to the third week of gestation. The somewhat higher fetal thiamine level of 3.0 μg/g reported by Barrett and Everson (13) was not the result of a higher maternal thiamine intake.

In the present study apparently both levels of thiamine fed during pregnancy provide intakes which support normal reproduction in the rat receiving an adequate ration prior to pregnancy. The basal diet provided a maximum of 50 μg/day during the final week. It thus appears that, for the last 3 days of gestation, the requirements for normal reproduction in the rat may be less than 80 μg/day as recommended by the NRC (14). The data further suggest that the dietary level of thiamine promoting maximal maternal liver and fetal thiamine reserves in the rat is probably no greater than 80 μg/day.

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# Fatty Livers Produced in Albino Rats by Excess Niacin in High Fat Diets

## II. EFFECT OF CHOLINE SUPPLEMENTS [1]

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*ABSTRACT* A study was conducted to determine the extent to which choline metabolism is involved in the production of fatty livers in rats fed diets high (40%) in fat and high (0.1%) in niacin. Fatty livers produced in animals fed excess niacin and non-protective levels of choline could be reversed by the addition of choline to the diet. Data from determination of $N^1$-methylnicotinamide excretion, serum alkaline phosphatase activity, and liver lipids suggested that the appearance of fatty livers in animals fed excess niacin resulted from an induced choline deficiency.

The effects of the addition of 0.1% of niacin to casein diets containing 5 or 40% of fat on the metabolism of the albino rat were studied in this laboratory (1). The excess niacin entered metabolic pathways to produce at least 2 unrelated effects, 1) increased concentrations of pyridine nucleotides in both blood and liver tissues and 2) increased levels of fat in the liver. The first effect occurred regardless of the level of fat in the diet, but the second occurred only in conjunction with a high fat intake.

The appearance of fatty livers in animals fed diets high in fat and in niacin could reflect a choline deficiency precipitated by the presence of excess niacin in the high fat diet. This theory is supported by the evidence of Handler and Dann (2), who reported fatty livers produced by the introduction of 2% of niacin in a 10% fat diet could be reversed by the addition of choline, betaine, or methionine to the same diet. In addition, Griffith and Mulford (3) reported that the addition of 0.52% of niacin to diets containing only partially protective levels of choline, produced a higher percentage of renal lesions in young rats than the same diets without the niacin supplements. They concluded that niacin exerted a moderate "choline-opposing" action.

Both groups of workers (2, 3) proposed that choline is used to detoxify niacin. Nicotinamide, the active form of the vitamin, is detoxified in the liver by the transfer of a methyl group from methionine (4), which originates from choline (5), to produce $N^1$-methylnicotinamide (MNA) and related methylated compounds. Therefore an excess of niacin, serving as a drain on the choline pool, could decrease the choline available for phospholipid formation and thus allow fat to accumulate in the liver as a result of reduced fat transport.

A direct relationship between choline requirement and the fat content of the diet has also been demonstrated. Griffith (6) observed an increase in the severity of choline deficiency in young rats when the fat content of the diet was increased. Thus, the 40% fat diets used in these experiments would be more choline-limiting than those low in fat and therefore more conducive to fatty liver production when the niacin content of the diet was increased.

The following experiments were conducted to determine the extent to which choline is involved in the production of fatty livers in rats fed diets high in fat and in niacin.

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## METHODS

*Experiment 1.* Seventy male weanling albino rats of the Sprague-Dawley strain were divided into 7 groups with 10 rats per group. Rats in group 1 were fed a basal diet providing the following: (in g/100 g diet) casein, 20; salts,[3] 4; choline, 0.15; vitamin mix, 0.25;[4] corn oil, 40;[5] and sucrose, 35.6. The diets of the remaining groups differed from the basal diet and are shown in table 1.

The animals were fed the experimental diets ad libitum for 2 weeks, after which they were killed and the livers removed for analysis of fat and moisture by methods described in a previous paper (1).

*Experiment 2.* Seven groups of 10 animals each were fed diets that differed from the basal diet as shown in table 2. The animals were fed the experimental diets for 2 weeks and then were killed. Livers were removed and analyzed for fat and moisture.

*Experiment 3.* Five groups of 10 rats were fed diets that differed from the basal diet as shown in table 3. The vitamin mix used in experiment 3 differed slightly from the one used in previous experiments. The major difference was an increase in the content of vitamin A powder from 2.5 to 10 mg/100 g diet. The change in the vitamin content of the diet made no difference in the results.

Each group of 10 animals was fed the experimental diet ad libitum for 2 weeks. On the twelfth day the animals were transferred to metabolism cages and 24-hour urine collections taken for immediate analysis of MNA. On the fourteenth day the animals were lightly anesthetized with ether and blood samples were collected

[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.

[4] The basal diet contained 0.5 mg niacin/100 g diet. The composition of the vitamin mix has been described previously (1).

[5] Containing 7.5 mg of α-tocopheryl acetate.

TABLE 1

*Weight gain, food intake, and liver composition of rats fed diets high in niacin and various choline supplements (exp. 1)*

| Group [1] | Diet | Wt gain | Food intake | Liver composition | |
|---|---|---|---|---|---|
| | | | | Moisture | Fat [2] |
| | | *g/week* | *g/week* | % | % |
| 1 | 0.15% Choline [3] | 21.2 ± 1.5 [4] | 36.7 ± 2.0 | 70.3 ± 0.5 | 15.0 ± 0.8 |
| 2 | 0.15% Choline + 0.10% niacin | 24.4 ± 0.4 | 41.4 ± 1.2 | 67.9 ± 0.9 | 22.5 ± 2.1 |
| 3 | 0.30% Choline [3] | 29.3 ± 1.6 | 48.4 ± 1.8 | 71.1 ± 0.2 | 10.5 ± 0.4 |
| 4 | 0.30% Choline + 0.10% niacin | 30.3 ± 1.6 | 52.3 ± 2.1 | 71.0 ± 0.2 | 13.7 ± 0.7 |
| 5 | 0.50% Choline [3] | 28.9 ± 1.0 | 47.3 ± 0.8 | 71.7 ± 0.3 | 8.8 ± 0.3 |
| 6 | 0.50% Choline + 0.10% niacin | 28.2 ± 1.8 | 47.8 ± 2.3 | 71.4 ± 0.2 | 11.4 ± 0.5 |
| 7 | 0.50% Choline + 0.33% niacin | 26.8 ± 1.6 | 45.4 ± 1.9 | 71.0 ± 0.5 | 11.4 ± 0.5 |

[1] Each group consisted of 10 animals.
[2] Based on dry weight of liver.
[3] Containing 0.5 mg niacin/100 g diet.
[4] SE of mean.

TABLE 2

*Weight gain, food intake, and liver composition of rats fed diets high in niacin and various choline supplements (exp. 2)*

| Group [1] | Diet | Wt gain | Food intake | Liver composition | |
|---|---|---|---|---|---|
| | | | | Moisture | Fat [2] |
| | | *g/week* | *g/week* | % | % |
| 1 | 0.25% Choline [3] | 22.0 ± 1.3 [4] | 44.2 ± 2.2 | 69.1 ± 0.3 | 11.7 ± 0.5 |
| 2 | 0.50% Choline [3] | 23.0 ± 2.0 | 44.8 ± 1.7 | 69.3 ± 0.3 | 10.5 ± 0.5 |
| 3 | 0.50% Choline + 0.10% niacin | 25.5 ± 1.9 | 47.0 ± 2.2 | 70.2 ± 0.4 | 13.1 ± 0.3 |
| 4 | 0.75% Choline [3] | 23.4 ± 1.2 | 43.4 ± 1.5 | 71.9 ± 0.4 | 11.4 ± 0.5 |
| 5 | 0.75% Choline + 0.10% niacin | 22.6 ± 2.0 | 44.2 ± 2.4 | 72.0 ± 0.2 | 14.6 ± 0.7 |
| 6 | 1.00% Choline [3] | 22.8 ± 0.9 | 44.0 ± 1.0 | 72.3 ± 0.3 | 10.0 ± 0.8 |
| 7 | 1.00% Choline + 0.10% niacin | 20.2 ± 1.5 | 41.0 ± 1.5 | 72.5 ± 0.1 | 8.4 ± 0.3 |

[1] Each group consisted of 10 animals.
[2] Based on dry weight of liver.
[3] Containing 0.5 mg niacin/100 g diet.
[4] SE of mean.

TABLE 3

*Effect of excess dietary niacin in rats fed diets high or low in choline (exp. 3)*

| Group [1] | Diet | Wt gain | Food intake | MNA excretion [2] | Serum alkaline phosphatase |
|---|---|---|---|---|---|
| | | *g/week* | *g/week* | *μg/g body wt* | *mg P/ml/hr* |
| 1 | 0.25% Choline [3] | 22.4 ± 2.0 [4] | 38.0 ± 2.4 | 5.9 ± 0.3 | 1.21 ± 0.10 |
| 2 | 0.25% Choline + 0.10% niacin | 25.4 ± 1.1 | 42.9 ± 1.3 | 33.6 ± 2.0 | 1.30 ± 0.04 |
| 3 | 1.00% Choline [3] | 25.2 ± 1.3 | 41.2 ± 1.7 | 5.4 ± 0.6 | 1.01 ± 0.08 |
| 4 | 1.00% Choline + 0.10% niacin | 22.8 ± 1.7 | 40.4 ± 2.1 | 45.1 ± 3.7 | 1.22 ± 0.05 |
| 5 | 1.00% Choline + 0.20% niacin | 22.3 ± 2.3 | 38.8 ± 2.1 | 52.5 ± 3.5 | 1.34 ± 0.11 |

[1] Each group contained 10 animals.
[2] $N^1$-Methylnicotinamide excretion during a 24-hour period.
[3] Containing 0.5 mg niacin/100 g diet.
[4] SE of mean.

from a tail snip. The animals were then decapitated and the livers excised and analyzed as described below.

The MNA concentrations were determined in urine samples by the method of Pelletier and Campbell (7). The 24-hour urine samples, collected under toluene, were diluted to 250 ml (rats fed normal amounts of niacin) or 1000 ml (rats fed excess niacin) and 1.0-ml aliquots used for analysis of MNA.

The blood samples were allowed to clot for one hour before centrifuging. The serum was drawn off and frozen immediately. Within 1 week the samples were thawed and 0.1 ml was used for determination of alkaline phosphatase activity by a modification of the Bessey-Lowry method (8). The serum was incubated at 38° with 1.0 ml of buffered substrate solution [6] (*p*-nitrophenyl phosphate and glycine buffer, adjusted to pH 9.4). Exactly 15 minutes after adding the serum, 10 ml of 0.02 M sodium hydroxide were added to each tube to stop the activity of the enzyme. The volume of each tube was brought to 40 ml with sodium hydroxide and the contents were read at 410 mμ. The color due to *p*-nitrophenol was removed by the addition of 0.4 ml hydrochloric acid and the contents again read as a serum blank. Activity of alkaline phosphatase was reported as milligrams of phosphorus liberated per milliliter per hour.

The methods for preparing liver samples for fat extraction have been described previously (1). Ether was used as the solvent in earlier experiments, but because a more quantitative recovery of the fat was desired in this experiment, a solvent of ethyl ether: Skellysolve B:ethyl alcohol (5:5:2) was used (9). One-gram samples of dried ground liver were extracted for 3 hours on the Goldfisch apparatus and total lipids determined gravimetrically.[7]

Lipid extracts were redissolved in alcohol:ether (3:1), diluted to 20 ml with ether, and 0.5-ml aliquots taken for phospholipid analysis. The aliquots were digested by the method of Youngberg and Youngberg (10). The Fiske and Subbarow method (11) was used for phosphorus analysis. The lipid fraction designated as neutral fat was calculated by subtracting phospholipid from total fat.

Standard errors of the means were calculated for all data and Student's *t* test was used as a measure of significance.

## RESULTS

*Experiment 1.* The results of experiment 1 are presented in table 1. Animals in groups fed 0.30 and 0.50% of choline showed significantly greater ($P < 0.01$) weight gains and food intakes than those fed 0.15% of choline (groups 3 and 5 vs. group 1). There were no significant differences in growth or food intake between those fed 0.5 mg and 0.10 g of niacin/100 g of diet at the same choline level.

Both the choline level and the presence of excess niacin in the diets affected the fat content of the livers. The percentage of liver fat was significantly decreased ($P < 0.01$) as choline was increased from 0.15 to 0.30% and from 0.30 to 0.50% regardless of the level of niacin. The addition of 0.10% of niacin to the diet caused

[6] Obtained from Sigma Chemical Company, St. Louis.
[7] All data for fat cited in the text are based on the dry weight of liver.

a significant increase in liver fat ($P < 0.01$) regardless of the level of choline. However, liver fat concentrations in animals fed 0.33% of niacin (group 7) were not significantly different from those in animals fed 0.10% of niacin (group 6).

*Experiment* 2. There were no significant differences in growth or food intake among any of the groups in this experiment (table 2). The addition of 0.10% of niacin to diets containing 0.50% (groups 2 vs. 3) or 0.75% (groups 4 vs. 5) of choline caused a significant increase ($P < 0.01$) in liver fat concentrations. However liver fat was not increased in animals fed 0.10% of niacin when the diet also contained 1.00% choline (groups 6 and 7). Increasing the choline supplement to 0.50%, or 0.75%, or 1.00% did not alter liver fat levels in animals fed either adequate or excessive amounts of niacin.

*Experiment* 3. Weight gain and food intake did not differ significantly among any of the groups in experiment 3 (table 3).

A marked difference (greater than fivefold) in urinary excretion of MNA was apparent between animals fed adequate amounts of niacin and those fed excess niacin (table 3). Rats fed excess niacin (groups 2, 4, and 5) excreted significantly greater quantities ($P < 0.01$) of MNA in 24 hours than did the control animals fed adequate niacin (groups 1 and 3). When the choline content was increased from 0.25 to 1.00% in diets containing 0.10% of niacin, a slight but significantly greater amount ($P < 0.02$) of MNA was excreted by the animals (groups 4 vs. 2). There were no differences in excretion of MNA between the groups fed adequate niacin (groups 1 and 3) or between groups fed 0.10 and 0.20% niacin at the same choline level (groups 4 and 5). Serum alkaline phosphatase activity was significantly elevated in those animals fed diets containing low choline (0.25%) or high niacin (0.10%).

Liver composition data are summarized in table 4. The total fat content of livers from animals fed 1.00% of choline was significantly lower than that from animals fed 0.25% of choline regardless of the amount of niacin present (group 3 vs. 1, $P < 0.02$; group 4 vs. 2, $P < 0.01$).

A difference in neutral fat was observed between the animals fed 0.10% of niacin and adequate (0.5 mg/100 g diet) niacin at the 0.25% choline level. Livers from animals in group 2 contained significantly greater ($P < 0.05$) amounts of neutral fat than those in group 1. There were no significant differences in percentage of phospholipids of dry weight of liver or in percentage of phospholipids of total liver fat between comparable groups.

## DISCUSSION

Results from experiments 1 and 2 indicate that 0.25% of choline in the diet is needed to produce optimal growth in rats fed the 40% corn oil diets described here. However, when the diet contains excess (0.10%) niacin, dietary choline must be increased to 1.00% to maintain liver fat levels in a range comparable to those in control animals. The reversal of the fatty liver syndrome by incorporating sufficient quantities of choline in the high niacin diets thus provides evidence that the fatty livers in rats fed excess niacin result from

TABLE 4

*Liver composition of rats fed diets high in niacin and high or low choline (exp. 3)*

| Group [1] | Diet | Moisture | Total fat [2] | Phospholipid [2] | Neutral fat [2] | Phospholipid in fat |
|---|---|---|---|---|---|---|
| | | % | % | % | % | % |
| 1 | 0.25% Choline [3] | 71.7±0.5 [4] | 15.9±0.7 | 4.59±0.32 | 11.3±0.5 | 28.8±1.3 |
| 2 | 0.25% Choline+0.10% niacin | 70.9±0.2 | 17.5±0.8 | 4.34±0.37 | 13.2±0.7 | 24.7±1.6 |
| 3 | 1.00% Choline [3] | 72.7±0.3 | 13.4±0.6 | 3.92±0.45 | 9.5±0.3 | 28.4±2.5 |
| 4 | 1.00% Choline+0.10% niacin | 71.8±0.2 | 14.1±0.5 | 3.75±0.39 | 10.3±0.6 | 26.9±2.9 |
| 5 | 1.00% Choline+0.10% niacin | 72.4±0.1 | 13.7±0.6 | 3.13±0.36 | 10.6±0.6 | 22.7±2.4 |

[1] Groups 1 through 4 contained 10 animals each; group 5 contained 9 rats.
[2] Based on dry weight of liver.
[3] Containing 0.5 mg niacin/100 g diet.
[4] SE of mean.

an induced choline deficiency. These results are in agreement with those of Handler and Dann (2).

Experiment 3 reinforces the observations of experiments 1 and 2. The amount of fat in the liver was inversely proportional to the amount of choline in the diet. Moreover, the lipid accumulated in livers of rats fed diets containing 0.25% of choline was neutral fat. It has been reported that fatty livers in choline deficiency are due to the accumulation of neutral fat (12). The theory of an induced choline deficiency in animals fed excess niacin is supported by MNA excretion data. Animals fed excess niacin excreted much greater amounts of MNA than the controls. Animals fed diets high in niacin (0.10%) and in choline (1.00%) excreted significantly more MNA than those fed diets high in niacin and low (0.25%) in choline. Apparently more methyl groups than are supplied by the low choline diet are needed for the methylation of nicotinamide in animals fed excess niacin.

Data from serum alkaline phosphatase determinations add further support to this theory. Elevated serum alkaline phosphatase activity in the rat is characteristic of choline deficiency (13). The relatively greater activity of this enzyme in serum from animals fed low levels of choline in this experiment suggest that a mild choline deficiency existed in animals from both the group fed adequate niacin and the group fed excess niacin. In the groups fed high levels of choline there was a significant increase in alkaline phosphatase activity when animals were fed excess niacin.

The effect of choline deficiency on the concentration of phospholipids in liver tissues is controversial. Some investigators report that total phospholipids are decreased in livers of choline-deficient rats (12, 14). Others report that total phospholipid level remains constant (15, 16), but that the rate of turnover decreases sharply in choline-deficient animals as compared with that in controls (15, 17). No evidence of a decrease in liver phospholipids in animals fed excess niacin was observed in the experiments reported here. However, no attempt was made to measure turnover rate. A reduction in phospholipid turnover in animals fed diets containing excess quantities of niacin could result in reduced fat transport and lead to increased accumulation of fat in liver tissues.

Animals fed 0.20 or 0.10% niacin showed similar responses. Those animals fed 0.20% of niacin did not differ from those fed 0.10% of niacin with respect to MNA excretion, alkaline phosphatase activity, or percentage of total fat, neutral fat, and phospholipid in the liver. At least 2 explanations can be postulated for these similarities: 1) that the extra niacin was not absorbed, or 2) that the extra niacin was excreted as niacin or some other non-methylated product. There is some evidence to support the latter. Lin and Johnson (18) investigated the excretion of radioactive metabolites in the urine after injections of labeled niacin in the rat. With normal doses of niacin the rats excreted MNA as the chief product, but with high doses of niacin the chief excretory products were nicotinuric acid and nicotinic acid. The excretion of niacin as either of these non-methylated compounds would not affect the supply of methyl groups.

It is suggested, therefore, that the inclusion of 0.10% of niacin in diets containing non-protective levels of choline induces a slight choline deficiency in young rats. The deficiency is not intensified in animals fed 0.20% of niacin probably because most of the larger supplement is excreted via metabolic routes which do not involve the metabolism of choline.

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# Partition of Excreted Nitrogen from Honey Bees Fed Various Proteins [1,2]

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*ABSTRACT* Newly emerged honey bees were fed diets containing 5% protein in the form of dandelion pollen, egg albumin, soybean meal hydrolysate or casein for 7 days, after which the recta were removed and the nitrogenous excretory products determined in the fecal material. Bees fed pollen showed the following as percentage of total nitrogen: uric acid, 62.06; creatine, 1.5; creatinine, 1.47; amino nitrogen, 6.11; urea, 0; and ammonia, 0. Amino nitrogen showed a direct relationship to diet adequacy: 1.36 and 6.11% for the negative controls and pollen-fed groups, respectively. No consistent pattern was noted for creatine or creatinine, except that both were lower for the pollen-fed groups than for the negative controls. When dietary protein sources were arranged in order of decreasing adequacy based on pharyngeal gland development, uric acid excretion varied inversely with diet adequacy; pollen, 48 (percentage of total nitrogen excreted); egg albumin, 62; soy hydrolysate, 62; casein, 96; and negative control, 96.

The natural diet of the honey bee (*Apis mellifera*) consists of honey, which furnishes carbohydrate for energy, and pollen, which supplies proteins and other nutrients necessary for growth, development and reproduction. Various proteinaceous materials have been substituted for pollen in the diet of the bee, among which are soybean flour, egg albumin and casein (1–5). The criteria for diet adequacy, such as pharyngeal gland development or longevity of the bee, have shown that none of these is as effective as pollen (1, 4), with the exception that Standifer (5) observed caged bees to respond equally well to egg albumin or pollen.

Little has been published concerning the catabolic fate of nitrogen in the honey bee. The present paper reports the determination of the forms in which nitrogen is excreted by the bee and also the influence of dietary protein source upon these forms or amounts.

## METHODS

Brood frames were taken from the hive, and all cells containing honey and pollen were covered with beeswax so that, upon emergence, the adult bees were prevented from obtaining food. The brood frames were incubated at 33 ± 1° and a relative humidity of 25 to 35%. About 75 adult bees which had emerged in the preceding 24 hours were collected each day, put into cages (5) and supplied with test diets [3] and water ad libitum. Cage floors were lined with waterproof paper to facilitate collection of feces.

In experiment 1 caged bees were fed various diets (table 1) containing 5 or 10% protein (furnished by egg albumin, sesame meal, soy α-protein, soy hydrolysate (enzymatic), liver extract, skim milk, castor bean meal, cottonseed meal or zein) and 75% carbohydrate,[4] with non-nutritive cellulose and water to give a palatable consistency. Bees normally defecate during free flight; however, caged bees will defecate when the gut has filled to capac-

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[1] Arizona Agricultural Experiment Station Technical Paper no. 889.

[2] Taken from a thesis submitted by J. B. McNally to the Graduate College, the University of Arizona as a requirement for the M.S. degree. Presented in part at the 1961 meeting of the Federated Societies for Experimental Biology and Medicine (Federation Proc., 20: 350e).

[3] Egg albumin, soy α-protein, soy hydrolysate (enzymatic), liver extract and zein were obtained from Nutritional Biochemicals Corporation, Cleveland. Sesame meal, skim milk and castor bean meal were obtained from a local supply house. The cottonseed meal was obtained through the courtesy of Producer's Cotton Oil Company, Phoenix, Arizona.

[4] Nulomoline: a commercial syrup containing 76% dextrose-levulose (1:1), 24% water, and traces of iron and copper. Nulomoline Company, Los Angeles, California.

TABLE 1

*Total fecal nitrogen of caged bees fed various proteinaceous diets (exp. 1)*

| Dietary source of protein | Total nitrogen |
|---|---|
| | *g/100 g feces* |
| Dandelion pollen (5) [1] | 4.53 |
| Dandelion pollen (10) | 5.35 |
| Average of all pollen diets (10) | 5.31 |
| Castor bean (10) | 4.32 |
| Egg albumin (10) | 5.49 |
| Cottonseed meal (10) | 5.57 |
| Skim milk (10) | 6.05 |
| Liver extract (10) | 6.64 |
| Soy α-protein (10) | 6.66 |
| Soy hydrolysate (10) | 6.82 |
| Zein (10) | 7.06 |

[1] Numbers in parentheses indicate grams of protein/100 g of diet.

ity, usually on the sixth day of adult life. On the seventh day the fecal material was collected from the cage floors, dried at 80° overnight, and ground into a fine powder with a mortar and pestle. Total nitrogen was determined by the micro-Kjeldahl method according to Kirk (6).

In experiment 2 pollen and non-pollen diets (table 2) contained 5% protein. Bee-gathered dandelion *(Taraxacum officinale)* pollen was used for the positive control. Egg albumin, soy hydrolysate and casein were obtained commercially. Each diet was fed ad libitum to groups of 75 newly emerged adult bees in cages as described above and replicated 5 times. After 7 days the bees in each cage were killed, decapitated and the fecal-containing recta were removed, combined with any fecal material from the cage floor, dried overnight and ground as in experiment 1. In both experiments difficulty was encountered in completely recovering the fecal material from the paper lining the cage floors. Because of this, all results were calculated as percentages rather than as total constituents excreted.

Extracts were prepared for analysis by grinding 250 mg of the fecal powder in a mortar together with 1 ml of saturated lithium carbonate solution and diluting to 25 ml with water (7). Uric acid was determined as the arsenophosphotungstic acid complex (8) and read at 520 mμ in an Evelyn colorimeter. α-Amino nitrogen was measured by the ninhydrin method (9) with leucine as a standard read at 620 mμ. Creatine and creatinine were analyzed by the Folin technique according to Hawk et al. (10) and read at 490 mμ. Total nitrogen was determined as described previously (6). Urea was converted to ammonia by urease and the resulting ammonia was determined by nesslerization (11). Analysis for free ammonia was carried out by direct nesslerization (11).

## RESULTS

Total nitrogen content of the fecal samples collected in experiment 1 is shown in table 1. Since most of the values represent means of only 2 replicates, standard error of the mean was not calculated. The data are not conclusive and are intended only to indicate possible trends. At the 10% protein level, bees fed the egg albumin diet showed substantially the same nitrogen excretion as those fed pollen; however, all other non-pollen protein sources, with the exception of castor bean, resulted in higher excreted nitrogen. The data imply that castor bean, egg albumin and cottonseed meal protein are equal to pollen and somewhat better than the remaining non-pollen protein sources in their utilization by the bee. This conclu-

TABLE 2

*Composition of diets (exp. 2)*

| Diet | Protein source | Sugar syrup [1] | Cellulose [2] | Water |
|---|---|---|---|---|
| | *g/100 g diet* | *g/100 g diet* | *g/100 g diet* | *g/100 g diet* |
| Pollen (10.2) [3] | 48.26 | 42.10 | 8.00 | 1.64 |
| Egg albumin (71.5) | 7.00 | 75.00 | 8.00 | 10.00 |
| Soy hydrolysate (52.6) | 9.50 | 75.00 | 6.50 | 9.00 |
| Casein (95) | 5.30 | 75.00 | 10.50 | 9.20 |
| Negative control | — | 75.00 | 13.00 | 12.00 |

[1] Nulomoline Company, Los Angeles, California.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Numbers in parentheses indicate grams of protein/100 g protein source.

sion may be open to question since the protein level fed (10%) exceeded the protein requirement of 2.5 to 5.0% according to Standifer et al. (5). It is felt that the following experiment provides a more valid relationship between excreted nitrogen and dietary protein adequacy or availability, since the bees received a more marginal amount of protein in experiment 2.

Data concerning partition of nitrogen excreted by the honey bee are shown in table 3, arranged from left to right in order of decreasing protein availability based on pharyngeal gland development according to Standifer et al. (5). The values are calculated both as a percentage of total solids and as a percentage of total nitrogen. The latter appear to be of greater validity since significant differences in non-nitrogenous solids would be eliminated. Analyses for ammonia and urea were negative for all samples and groups, which is in accord with data reported for most other insect species (12).

Haydak (13), Ludwig (14) and others have shown that over 80% of the nitrogen excreted by most insects is in the form of uric acid. The data (table 3) show that the honey bee excreted between 48 and 96% of nitrogen as uric acid, depending upon the dietary protein source. Since the negative controls had the highest excretion of uric acid while ingesting no nitrogen, catabolism of body protein or other nitrogen-storage forms is indicated. Casein-fed bees showed uric acid excretion of the same magnitude (96%), and thus casein appears to be inadequate as a source of nitrogen. Since casein and egg albumin have very similar amino acid compositions, it is difficult to account for the difference in availabilities noted here. A possible explanation may be that casein is marginal in tryptophan for the bee, whereas egg albumin provides a slight excess over the requirement of 1.0% (1). Since bees fed pollen or soy hydrolysate excreted about equal amounts of uric acid (62%) and those fed egg albumin even less (48%), it is probable that the first 2 protein sources are equally well-utilized and albumin more so. Consequently, uric acid excretion appears to show an inverse relationship to dietary protein availability for the honey bee.

Fecal amino acids are not considered a normal end product of nitrogen metabolism. According to Powning (15), they represent food ingested in excess of the dietary requirement. However, it is possible that they reflect the ability of the bee to enzymatically cleave dietary protein. This is supported by the observation (table 3) that the excreted α-amino nitrogen, as

TABLE 3

*Partition of fecal nitrogen of caged bees fed diets at 5% protein content (exp. 2)*

| Constituent | Egg albumin | Pollen | Soy hydrolysate | Casein | Negative control |
|---|---|---|---|---|---|
| Uric acid N | | | | | |
| g/100 g excreta | 1.55 ±0.05 [1] | 1.62 ±0.06 | 2.48 ±0.05 | 2.22 ±0.07 | 3.09 ±0.05 |
| g/100 g total N | 47.72 ±2.06 | 62.06 ±2.63 | 62.03 ±1.34 | 95.95 ±2.91 | 96.38 ±2.46 |
| α-Amino N | | | | | |
| g/100 g excreta | 0.214±0.009 | 0.160±0.006 | 0.167±0.005 | 0.067±0.004 | 0.044±0.002 |
| g/100 g total N | 6.60 ±0.32 | 6.11 ±0.21 | 4.19 ±0.17 | 2.93 ±0.23 | 1.36 ±0.08 |
| Creatine N | | | | | |
| g/100 g excreta | 0.200±0.004 | 0.040±0.004 | 0.044±0.008 | 0.104±0.006 | 0.248±0.012 |
| g/100 g total N | 6.16 ±0.15 | 1.51 ±0.14 | 1.09 ±0.20 | 4.54 ±0.40 | 7.72 ±0.41 |
| Creatinine N | | | | | |
| g/100 g excreta | 0.157±0.006 | 0.038±0.003 | 0.139±0.004 | 0.096±0.005 | 0.151±0.005 |
| g/100 g total N | 4.85 ±0.24 | 1.47 ±0.11 | 3.47 ±0.12 | 4.12 ±0.15 | 4.72 ±0.20 |
| Total N | | | | | |
| g/100 g excreta | 3.25 ±0.06 | 2.61 ±0.04 | 4.00 ±0.06 | 2.32 ±0.08 | 3.21 ±0.06 |
| g accounted N/100 g total N | 65.32 ±2.35 | 71.16 ±2.80 | 70.78 ±0.45 | 107.54 ±1.02 | 110.19 ±2.78 |

[1] Mean ± SE for 5 replicates.

a percentage of total nitrogen, is lowest for the negative control and highest for pollen and egg albumin. The inadequacy of casein for the bee is re-emphasized when its α-amino nitrogen value (table 3) is compared with that of pollen (2.93 vs. 6.11%). Although α-amino nitrogen was measured rather than the amino acid content and other ninhydrin-reactive substances may occur in the fecal material, unpublished results in this laboratory show the presence of at least 40 μmoles of free amino acids per gram of oven-dried sample, or about 0.6%

Little is known about the excretion of creatine and creatinine by insects. In mammals urinary creatinine is related to muscle mass, and the creatine-to-creatinine ratio increases with severe muscle wasting under conditions of extreme, negative nitrogen balance (16). According to the data in table 3, creatine and creatinine account for about 12% of the total excreted nitrogen for bees fed no protein, but for only about 3% for bees receiving pollen. In general, excretion of either creatine or creatinine is lowest for pollen and soy hydrolysate and highest for egg albumin and non-protein diets. If the creatine-to-creatinine ratio is computed, the numerical sequence is similar to that observed for either constituent alone; soy, 0.314; pollen, 1.03; casein, 1.10; egg albumin, 1.27; and negative control, 1.64. Thus, neither creatine nor creatinine excretion reflects the adequacy of dietary protein.

Total nitrogen was determined in the excised rectal tracts and fecal contents, rather than in defecated excreta, in the same manner as described for the nitrogen-containing compounds. Analysis of rectal tissue of pollen-fed bees showed only 0.08% nitrogen, which was negligible when compared with fecal nitrogen. According to the data in table 3, the total excreted nitrogen showed no discernible relationship to dietary adequacy. However, if the nitrogenous compounds determined are added together and expressed as a percentage of total nitrogen, the result is a meaningful indication of nitrogen forms accounted for in the fecal sample. For both casein and the negative control all of the nitrogen is accounted for by the compounds measured; but approximately one-third of the total cannot be accounted for when bees are fed pollen, egg albumin or soy. A possible explanation for this is that when dietary protein does not satisfy the nitrogen requirement of the bee, the uric acid excretion is increased by tissue catabolism to the point where compounds not determined in this work are negligible by comparison.

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# Dietary Fat Supplements, Body Weight and Osteoarthritis in DBA/2JN Mice

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*ABSTRACT* Dietary supplements of saturated fats, either in the form of lard or hydrogenated safflower oil, had no demonstrable deleterious effect on the development of degenerative joint disease in male DBA/2JN mice. Although the lard resulted in greater obesity than did the cottonseed oil supplement, the weight gain was more rapid and greater when safflower oil was used rather than 2 hydrogenated products of safflower oil having iodine numbers equal to that of the cottonseed oil and lard, respectively.

A proposed osteoarthritis-enhancing action of dietary fat supplements has been the subject of a number of investigations. Silberberg and co-workers (1–4) reported that lard has a specific deleterious effect on the joint disease in mice. The action of lard was attributed to its non-specific arthritogenic influence in mice by virtue of its high caloric content and, in the case of the Y strain, through production of extreme obesity. Others reported, however, that a dietary regimen in which a vegetable shortening provided 87% of the metabolizable calories had no influence on the development of degenerative joint disease in 3 inbred strains of mice (5). Although this was a high fat ration, it had been diluted with an inert filler to the same caloric density as the stock chow. One possible explanation for the disparity between these observations in the articular changes was the type of fat used in the supplement — cottonseed oil was reported to be less injurious than lard (6). The following experiments were carried out to test this explanation.

## METHODS AND MATERIALS

The animals used were male DBA/2JN mice. The experimental procedures, including evaluation of the joint disease by the papain-maceration method, were the same as those described in previous studies from this laboratory (5).

Experiment 1 was performed in 1961–62. The diets were the same as the three used by Silberberg and Silberberg (6). One was a control ground laboratory chow [2] (diet 10,000); the two others consisted of 74 parts of ground chow, 1 part of a commercial vitamin B mixture [3] and 25 parts of lard (diet 10,063) or cottonseed oil [4] (diet 10,062). Because of an impending high mortality in the lard-enriched group, the experiment was terminated when the mice were 14.5 months old, rather than 16 as originally planned.

Experiment 2 was conducted in 1962–63. Although the difference in the degree of unsaturation of the lard and cottonseed oil is considerable, there is little variation in the number of carbon atoms in their constituent fatty acids. To obviate any problems that might be associated with the slight differences in fatty acid chain lengths and the presence of trace substances that might influence the results, a highly unsaturated fat, safflower oil,[5] was chosen for this study (diet 10,080). One batch of the refined safflower oil, edible

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[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[3] This vitamin mixture contained in each gram: (in milligrams) inositol, 5; choline chloride, 75; menadione, 2.25; *p*-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vitamin $B_{12}$, 0.0012; ascorbic acid, 45; α-tocopherol, 5; and vitamin A (as the acetate), 900 IU; and vitamin D, 100 IU (obtained from Nutritional Biochemicals Corporation, Cleveland). No odor of rancidity was ever apparent in any of our rations, and they were routinely checked for this point throughout the experiment.

[4] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[5] Obtained from Drew Chemical Corporation, Boonton, New Jersey.

grade, was hydrogenated to an iodine number of 110 (diet 10,081) which is the same as that of the cottonseed oil used in the first experiment (table 1). Another batch was hydrogenated to an iodine number of 55 (diet 10,082) which is the same as that of the lard (table 1). The orginal safflower oil and the hydrogenated fats were used in the diets at the same levels as in experiment 1.

The 3 safflower oils were analyzed for fatty acids by means of the gas chromatograph and found to have the composition listed in table 2.

TABLE 1

*Fat supplements used in rations*

| Diet no. | Fat [1] | Iodine no. of fat |
|---|---|---|
| 10,000 | none | — |
| 10,062 | cottonseed oil | 110 |
| 10,063 | lard | 55 |
| 10,080 | safflower oil | 145 |
| 10,081 | hydrogenated safflower oil | 110 |
| 10,082 | hydrogenated safflower oil | 55 |

[1] The fats were added at a level of 25% of the ration. The remainder consisted of 1% vitamin fortification mixture and 74% ground commercial laboratory chow (Purina Laboratory Chow, Ralston Purina Co., St. Louis).

TABLE 2

*Fatty acid composition of dietary fats* [1]

| Fatty acid | No. of safflower oil | | |
|---|---|---|---|
| | 10,080 | 10,081 | 10,082 |
| | % | % | % |
| 16:0 [2] | 5.8 | 6.4 | 6.3 |
| 18:0 | 1.7 | 3.0 | 30.9 |
| 18:1 | 11.7 | 51.5 | 62.8 |
| 18:2 | 80.8 | 39.1 | 0.0 |

[1] Values are percentages of total peak area.
[2] The first number represents the number of carbon atoms; the second the number of double bonds.

## RESULTS

*Experiment 1.* Mice receiving the fat supplements became significantly heavier ($P < 0.01$) than the control animals, more so with lard than with cottonseed oil (fig. 1, table 3). Bone length, as measured in the femur, was not affected by the diets. The osteoarthritis scores of the mice fed the lard ration were close to those of the mice fed the stock ration; there was a questionable reduction ($0.05 < P > 0.01$) in the amount of joint disease in the mice

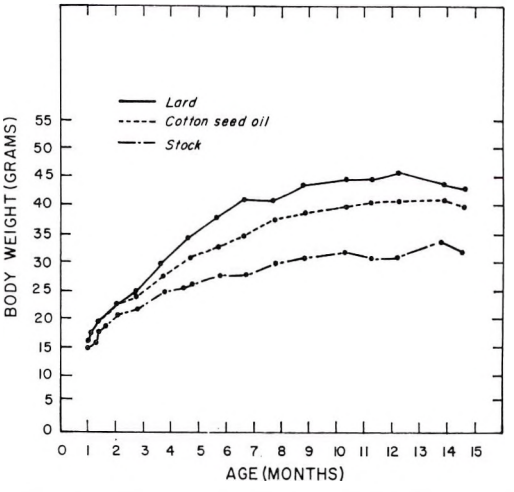


Fig. 1 Weight curves for rats fed lard, cottonseed oil or stock rations (exp. 1).

receiving the cottonseed oil-enriched ration when compared with the animals fed the basal stock ration (table 3).

*Experiment 2.* The mice fed the fat-supplemented rations attained significantly higher body weights than did the animals fed the basal stock diet (table 3). The weight gain was most rapid as well as greatest in the mice receiving the ration containing the unhydrogenated safflower oil (ration 10,080). The rate of increase and the maximal weights attained were less the greater the hydrogenation of the safflower oil (fig. 2). The femur lengths

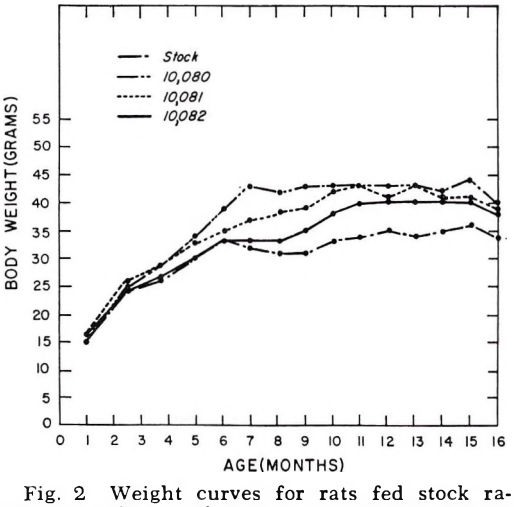


Fig. 2 Weight curves for rats fed stock ration, or safflower oil rations nos. 10,080—10,082 (exp. 2).

TABLE 3

*Summary of observations (exps. 1 and 2)*

| | Diets, exp. 1 | | | Diets, exp. 2 | | | |
|---|---|---|---|---|---|---|---|
| | Stock | Lard | Cottonseed oil | Stock | No. 10,080 | No. 10,081 | No. 10,082 |
| Peak body weight, g [1] | 33.6 ±0.4 [2] (20) [3] | 48.2 ±1.5 (20) | 42.8 ±1.5 (20) | 37.1 ±1.0 (22) | 46.8 ±1.6 (26) | 45.7 ±1.4 (27) | 43.0 ±1.0 (25) |
| Final body weight, g | 33.0 ±0.5 (20) | 42.9 ±2.2 (20) | 39.9 ±1.5 (20) | 34.3 ±1.1 (22) | 40.4 ±1.9 (26) | 38.2 ±1.8 (27) | 38.1 ±1.4 (25) |
| Epididymal pad weight, g | 1.07±0.08 (20) | 3.24 ±0.34 (20) | 3.00 ±0.27 (20) | 1.40±0.19 (13) | 3.10 ±0.36 (17) | 2.68 ±0.38 (14) | 2.80 ±0.25 (20) |
| Femur length, mm | 15.26±0.04 (20) | 15.37 ±0.04 (20) | 15.44 ±0.04 (20) | 15.65±0.06 (22) | 15.45 ±0.04 (25) | 15.62 ±0.06 (27) | 15.57 ±0.08 (25) |
| Osteoarthritis mean score [4] | 0.41 (40) | 0.48 (40) | 0.21 (40) | 0.45 (44) | 0.46 (52) | 0.48 (54) | 0.26 (50) |
| Osteoarthritis ridit [5] | 0.500 (40) | 0.519±0.041 (40) | 0.424±0.032 (40) | 0.500 (44) | 0.522±0.032 (52) | 0.549±0.032 (54) | 0.463±0.028 (50) |
| Survivors | 24/24 | 20/26 | 23/26 | 23/30 | 23/30 | 28/40 | 28/40 |

[1] Computed from peak weight of each animal rather than peak month value as in figures 1 and 2.

[2] Mean ± SE.

[3] The numbers in parentheses represent the number of measurements tested.

[4] Based on a severity scale of 0 to 4 (5), recorded for each knee separately.

[5] Because the arthritis scores are discontinuous, the ridit method of Bross (9), as previously adapted (5), has been employed for statistical evaluation of the joint disease. The stock group has been chosen as the identifiable distribution in each experiment; no confidence limits are attached to it.

were comparable in all groups except for a slight reduction in the animals fed the safflower ration. There were no significant differences in the osteoarthritis scores (table 3).

## DISCUSSION

Under the conditions of these experiments, no evidence was found that supplementation of the diet with lard or with saturated safflower oil potentiates the development of degenerative joint disease in DBA/2JN mice. This was true even though the mice fed the various high fat diets became obese. The latter was evident in both the increased weight of the epididymal fat pad and the increased body weights with no change in skeletal size as indicated by bone lengths (table 3). The failure of obesity to have a deleterious effect on the joints of mice has been a consistent observation in numerous previous studies in this laboratory (5).

The lack of agreement between our previous observations that high fat diets per se do not have an arthritis-promoting effect in mice and the observations of others that they do have an effect has not been resolved by the present experiments. This difficulty exists even though we attempted to make the experimental design as uniform as possible. The only dietary variable in our last experiment was the degree of hydrogenation of the fat.

Other factors such as the strain of mice, variations in experimental design or genetic drift may be responsible for the differences in results secured in our laboratory and that of the Silberbergs. That the strain of mice has to be considered is evident from the observation of Silberberg and Silberberg (4) that lard supplements increased osteoarthritis in C57BL but had only a slight effect in DBA mice.

The suggested difference in experimental design stems from the presumption that the experiment in which the mice were fed the original vegetable fat was performed by others without simultaneous controls for the lard and stock diets. The arthritis scores for the mice fed the vegetable oil diets appear to have been compared with data from experiments published some years earlier (6). The weights of the mice at one month of age as shown by the weight curves in that report suggest that the animals were not of uniform body weights at the start of the study.

Changes may have occurred in the mice during the past 10 years which could help in explaining the differences in results secured in the 2 laboratories. This suggestion is supported by the low mean arthritis scores in our experiments for the mice fed the stock diet. The difference is apparent when the data from the present experiments are compared with those secured in previous years. The mice used in the present study as well as those in three other groups studied at the same time had significantly lower arthritis scores than those consistently observed in previous years in the DBA/2JN animals. All mice were obtained from the same source — the Animal Production Unit of the National Institutes of Health (5).

Presumably, the above changes resulted from some genetic drift. Degenerative joint disease in mice is a genetically influenced disorder in which the inheritance is governed by multiple genes whose over-all effect is recessive in nature. This fact makes it impractical to use non-inbred mice for investigating nutritional or other factors that may contribute to the lesion unless such factors are of overwhelming importance. The DBA/2JN mice have been used here because they have an intermediate susceptibility to the lesions. Furthermore, they are not subject to certain intercurrent diseases that have interfered with previous studies. Several C57BL strains of mice have been avoided because they are at times affected by a pruritic dermatitis (5, 8) that influences the development of osteoarthritis.

It is recognized that obesity can be produced in certain strains of mice by feeding them a high fat diet (5, 10). The fat used in most of these earlier studies was a hydrogenated vegetable shortening. Other work suggests that the nature of the fat has a marked influence on the degree of obesity attained by rats fed high fat diets. The fats that were solid at room temperature produced much greater weight gains than the liquid fats or oils (11).

The observations of this study agree with the latter report only in part. We found that the mice fed the lard diets gained weight more rapidly than did those fed the cottonseed oil diets. These results are partially explainable on the consistency of the diet — the one containing large amounts of cottonseed oil was oily and separated on standing. The layer of oil on the surface of the feed made it difficult for the animal to eat the diet. With solid fats, the consistency of the ration was maintained indefinitely making it much easier for the animals to eat the diet.

The better growth of the mice fed the safflower oil compared with that of the animals fed the same oil after hydrogenation (fig. 2) is difficult to explain. On the basis of our own experience and that of Barboriak et al. (11), we would have expected the opposite effect.

To explain this anomaly, we might suggest that there was an alteration in the palatability of the ration as a result of hydrogenating the oil. Hydrogenation also may have changed the physical characteristics of the oil. This may explain why ration 10,083 containing safflower oil with an iodine number of 55 had a dry consistency when compared with the ration containing the less saturated oil (10,081) or the lard diet (10,063). Suggestive confirmation for a change in the palatability of the ration comes from the observation that the feed cups of the mice fed the safflower oil ration had to be filled more frequently than those of the animals fed rations containing the hydrogenated oil.

Furthermore, there is a possibility that the metabolic utilization of the hydrogenated fats may have been altered as a result of saturating some of the double bonds (table 2). The metabolic change may have stemmed from an increased proportion of stearic acid in the fat or a change in the normal *cis*, *trans* isomerization, or both.

The differential growth effect of the safflower oil and its hydrogenated products deserves further study.

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