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Contents

No. 1 SEPTEMBER 1963

R. Adams Dutcher — A Biographical Sketch. *N. B. Guerrant, D. E. H. Frear and R. V. Boucher* 1

Calcium Metabolism in Hybrid and Inbred Fetuses of Mice and in Those Co-existing in the Same Uterus. *Hiroshi Itoh and Takayoshi Ino* 5

Type and Quantity of 3 β-Hydroxysterols Excreted by Subjects Subsisting on Formula Rations High in Corn Oil. *H. L. Haust and J. M. R. Beveridge* 13

Vitamin K Deficiency and Oxidative Phosphorylation. *Anna Maria Paolucci, P. B. Rama Rao and B. Connor Johnson* 17

The Protein Quality of Waste-grown Green Algae. I. Quality of Protein in Mixtures of Algae, Nonfat Powdered Milk, and Cereals. *Bessie B. Cook, Esther W. Lau and Betty M. Bailey* 23

Lack of Effect of Urease Injection on Growth Rate and Feed Efficiency of Chicks and Rats. *G. R. Wagner, E. L. Nichols, R. H. Kohlmeier, S. L. Balloun and V. W. Hays* 30

Effect of Alternate Depletion — Repletion on the Laboratory Rat. *E. E. Howe and C. L. Dooley* 35

Effects of Cholesterol and Cholic Acid Supplements on Rats Fed Low Iodine Diets. *Takeo Mogami, S. B. Andrus, Anna Maria Paolucci and D. M. Hegsted* 39

Nutrition and Biochemistry of Survival During Newcastle Disease Virus Infection. I. Liver Nucleic Acid, Protein and Lipid Patterns in Chicks. *Robert L. Squibb* 48

Effect of Diet on the Uptake of Carbon-14-labeled Acetate, Glucose and Leucine into Lipids of the Chick. *J. E. Marion and H. M. Edwards, Jr.* 55

Interrelations among Magnesium, Vitamin B_6, Sulfur and Phosphorus in the Formation of Kidney Stones in the Rat. *F. F. Faragalla and S. N. Gershoff* 60

Effect of Linolenic Acid upon the Metabolism of Linoleic Acid. *Hans Mohrhauer and Ralph T. Holman* 67

Dietary Fat and the Structure and Properties of Rat Erythrocytes. I. Effect of Dietary Fat on the Erythrocyte Lipids. *Brian L. Walker and Fred A. Kummerow* 75

Effect of NaF on the Growth of Rats with Varying Vitamin and Calcium Intakes. *Robert W. Harkins, John B. Longenecker and Herbert P. Sarett* 81

Water Addition to Purified Diets. II. Effects of Water and Carbohydrate on Protein Efficiency Ratio in Rats. *Kenneth W. Keane, Charles J. Smutko, Carl H. Krieger and Arnold E. Denton* 87

Invitation for Nominations for 1964 American Institute of Nutrition Awards 91

Invitation for Nominations for 1964 American Institute of Nutrition Fellows 93

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition 94

No. 2 OCTOBER 1963

The Presence of Lactose in Intestinal Tissue. *F. W. Lengemann and C. L. Comar* 95

Effect of Diet on Serum and Muscle Chloride and Muscle Moisture. *C. H. Lushbough and M. C. Urbin* 99

Changes in Succinic Dehydrogenase Activity and Fatty Acid Composition of Rat Liver Mitochondria in Essential Fatty Acid Deficiency. *Takuya Hayashida and O. W. Portman* 103

Action of Linoleic and Arachidonic Acids upon the Eicosatrienoic Acid Level in Rat Heart and Liver. *Raúl O. Peluffo, Rodolfo R. Brenner and Osvaldo Mercuri* 110

Some Relationships between Caloric Restriction and Body Weight in the Rat. IV. Fecal Nitrogen, Fat, Energy, and Ash and Urinary Creatinine. *Melvin Lee and Salvatore Pablo Lucia* 117

Influence of Dietary Menhaden Oil on Growth Rate and Tissue Fatty Acids of the Chick. *Hardy M. Edwards, Jr. and J. E. Marion* 123

Effects of Several Low Protein Diets on the Serum Iron, Total Iron-binding Capacity, and Serum Cholesterol of Rats. *Richard R. Roehm and Helen L. Mayfield* 131

Comparative Protein Evaluation Studies by Carcass Retention and Nitrogen Balance Methods. *William Chalupa and Hans Fisher* 139

Distribution and Phosphorylation of Oxythiamine in Rat Tissues. *G. Rindi, L. de Giuseppe and U. Ventura* 147

Metabolism of Sodium Acetate-1-C^{14} and Palmitic Acid-1-C^{14} in the Pantothenic Acid-deficient Rat. *Fudeko T. Maruyama and Agnes Fay Morgan* 155

Human Metabolism of L-Ascorbic Acid and Erythorbic Acid. *Jerry M. Rivers, Elizabeth D. Huang and Mary L. Dodds* 163

Effect of Lysine Deficiency on Chagas' Disease in Laboratory Rats. *Robert G. Yaeger and O. Neal Miller* 169

Relationship of Excess Calcium and Phosphorus to Magnesium Requirement and Toxicity in Guinea Pigs. *E. R. Morris and B. L. O'Dell* 175

No. 3 NOVEMBER 1963

Availability of Microbially Synthesized Thiamine in the Rat. *John Yudkin* 183

Effect of Iron Supplementation of Sow Rations on the Prevention of Baby Pig Anemia. *C. H. Chaney and C. E. Barnhart* 187

Studies on Chloride Deficiency in Chicks. *R. M. Leach, Jr. and M. C. Nesheim* 193

Cardiovascular Lesions in Experimental Copper Deficiency in Chickens. *W. W. Carlton and Wilson Henderson* 200

Influence of Low, Intermediate and High Levels of Dietary Protein on Heat Production of Rats. *E. W. Hartsook and T. V. Hershberger* 209

Effect of Egg White Diets on Calcium Metabolism in the Rat. *George W. Engstrom and Hector F. DeLuca* 218

Effect of Certain Factors on Nitrogen Retention and Lysine Requirements of Adult Human Subjects. IV. Total Nitrogen Intake. *Helen E. Clark, Mary Alice Kenney, Alice F. Goodwin, Kamla Goyal and Edwin T. Mertz* 223

Amino Acid Balance in the Adult: High Nitrogen — Low Tryptophan Diets. *Hans Fisher, M. K. Brush, R. Shapiro, J. P. H. Wessels, C. D. Berdanier, P. Griminger and E. R. Sostman* 230

Evaluation of Protein in Middle Eastern Diets. I. Almond (*Prunus amygdalus*). *J. W. Cowan, Z. I. Sabry, F. J. Rinnu and J. A. Campbell* 235

Free and Total Cholesterol in Human Blood Fractions. *Betty E. Hawthorne, Elveda Smith and Josephine O. Pescador* 241

Studies in Infantile Malnutrition. II. Effect of Protein and Calorie Intake on Weight Gain. *George G. Graham, Angel Cordano and Juan M. Baertl* 249

Glutathione and the Antithyrotoxic Effect of Methionine. *L. W. Charkey and D. F. Hougham* 255

Dynamic Aspects of Cholesterol Ester Metabolism in Rabbits with Atherosclerosis. *Leon Swell, M. D. Law and C. R. Treadwell* 263

Possible Mechanisms for Dietary Molybdenum Toxicity in the Rat. *Herman L. Johnson and Russell F. Miller* 271

Nutritional Value of Proteins of Oriental Soybean Foods. *Bluebell R. Standal* 279

No. 4 DECEMBER 1963

Gross and Microscopic Pathology of Tocopherol-deficient Mink. *Howard D. Stowe and C. K. Whitehair* 287

Effect of Lysine on Calcium Metabolism in Man. *Herta Spencer and Joseph Samachson* 301

Effect of Dietary Fluoride upon the Magnesium Calcinosis Syndrome. *Y. Chiemchaisri and Paul H. Phillips* 307

Some Independent and Combined Effects of Copper, Molybdenum, and Zinc on the Placental Transfer of Zinc-65 in the Rat. *Kenneth E. Kinnamon* 312

Effect of Oleic and Linoleic Acids on the Absorption of Saturated Fatty Acids in the Chick. *R. J. Young and R. L. Garrett* 321

Periodic B-vitamin Supplementation of Growing Rats Fed a Vitamin-free Diet. *W. J. Stielau and J. H. Meyer* 330

Vitamin E Content of Vegetable Oils and Fats. *David C. Herting and Emma-Jane E. Drury* 335

Amino Acids in Dog Blood and Gut Contents after Feeding Zein. *E. S. Nasset, S. N. Ganapathy and D. P. J. Goldsmith* 343

Factors Influencing the Induction of Fatty Liver by Orotic Acid. *Herschel Sidransky, Ethel Verney and Benito Lombardi* 348

Fatty Acid Composition of Plasma and Liver Lipid Components as Influenced by Diet in the Growing Chick. *G. A. Leveille, J. A. Tillotson and H. E. Sauberlich* 357

Amino Acid Balance and Imbalance. XII. Effect of Amino Acid Imbalance on Self-selection of Diet by the Rat. *Juan C. Sanahuja and Alfred E. Harper* 363

Vitamin B_{12} and DNA Biogenesis. *Rogene F. Henderson and James S. Dinning* 372

Effect of Delayed Supplementation of Wheat Gluten with Lysine and Threonine on its Capacity to Promote Growth in the Weanling Rat. *E. E. Howe and C. L. Dooley* 379

Action of Vitamin A on Liver Homogenate Oxidation of Tricarboxylic Acid Cycle Intermediates. *H. F. DeLuca, M. R. Manatt, N. Madsen and E. B. Olson* 383

Effect of Fasting on the Incorporation in Vitro of Palmitate-C^{14} into Glycerolipids of Mitochondria. *Amornrat Chareonchai and Ralph M. Johnson* 387

Effect of Soybean Trypsin Inhibitor on Methionine and Cystine Utilization. *Eva Kwong and Richard H. Barnes* 392

Supplementation of Cereal Proteins with Amino Acids. VI. Effect of Amino Acid Supplementation of Rolled Oats as Measured by Nitrogen Retention in Young Children. *R. Bressani, D. L. Wilson, M. Chung, M. Béhar and N. S. Scrimshaw* 399

Effects of Food Restriction and Realimentation on Serum Proteins: Complement Levels and Electrophoretic Patterns. *Henry E. Weimer, James F. Godfrey, Roberta L. Meyers and James N. Miller* 405

Adenosine Triphosphatase and ATP-P_i Exchange in Mitochondria of Essential Fatty Acid-deficient Rats. *Ralph M. Johnson* 411

Interrelationships of Ascorbic Acid and Pantothenic Acid in the Young Guinea Pig. *Cecelia Pudelkewicz and Charlotte Roderuck* 415

Antimetabolites in the Nutrition of *Aedes aegypti* L. Larvae. Thiamine, Riboflavin, and Pantothenic Acid Antagonists. *S. Akov and K. Guggenheim* 419

Dietary Protein Level and the Turnover Rate of Tissue Proteins in Rats. *Keiichiro Muramatsu, Takahiko Sato and Kiyoshi Ashida* 427

Index to Volume 81 435

ERRATUM

Follis, R. H., Jr., K. Vanprapa and D. Damrongsakdi 1962 Studies on iodine nutrition in Thailand. J. Nutrition, *76:* 159.

In table 2, the columns headed Mean and SD contain erroneous values in lines 3, 5, 9, and 11.

To correct these columns in your copy of volume 76, number 2, please cut along lines of reprinted columns below and paste over the appropriate columns in table 2, page 166.

Mean	SD
70.3	± 16.5
74.5	± 19.9
29.0	± 5.6
38.2	± 16.1
15.0	± 12.3
31.2	± 14.3
14.5	± 8.0
58.7	± 15.8
40.5	± 19.5
36.4	± 15.3
17.4	± 6.0
123.5	± 27.7

R. ADAMS DUTCHER

(1886 – 1962)

R. ADAMS DUTCHER

R. Adams Dutcher

— *A Biographical Sketch*

(March 28, 1886 – April 20, 1962)

Raymond Adams Dutcher, Professor Emeritus and former head of the Department of Agricultural and Biological Chemistry at The Pennsylvania State University, died suddenly in Fort Meyers, Florida on April 20, 1962. He was born on March 28, 1886, at Raymond, South Dakota, the son of Paul and Susie (Adams) Dutcher. His father was the owner and publisher of a local newspaper in Brookings, South Dakota, and Dr. Dutcher spent his boyhood in that city. He recived his B.S. degree in Chemistry from South Dakota State College in 1907, and immediately joined the staff as an instructor, completing the requirements for his M. S. Degree in 1910.

From 1910 to 1912 he did graduate work in biochemistry and nutrition at the University of Missouri, and received an M.A. degree in 1912. He was instructor in biochemistry at the University of Illinois for the academic year 1912–1913, and then joined the agricultural chemistry faculty at Oregon State College as an assistant professor. He remained at Oregon for four years, then went to the University of Minnesota where he joined the Department of Agricultural Biochemistry. During World War I he served as a Captain in the Sanitary Corps.

In 1921 he left Minnesota to become the head of the Department of Agricultural and Biological Chemistry at The Pennsylvania State College. He held this position for 30 years, retiring in 1951. Following his retirement at Pennsylvania State College, he taught for several years at the University of Florida, on a part-time basis, and engaged in consulting work.

Dr. Dutcher was one of the pioneers in the field of vitamin research, and much of his early work laid the foundation for later discoveries in human and animal nutrition. He was an inspiring teacher, who guided many undergraduate and graduate students through the intricacies of organic and biological chemistry. An extremely capable administrator, he raised his department at Pennsylvania State College to national prominence, with many of its graduates assuming responsible positions in teaching, research, and industry.

Many important scientific assignments and honors came to Dr. Dutcher during and following his active career. He traveled in Germany as an Oberlaender Fellow in 1934, and returned to that country in 1945 as a scientific consultant to the Field Intelligence Agency. He served as a member of the White House Conference on Child Health; member of the research advisory committee to the Farm Chemurgic Council; member of the Food and Nutrition Board, National Research Council; member of the U. S. P. Vitamin Advisory Board; member and counselor of the Institute of Food Technologists; and a member of the General Board of Dairy Research. The University of Puerto Rico and his alma mater, South Dakota State College, awarded him honorary doctorates. He received the Grocery Manufacturer's Award in 1955, and the Honor Scroll of the Pennsylvania Chapter of the American Institute of Chemists was given to him in 1960.

Dr. Dutcher was a member of many professional societies and fraternities, including the American Association for the Advancement of Science, American Institute of Nutrition, American Society of Biological Chemists, Pennsylvania and New York Academies of Science, British Biochemical Society, Sigma Xi, Gamma Sigma Delta, Alpha Zeta, Phi Lambda Upsilon, Alpha Chi Sigma, Theta Chi, Accacia, and Phi Kappa Phi. He was a member of the American Chemical Society for over 50 years, and served in various Section and Division offices. For

many years Dr. Dtucher was a member of the Chemist's Club of New York City. He was an ardent golfer, and a member of Centre Hills Country Club of State College, Pennsylvania. He enjoyed photography and possessed an extensive collection of figurines.

During his career Dr. Dutcher published numerous scientific and technical papers and was co-author of three textbooks.

In 1912, Dr. Dutcher married Mary Marguerite Wright. She and their two children, Adams Wright Dutcher of Salisbury, Maryland, and Rachel Dutcher Maloney of Stamford, Connecticut survive. Six grandchildren and one great-grandson also survive.

N. B. GUERRANT
D. E. H. FREAR
R. V. BOUCHER
University Park, Pennsylvania

Calcium Metabolism in Hybrid and Inbred Fetuses of Mice and in Those Co-existing in the Same Uterus

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ABSTRACT Uptake of radiocalcium by fetuses conceived in dams of inbred mice, DD and RR, by competitive mating, was determined at 24 hours after intraperitoneal injection. Average dry weight of 269 fetuses in 52 dams at the 18th day of gestation was 134 mg and the fetal Ca^{45} uptake averaged 6.7% of the total dose. Fetal Ca^{45} uptake decreased with increase in litter size generally. Average litter sizes for the dams having fetuses of different kinds were approximately the same. However, of the 2 horns of the uterus, the higher fetal uptake of Ca^{45} was observed in the uterine horn having the larger number of fetuses. The fetuses were heaviest and the fetal Ca^{45} uptake was highest in the hybrids DR and RD, followed by the inbred DD and then by the inbred RR strain. When inbred and hybrid fetuses co-existed in the same uterus of inbred mice, hybrids deposited significantly more Ca^{45} than did the inbreds. It is considered that these facts indicate heterosis on calcium metabolism in hybrid fetuses when both inbreds and hybrids are maintained under the same environment during prenatal growth.

It has been suggested that nutritive requirements of mice differ with each strain (1–6).[2] Knowledge of the genetical characteristics for the requirement of various nutrients in each strain and hybrid of mice, however, is necessary when mice are to be used for nutritional and genetical studies. Itoh and Ino (7) reported on calcium metabolism during growth in 2 strains of inbred mice (DD and RR) and their reciprocal hybrids (DR and RD), measuring the calcium content of the femur and its Ca^{45} uptake. The total calcium values and the radiocalcium uptakes in DD mice increased markedly at the earlier period of growth as compared with those observed in RR mice, and hybrid mice showed greater utilization of calcium than the RR mice. Pecher (8) and Pecher and Pecher (9) reported the distribution of Ca^{45} in the tissues, skeleton, fetus and excreta of mice after the injection of radiocalcium. However, little is known concerning calcium metabolism in the fetus of various strains of mice which may have different characteristics.

The possibility of producing pregnancy in dams that will result in both inbred and hybrid fetuses in the same uterus, by artificial insemination or by competitive mating, provides a means for direct comparison of growth, and the metabolism of various materials in inbred and hybrid fetuses under identical intra-uterine conditions.

In the study reported here, distribution of radiocalcium (by the placental transfer after the injection of Ca^{45} into the dam) in individual fetuses conceived following competitive mating of mice of 2 inbred strains, DD and RR, is presented. The relationships of fetal Ca^{45} uptake to maternal body weight, fetal body weight, number of fetuses (and that in each horn of uterus) and the kinds of fetuses co-existing in the same uterus are discussed.

EXPERIMENTAL

Thirty female mice of the inbred DD and 26 female RR inbred mice from Faculty of Agriculture Colony, and their 285 fetuses were used in this experiment. The genetic constitution and characteristics of both DD and RR inbred strains of mice were as follows (10): The DD strain were strong and docile, grew rapidly, and were heavy on maturing. They were albino with color genes ccSSaaBB. The RR strain could not be called docile, were not very prolific, did

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[1] Present address: National Institute of Animal Health, Tokyo.

[2] Lyon, J. B., Jr. 1957 Difference in glucose utilization among inbred strains of mice. Federation Proc., *16*: 215 (abstract).

not grow rapidly, and had chocolate-colored fur with color genes CCSSaabb. They were fed the stock ration which had the following composition: polished barley, 40%; ground corn, 25%; fish meal, 15%; NaCl, 0.5%; and calcium carbonate 0.5% to provide an estimated 0.92% calcium in the total diet.

To produce as many gestational mice having the co-existing inbred and hybrid fetuses in their uterus as possible, all mice were competitively mated. One or two female mice in estrus, of DD or RR strains, were successively caged with each 2 mature male mice of DD and RR strain. On the 17th day following confirmation of gestation by the extrusion of the vaginal plug, each dam was injected intraperitoneally with a tracer dose of Ca^{45} chloride (6 μc contained in about 0.08 mg calcium), and placed in individual metabolism cages. Twenty-four hours after dose administration each dam was killed, the uterus removed, and the litter size and the implanted position of each fetus in both horns of the uterus were recorded. The genetic type of each fetus was then determined and tabulated.

To distinguish between the inbred and hybrid mouse, the pigment ring in the iris (around the eyeball), which was clearly visible through the closed eyelids, of each fetus was used (fig. 1). The DD fetus, conceived in the DD dam, showed white both in the eyeball and in the eye ring, whereas the DR fetus had a wide and grayish black ring around the white eyeball. Both the RR and RD fetuses in the uterus of the RR mouse had a black ring around their eyeball, but the width of eye ring and the depth of pigment varied. Whereas the RR fetus showed a narrow and dark black ring, the RD fetus had a wide, grayish black belt around the narrow and dark eye ring.

The body weight of the dam without uterus or fetuses was recorded. Each fetus was dried at 105°C for about 5 hours, weighed, placed in a small porcelain crucible and ashed in an electric furnace at 600°C. Ash was dissolved in 2 N HCl and aliquots were taken for radioactivity measurement (11).

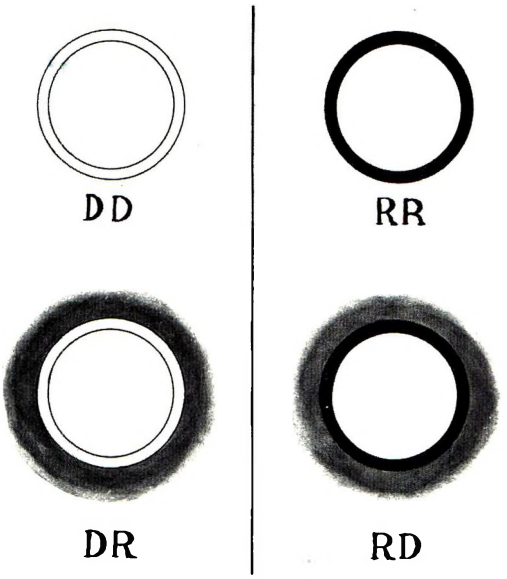

Fig. 1 Pigment rings in the iris around the eyeballs used for discrimination of the kinds of fetuses.

RESULTS AND DISCUSSION

Fifty-two out of the 56 dams were injected with isotope on the 17th day after conception and 2 others were administered radiocalcium on the 13th and 19th day, respectively. It was found on the 18th day of gestation that 11 and 13 DD dams were carrying inbred (DD) and hybrid (DR) fetuses, respectively, 11 and 8 RR dams carrying inbred (RR) and hybrid (RD) fetuses, respectively, and 9 inbred dams having the co-existing inbred and hybrid fetuses in the same uterus (table 1). The litter size varied from one to 9 young. The average litter size for 52 dams was 5.2, and the average value (6.1) for the 9 inbred dams having the co-existing inbred and hybrid fetuses in the same uterus was higher than that for the other 43 dams (5.0). The smallest litter size was observed in RR dams having inbred RR fetuses and the largest litter size was found in DD dams having DD fetuses. These differences in litter size, however, were not significant. The average litter sizes for each inbred strain of dams (DD or RR), that for the dams carrying inbred fetuses (DD or RR), and that for the dams having hybrid fetuses (DR or RD), were

approximately the same. It appears, therefore, that the results obtained from each group of the litters having fetuses of different kinds are not affected by the litter size.

Gestation age and Ca^{45} uptake. The fetal uptake of Ca^{45} 24 hours after dose administration is compared 14, 18 and 20 days after conception (table 2). It appears that the litter size in these age groups does not affect the fetal body weight and Ca^{45} uptake. The radiocalcium concentration in the 18-day-old fetuses, almost half the number of which are hybrids and which show higher fetal Ca^{45} uptake as compared with the inbred fetuses (table 3), was considerably higher than that in the 14-day-old fetuses, but lower than that in the 20-day-old inbreds. However, on the dry weight basis, fetal uptake of Ca^{45} at 18 and 20 days of age was approximately the same in both age groups, and was more than twice that of the 14-day-old fetuses. These results indicate that calcium laydown in fetuses increases rapidly during the last trisection of pregnancy and especially during the last fifth of the gestation period (12).

Maternal body weight and Ca^{45} laydown. It is usually assumed that the retention of

TABLE 1

Number of dams having various kinds of fetuses in various litter sizes

Kinds of dams	DD		RR		DD	RR	Total
Kinds of fetuses	DD	DR	RR	RD	DD + DR	RR + RD	
			Number of dams				
Litter size							
1	0	1	1	0	0	0	2
2	1	1	1	0	0	0	3
3	0	2	2	1	0	0	5
4	2	2	2	1	0	0	7
5	2	1	1	1	2	2	9
6	3	4	3	3	1	0	14
7	1	2	0	2	0	3	8
8	1	0	1	0	1	0	3
9	1	0	0	0	0	0	1
Total no. of dams	11	13	11	8	4	5	52
Total no. of fetuses	62	60	48	44	24	31	269
Average litter size	5.6 ± 1.9[1]	4.6 ± 1.9	4.4 ± 2.0	5.5 ± 1.3	6.0 ± 1.2	6.2 ± 1.0	5.2 ± 1.8
	5.1 ± 1.4[2]		4.8 ± 1.4[2]		6.1 ± 1.1[3]		
	5.0 ± 2.0[4]		5.0 ± 1.7[5]				
	5.0 ± 1.9[6]						

[1] SD of mean.
[2] Average litter size in the same strain of dams having inbred or hybrid fetuses.
[3] Average litter size in the total number of dams having the co-existing inbred and hybrid fetuses in the same uterus.
[4] Average litter size in the 2 strains of dams having inbred fetuses.
[5] Average litter size in the 2 strains of dams having hybrid fetuses.
[6] Average litter size in the total number of dams having inbred or hybrid fetuses alone.

TABLE 2

Fetal uptake of Ca^{45} at various ages of gestation

Gestation days	No. of dams	No. of fetuses	Dry weight	Uptake of Ca^{45}	Ca^{45} % dose/ 100 mg of dry wt
			mg	*% of dose*	
14	2	9[1]	48 ± 9[2]	1.1 ± 0.2	2.29 ± 0.65
18	43[3]	214	136 ± 28	6.8 ± 2.4	5.02 ± 1.02
20	2	7[1]	204 ± 44	10.1 ± 3.3	4.95 ± 1.30

[1] All fetuses are inbred, and 4 are DD and the others are RR fetuses.
[2] SD of mean.
[3] Exclusive of dams having the co-existing inbred and hybrid fetuses in the same uterus.

radiocalcium in the skeleton of the dams differs with their body weight (less that of the uterus and fetuses), and that fetal uptake of Ca^{45} is thus influenced. However, there were no positive correlations between the body weight of dams (25.6 ± 6.6 g) and either the dry weight of the litter (695 ± 430 mg, r = − 0.14) or the fetal uptake of Ca^{45} per litter (34.8 ± 21.6%, r = − 0.18). Neither were such correlations observed among the dams having the same number of fetuses. It is indicated, therefore, that calcium metabolism in the 18-day-old fetus is not affected by maternal body weight and that there is no relationship between litter size or body weight of fetuses and the body weight of the dams.

Effects of fetal body weight and litter size on Ca^{45} uptake. The average dry weight of 269 fetuses at 18 days of gestation (including 55 fetuses in 9 dams containing co-existing inbred and hybrid fetuses) shown in table 4, was 134 mg and the radiocalcium content averaged 6.7% of the total dose. The correlation observed between the fetal uptake of Ca^{45} and the dry weight of fetus, was significant ($P < 0.05$) and showed a higher confidence (r = 0.73) than when compared with the fresh weight of the fetus. The coefficient of correlation (r = 0.89) between the radiocalcium content per litter and the dry weight of the litter was highly significant ($P < 0.01$). It is considered, therefore, that the fetal uptake rate of Ca^{45} increases in proportion to body weight at the same age of gestation and that calcium laydown increases with increasing skeletal capacity.

Fifty-two litters were divided into 7 groups according to litter size. Mice in group 1 had 1 or 2 fetuses and group 7 contained those mice conceiving 8 or 9 fetuses. Body weight and distribution of Ca^{45} in individual fetuses and litters in the 7 groups are shown in table 4. In each group, the dams having either inbred or

TABLE 3

Comparisons of body weight and Ca^{45} uptake in the inbred and hybrid fetus[1]

Kind of fetus		No. of litters	No. of fetuses	Fresh wt of fetus	Dry wt of fetus	Uptake of Ca^{45} by fetus	Ca^{45} % dose/ 100 mg of dry wt
				mg	*mg*	*% of dose*	
Inbred	DD	11	62	943 ± 198[2]	133 ± 29	6.5 ± 2.8	4.90 ± 1.20
	RR	11	48	892 ± 149	124 ± 21	5.9 ± 2.5	4.72 ± 1.14
	Total	22	110	922 ± 161	129 ± 23	6.2 ± 2.6	4.83 ± 1.17
Hybrid	DR	13	60	993 ± 229	142 ± 39	7.5 ± 2.3	5.27 ± 1.10
	RD	8	44	1008 ± 156	142 ± 25	7.3 ± 1.8	5.16 ± 0.72
	Total	21	104	1001 ± 188	142 ± 31	7.4 ± 2.0	5.22 ± 0.93

[1] The identical kind of fetus is implanted in each dam.
[2] SD of mean.

TABLE 4

Relation of litter size and the fetal uptake of Ca^{45}

Group	Litter size	No. of dams	Total no. of fetuses	Dry weight		Uptake of Ca^{45}		Ca^{45} % dose/100 mg of dry wt
				Litter	Fetus	Litter	Fetus	
				mg	*mg*	*% dose*	*% dose*	
1	1 or 2	5	8	213	131 ± 37[1]	13.3	8.3 ± 3.5	6.25 ± 1.78[2]
2	3	5	15	473	158 ± 39	26.4	8.8 ± 1.8	5.58 ± 0.68
3	4	7	28	484	121 ± 19	25.5	6.4 ± 1.2	5.28 ± 0.45
4	5	9	45	672	134 ± 22	35.5	7.1 ± 1.7	5.29 ± 0.65
5	6	14	84	797	133 ± 25	41.2	6.9 ± 1.0	5.16 ± 0.83
6	7	8	56	962	137 ± 25	45.3	6.5 ± 0.8	4.71 ± 0.79
7	8 or 9	4	33	1110	135 ± 11	43.6	5.3 ± 1.1	3.95 ± 0.25[2]
Average	5.2 ± 1.8	(52)[3]	(269)	695 ± 430	134 ± 25	34.8 ± 21.6	6.7 ± 1.5	5.01 ± 0.90

[1] SD of mean.
[2] Significant difference $P < 0.05$.
[3] Total number of animals is indicated in parentheses.

hybrid fetuses were approximately the same in number (table 1). Almost no difference was observed in fetal dry weight and Ca^{45} uptake between the DD and RR dams carrying the co-existing inbred and hybrid fetuses in the same uterus (table 5). It is assumed, therefore, that breeding has no influence on fetal dry weight and Ca^{45} uptake, nor does position of litter in the horns of uterus (table 6). There was no correlation between fetal dry weight and litter size, except in group 2 (3 littermates) with heavier dry weight of fetuses and group 3 (4 littermates) with lighter fetuses. In general, the radiocalcium content of fetuses decreased with increase in litter size. A fairly high transfer of Ca^{45} (13.3%) was observed in those litters of group 1. Dams having 6 to 9 fetuses transferred more than 40% of the Ca^{45} dose to their litters. These values are higher than those reported for mice by Pecher and Pecher (9) but are comparable to those observed in rats by Feaster et al. (12) where 36% of the dose traversed the placental barrier to the litters during 24 hours.

Concentration of Ca^{45}/mg dry weight in individual fetuses decreased with increase in litter size. It appears that calcium laydown in individual fetuses is affected by increases of both litter size and total body weight of litter. There were no significant differences, however, in the Ca^{45} laydown in fetus among the groups having 3 to 7 littermates. Nevertheless, fetuses in group 1 (1 or 2 littermates) contained a significantly higher percentage of the Ca^{45} dose ($P < 0.05$), than that in those groups having more than 3 fetuses. On the other hand, fetuses in group 7 (8 or 9 littermates), showed a significantly lower deposition of Ca^{45} ($P < 0.05$), than those having less than 8 young. It is considered, therefore, that the correlations between body weight and Ca^{45} distribution in fetuses are low in groups 1 and 7, which have the smallest or largest litter size. In a preliminary experiment, calcium concentration/100 mg of fetal dry weight was estimated to be 1.4 ± 0.2 mg, in 23 fetuses (14 inbreds and 9 hybrids) in 4 dams on the 18th day of gestation. It is presumed that the Ca^{45} laydown in fetuses is affected

TABLE 5

Comparison of Ca^{45} uptake in the inbred and hybrid fetus, co-existing in the same uterus

Dams		Fetuses		Fetal dry weight	Uptake of Ca^{45} by fetus	Ca^{45} % dose/100 mg of dry wt
Kind	No.	Kind	No.			
				mg	*% of dose*	
DD	4	DD	12	118 ± 26[1]	5.3 ± 2.0	4.44 ± 1.19
		DR	12	143 ± 21[2]	7.9 ± 2.4[3]	5.47 ± 1.57[2]
		Total	24	130 ± 24	6.6 ± 2.1	4.95 ± 1.36
RR	5	RR	14	130 ± 26	5.4 ± 1.6	4.10 ± 0.73
		RD	17	131 ± 27	7.1 ± 2.2[2]	5.47 ± 1.60[3]
		Total	31	131 ± 26	6.3 ± 1.9	4.86 ± 1.19

[1] SD of mean.
[2] Significant difference $P < 0.05$.
[3] Significant difference $P < 0.01$.

TABLE 6

Relation of fetal Ca^{45} uptake and the number of fetuses in each uterine horn

Group	No. in each horn[1]	No. of dams					Total fetuses in each horn[1]	Ca^{45} % dose/100 mg of dry wt	
		DD[2]	DR	RR	RD	Total		Horn with few fetuses	Horn with many fetuses
A	0:3 or 4	1	2	2	1	6	0:19		5.43 ± 0.86[3]
B	1:3,5 or 6	2	1	1	0	4	4:17	4.75 ± 0.61	4.96 ± 0.58
C	2:4 or 5	1	2	2	3	8	16:33	4.92 ± 0.91	5.40 ± 0.98

[1] One side of uterine horn with few fetuses; the other side of horns with large number of fetuses.
[2] Kind of fetus.
[3] SD of mean.

by the total calcium content of litter, if calcium concentration per dry weight of fetuses is approximately constant.

Effects of fetus location on Ca^{45} deposition. Variations in fetal uptake of Ca^{45} in individual litters were observed in several cases, depending upon the uterine position of each fetus and the number of fetuses in each uterine horn. Various fetus locations in both uterine horns are shown in table 7. Eighteen dams (9 dams conceiving inbred fetuses and 9 others having hybrid fetuses) implanted the disparity of 2 to 5 fetuses in each uterine horn. These dams were divided into 3 groups on the basis of the number of fetuses in each horn of the uterus (table 6). Mice in group A, having fetuses in only one horn of their uterus, did not deposit a significantly higher percentage of the Ca^{45} dose in each fetus, than those of groups B and C, having a larger number of fetuses. The higher fetal uptake of Ca^{45} was observed in a horn of the uterus which had the larger number of fetuses. This difference, however, was not significant. Since these results are contradictory to the observation that fetal Ca^{45} uptake decreased with increase in litter size, it is considered that distribution of blood vessels in the uterus is of primary importance. It is assumed that distribution of blood vessels in the uterine horn having the larger number of fetuses, is greater than that in the other horn which has 1 or 2 fetuses. When the same number of fetuses (in 14 dams), or the disparity of one fetus (in 11 dams), were implanted in each horn of uterus, Ca^{45} was approximately the same in both horns.

TABLE 7

Number of dams in various fetus locations in both horns of the uterus

Litter size	Fetus location[1,2]	No. of dams
1	0 : 1	2
2	1 : 1	3
3	0 : 3	5[3]
4	0 : 4	1[3]
4	1 : 3	2[3]
4	2 : 2	4
5	2 : 3	5
6	1 : 5	1[3]
6	2 : 4	7[3]
6	3 : 3	5
7	1 : 6	1[3]
7	2 : 5	1[3]
7	3 : 4	3
8	4 : 4	2
9	4 : 5	1

[1] Number of fetuses in one side of uterine horn: that in the other side of horns (with large number of fetuses).

[2] Combinations other than the following are discarded because they did not occur in any dam in this study.

[3] Eighteen dams had the disparity of 2 to 5 fetuses in each horn of the uterus. For details on the breeding, see table 6.

Laydown of Ca^{45} in hybrid and inbred fetuses. It has been reported that calcium metabolism in mice differs with individual strains of inbred mice and their hybrids (7). It seemed pertinent, therefore, to note the distribution of Ca^{45} in the fetus of 2 strains of inbred mice (DD and RR) and their reciprocal hybrids (DR and RD), and that of the inbred and hybrid fetus in the same uterus.

Comparisons of average body weight, dry weight and the Ca^{45} uptake by the inbred and hybrid fetus are shown in table 3. Both fresh and dry weight of the inbred fetus was lower than that of the hybrid fetus, although the water content in both groups showed approximately similar values (86%). The highest values for body weight, Ca^{45} uptake and turnover rates in fetus were observed in the hybrid DR and RD mice. The lowest values were noted for the inbred RR mice. It appears that calcium metabolism in the hybrid fetus is more active than that in the inbred fetus, although the difference is not significant. The results shown for the inbred fetuses and for the hybrid fetuses are in good agreement with those reported previously for growing mice (7).

Ca^{45} laydown in the inbred and hybrid fetus, co-existing in the same uterus. The implanted positions of individual fetuses in both uterine horns of the 4 DD dams carrying inbred (DD) and hybrid (DR) fetuses, and the 5 RR dams carrying inbred (RR) and hybrid (RD) fetuses are shown in figure 2. Comparisons of body weight and Ca^{45} distribution in these 4 kinds of fetuses are presented in table 5. These values for the hybrid fetuses (DR and RD) were higher than those for the inbred fetuses (DD and RR), with the exception of the similar body weight observed for the RR and RD fetuses. The DR fetus in DD dams contained a significantly higher percentage of the Ca^{45} dose

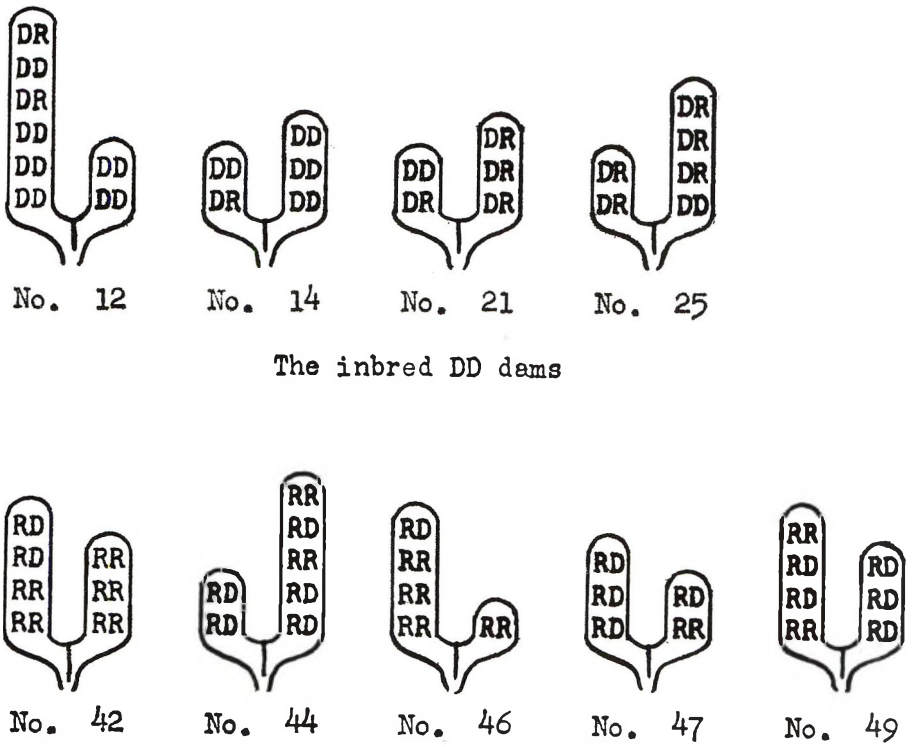

Fig. 2 The implanted position of the co-existing inbred and hybrid fetuses in the same uterus.

($P < 0.01$) than did the DD fetus. The RD fetus in RR mice had a significantly greater uptake of Ca^{45} ($P < 0.01$) than did the RR fetus implanted in the same dam. However, the body weight of inbred RR fetuses co-existing with hybrid RD fetuses in RR mice was heavier than that of the inbred DD fetuses co-existing with hybrid DR fetuses in DD dams. These results were contradictory to the data described above.

It is supposed that fetal body weight and calcium metabolism in those litters has not been influenced by litter size and the implanted position of fetuses in both horns of the uterus, if the kinds of fetuses implanted in individual dams are identical. Therefore, under these conditions of co-existence of both inbred and hybrid fetuses in the same uterus, it is considered that Ca^{45} deposition in the hybrid fetus being greater than that in the inbred fetus, indicates effects of heterosis ("hybrid vigor") on calcium metabolism in the hybrid fetus when both kinds of fetuses are in the same environment during prenatal growth.

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Type and Quantity of 3 β-Hydroxysterols Excreted by Subjects Subsisting on Formula Rations High in Corn Oil[1,2]

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ABSTRACT A normal and a diabetic subject were given a fat-free formula diet for 8 days and then continued for a similar period of time with a ration providing corn oil at a level of 60% of calories in place of an equicaloric amount of carbohydrate. By a differential-spectrophotometric system of analysis, 3 β-hydroxysterols excreted in the feces during the last 4 days of each regimen were determined. During the corn oil regimen, total 3 β-hydroxysterols in feces increased 11- and 17-fold, respectively; β- plus γ-sitosterol accounted for about 80% of the sitosterol intake and comprised about 60% of the 3 β-hydroxysterol output. Cholesterol excretion increased four- and fivefold. In the case of the diabetic subject, this was accompanied by a tenfold increase in the elimination of coprostanol. These data demonstrate that endogenously derived sterols contribute to the well-known increase in fecal unsaponifiable matter observed during the consumption of vegetable fats of high plant sterol content. This observation, considered in the light of the decrease in plasma cholesterol observed when corn oil is added to a fat-free diet, suggests a cause and effect relationship between the 2 observations.

Earlier work from this laboratory indicated that introduction of corn oil into a fat-free formula diet at a level of 60% of calories for an equicaloric amount of carbohydrate decreased plasma cholesterol concentrations of volunteer subjects below the low levels attained during the consumption of the basal ration (1). Depressed absorption of cholesterol, due to altered bile acid economy, was one of the mechanisms proposed to explain the hypocholesterolemic effect of the vegetable fat. This suggestion received support from the observation that transfer from a diet high in butter fat to one high in corn oil led to an increased fecal output of this group of steroids (2). The present report is concerned with changes in the fecal elimination of 3 β-hydroxysterols when using formula rations free from cholesterol.

EXPERIMENTAL

Two adult male volunteers, one normal and one a controlled diabetic, were given for 8 days a fat-free formula diet and continued for a similar period of time with a ration modified by the introduction of corn oil at a level of 60% of calories at the expense of an equicaloric amount of carbohydrate. The only other items permitted during the experimental period were water, tea or coffee without sugar or milk. Body weight was maintained constant by increasing or decreasing the daily food intake. Feces were collected during the last 4 days of each dietary regimen, and fasting levels of plasma cholesterol determined at days zero, 4, 8, 12 and 16 by a slight modification of the Schoenheimer-Sperry procedure (3).

Details about the preparation and storage of the diets[3] have been described elsewhere (2, 4). The diets were dispensed in 950-kcal batches which, in the case of the basal ration, contained the following ingredients: skim milk powder,[4] 28.1 g; calcium caseinate,[5] 28.4 g; sucrose, 20.0 g;

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[4] Mil-ko, Mil-ko Products, Ltd., Canada.

[5] Casec, Mead Johnson of Canada, Ltd., Belleville, Ontario.

maltose and dextrins,[6] 169.8 g; thiamine, 0.6 mg; riboflavin, 0.6 mg; niacin, 5.0 mg; Ca pantothenate, 5.0 mg; ascorbic acid, 25 mg; vitamin A, 1700 IU.

Corn oil was found to contain about 0.93% of digitonin-precipitable sterol, based upon closely agreeing results obtained by the anthrone procedure of Vahouny et al. (5), the Liebermann-Burchard[7] and Tschugaeff reactions.[8,9] In each instance β-sitosterol was used as a standard of reference.

Feces were stored and fractionated as described elsewhere (2, 6). An estimate of the total unsaponifiable fraction was obtained by saponifying an aliquot of the alcoholic extract of feces and extracting with petroleum ether. The latter was washed with 10% aqueous ethanol and evaporated to dryness. The residue was dried at 100°C, weighed and discarded. Other aliquots of the alcoholic extracts of the fecal lipids were treated as above, but the petroleum ether extracts were taken to dryness on a steam bath under a stream of nitrogen. The residue was dissolved in absolute alcohol. The 3 β-hydroxysterols that were present were isolated as, and regenerated from, their digitonides. A system of differential-photometric analysis[10,11] was applied which permitted quantitative differentiation between coprostanol, cholesterol, sitosterol (β plus γ), 7-dehydrocholesterol, Δ^7-cholestenol, and cholestanol plus stigmastanol.

RESULTS AND DISCUSSION

The changes occurring in the fecal excretion of unsaponifiable matter and of 3 β-hydroxysterols after transfer of subjects H and E from the fat-free regimen to the diet high in corn oil are shown in table 1. Values for plasma cholesterol at days zero, 4, 8, 12 and 16 are also shown. The consumption of the fat-free diet in the case of both subjects led to a decrease in the level of plasma cholesterol which dropped further as a result of the isocaloric substitution of corn oil for carbohydrate. These observations are consonant with the results obtained in previous large-

[6] Dextri Maltose, Mead Johnson of Canada, Ltd.

[7] Haust, H. L., and J. M. R. Beveridge 1963 The differential-photometric estimation of coprostanol in the presence of cholesterol, sitosterol and other 3 β-hydroxysterols. Canad. J. Biochem. Physiol. (submitted for publication).

[8] See footnote 7.

[9] Haust, H. L., and J. M. R. Beveridge 1963 Differential-photometric estimation of cholesterol and sitosterol in the presence of certain other 3 β-hydroxysterols. Canad. J. Biochem. Physiol. (submitted for publication).

[10] See footnote 7.

[11] See footnote 9.

TABLE 1

Changes occurring in plasma cholesterol,[1] fecal unsaponifiable matter and fecal 3 β-hydroxysterols when subjects H and E consuming for 8 days a fat-free diet were transferred to one providing, for a similar period, 60% of calories from corn oil

Diet	Subject H		Subject E	
Analysis of feces excreted during last 4 days of each dietary period	Fat-free	Corn oil, 60% of calories	Fat-free	Corn oil, 60% of calories
	Days 4–8	Days 12–16	Days 4–8	Days 12–16
	g/4 days	*g/4 days*	*g/4 days*	*g/4 days*
Wet weight	407	371	272	467
Dry fat-free feces	85 ±0.3[2]	79 ±1.4	67 ±0.3	123 ±0.5
Unsaponifiable matter	1.83±0.00	15.56±0.32	1.40±0.05	21.4 ±1.1
Total 3 β-hydroxysterol	1.12±0.01[3]	12.52±0.00[4]	0.93±0.01[3]	15.88±0.40[4]
Sitosterol	—	7.35±0.24[5]	—	9.62±0.00[5]
Stigmastanol	—	nil	—	0.70±0.07
Cholestanol	nil		nil	
Coprostanol	0.08±0.01	0.20±0.08	0.36±0.01	3.34±0.23
Cholesterol	1.05±0.00	5.30±0.23	0.58±0.00	2.49±0.03
Δ^7-Cholestenol	nil	nil	nil	nil
7-Dehydrocholesterol	nil	nil	nil	nil

[1] The plasma cholesterol values for subjects H and E at days zero, 4, 8, 12 and 16 were, respectively, 209.2, 120.1, 145.4 109.6, and 107.3; and 165.0, 113.0, 108.5, 82.2 and 82.2 mg/100 ml.

[2] Variations are those between two wet fecal aliquots removed for analysis. Recoveries including standard deviations for the sterols listed here were: 7-dehydrocholesterol, 99.2 ± 0.4%; Δ^7-cholestenol, 105.1 ± 7.5%; coprostanol, 97.9 ± 6.4%; cholesterol, 100.7 ± 7.9%; sitosterol, 99.1 ± 7.6%; cholestanol and/or stigmastanol, 90.4 ± 10.1%; total 3 β-hydroxysterol, 100.3 ± 0.9%.

[3] Based upon the anthrone procedure (5) and an average molecular weight of 388.

[4] Based upon the anthrone procedure (5) and an average molecular weight of 397.

[5] The intake of sitosterol during this period was 9.5 g (subject H) and 12.7 g (subject E).

scale experiments performed on human subjects (1).

The large increases in the amount of fecal unsaponifiable matter excreted by the 2 subjects on the last 4 days of the corn oil period are due to 3 β-hydroxysterols. A major portion of these was comprised of sitosterol, i.e., 59% in the case of subject H, and 61% in the case of subject E who was the controlled diabetic. In either instance, this fraction of fecal 3 β-hydroxysterols accounted for nearly 80% of the sitosterol ingested (9.5 and 12.7 g, respectively) in the corn oil during the period of fecal collections. By gas-liquid chromatographic criteria,[12] 75% of the sitosterol excreted by subject H was present as the β-isomer; the remainder was comprised of γ-sitosterol and a trace amount of stigmasterol. Subject E, during the corn oil period, also excreted minor amounts of saturated Liebermann-Burchard-negative sterols, presumably cholestanol or stigmastanol, or both.

The question arises as to the fate of that portion of sitosterol ingested with the corn oil, but which remained unaccounted for in feces. Contrary to earlier belief (6), plant sterols have been shown to be absorbed to a significant degree in both animals (7) and man (8); bile acids have been observed among the radioactive metabolites when labeled plant sterols were administered intravenously (9). Part of the sitosterol ingested with the corn oil by the 2 subjects studied here may therefore have been absorbed and subjected to similar transformations. Its conversion to 24-ethyl-coprostanol, (10), although never demonstrated in man, must also be considered.

The major observation revealed by a comparison of the sterols excreted during the 2 periods is the remarkable increase in the fraction of 3 β-hydroxysterols derived from *endogenous* sources during the consumption of the regimen high in corn oil. Thus, 4 and 5 times as much cholesterol was eliminated by subjects H and E, respectively, during this period as was observed during the consumption of the antecedent fat-free regimen. In the case of subject E, this was accompanied by an almost tenfold increase in the fecal output of coprostanol. These data, for the first time as far as the authors have been able to determine, demonstrate that the overall increase in sterol excretion observed when subjects are transferred from a fat-free diet to one high in corn oil is due in large part to an increase in the output of sterols derived from endogenous sources.

The association of significant increases in the elimination of endogenous sterol with decreasing plasma cholesterol levels suggests that these 2 phenomena are causally related. Hellman and Rosenfeld (11) demonstrated a similar circumstance in the case of isotopically labeled cholesterol given to a hypercholesterolemic subject who was transferred from a diet containing corn oil to one containing butter fat. Due to inadequacies of the analytical criteria used, other workers have been unable to determine to what extent sterols excreted during the consumption of unsaturated fat of high plant sterol content are derived from dietary and endogenous sources (12–15).

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Vitamin K Deficiency and Oxidative Phosphorylation[1]

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ABSTRACT Severe vitamin K deficiency in the rat, produced by strictly nutritional means, does not impair the efficiency of oxidative phosphorylation in liver mitochondria, with either glutamate or β-hydroxybutyrate as substrate. Liver mitochondria from dicumarol-treated rats also exhibited a normal coupling of phosphorylation to oxidation. In vitro addition of vitamin K_1 or K_2 to liver mitochondria from vitamin K-deficient or dicumarol-treated rats with occasional low P/O ratios, did not restore the P/O ratio to maximal values. In vitro uncoupling of oxidative phosphorylation by dicumarol was not reversed by vitamin K_1. It does not appear that vitamin K exerts its action on prothrombin synthesis through participation in mitochondrial oxidative phosphorylation.

Studies of the respiratory chain in various organisms have indicated that vitamin K_1 and other closely related naphthoquinones, with antihemorrhagic properties, may participate in electron transport and oxidative phosphorylation processes (1–5). In bacterial systems, convincing evidence has been provided (6) suggesting a direct participation of a cyclized form of vitamin K_1 in the phosphorylative events coupled to electron transport. Studies conducted in animal systems have led to less clear-cut results. Anderson and Dallam (7) have reported that rat liver mitochondria, treated with UV light to destroy the light-sensitive quinone bound to these structures, exhibited a complete uncoupling of phosphorylation from oxidation which was reversed by vitamin K_1. Beyer (3), in similar studies, found that vitamin K_1, alone, partially restored oxidative phosphorylation, whereas a combination of cytochome c and vitamin K_1 restored the system to nearly original levels. Martius (8) had previously observed that liver mitochondria, isolated from vitamin K-deficient chicks, had P/O ratios one-third lower than normal mitochondria and that these depressed values could be increased by the in vitro addition of vitamin K_1 (9). However, Beyer and Kennison (10) in later studies were unable to show any correlation between vitamin K deficiency, as indicated by prolonged prothrombin time, and efficiency of oxidative phosphorylation, in chick liver mitochondria. Parmar and Lowenthal [4] noted no depression of mitochondrial oxidative phosphorylation following administration of 2-chloro-3-phytyl-1:4-naphthoquinone, a competitive inhibitor of vitamin K_1, which produced in the normal animal a clotting defect resembling vitamin K deficiency.[5] Similarly, Green et al. (11) have reported that mitochondria from dicumarol-treated rats show no detectable difference, from those from normal rats, in their ability to esterify phosphate during oxidation of β-hydroxybutyrate. Moreover this type of quinone has not been detected with certainty in mitochondria. It appears that the hypothesis that vitamin K is involved in oxidative phosphorylation, in higher animals, requires further proof.

Since we have standardized a procedure to produce, by dietary means, a severe and uncomplicated vitamin K deficiency in rats, we have re-examined the possibility that liver mitochondria, isolated from vitamin K-deficient rats, might show a lowering of their phosphorylative abilities coupled to the oxidation of glutamate and β-hydroxybutyrate. If vitamin K actually

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[5] Lowenthal, J., and J. A. MacFarlane 1961 2-Chloro-3-phytyl-1:4-naphthoquinone, an antivitamin of vitamin K_1 (2-methyl-3-phytyl-1:4-naphthoquinone). Federation Proc., 20: 53 (abstract).

occurs in mitochondria, feeding a vitamin K-free diet, such as we used in our experiments, should be sufficient to obtain a substantial portion of depleted mitochondria within about 4 weeks, considering that rat liver mitochondria turn over as a single entity with a half-life of approximately 10 days, as shown by Fletcher and Sanadi (12). Studies were also made on the effect of dicumarol administration, superimposed on a vitamin K-free diet, on mitochondrial oxidative phosphorylation.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain were fed ad libitum a vitamin K-free diet (13) and kept in tubular cages (14) to prevent coprophagy. The control animals were also fed the synthetic diet, supplemented with 20 μg of menadione, in corn oil, given orally on alternate days. After the animals had been fed the experimental diet for 4 weeks, or before if symptoms of severe hemorrhage were evident, they were decapitated and the livers were quickly removed and placed in ice-cold sucrose solution. Plasma prothrombin times were determined according to the one-stage method of Quick (15). For the experiments intended to test the effect of dicumarol on oxidative phosphorylation in vivo, the animals received dicumarol mixed with the vitamin K-free diet, at a 0.25% level, for a period varying from 5 to 9 days.

Reagents. All chemicals were of analytical reagent grade. Solutions were prepared in glass-distilled water and adjusted to pH 7.4. Hexokinase (crude, type II), ATP-Na_2 [6] salt, DPN^+, vitamin K_1 (2-methyl-3-phytyl-1, 4-naphthoquinone), and dicumarol (3, 3′- methylenebis-4-hydroxy - coumarin) were obtained commercially.[7,8]

Analysis. Oxygen consumption was measured by the conventional Warburg technique. Phosphate esterification was calculated on each flask following P_i determination, according to the method of Fiske and Subbarow (16), on cold trichloroacetic filtrates.

Mitochondrial preparation. Twice-washed mitochondria were prepared, by differential centrifugation, essentially according to Schneider and Hogeboom (17) in 0.25 M sucrose containing 0.001 M EDTA-Na_2, as recommended by Slater and Cleland (18) and adjusted to pH 7.4. Homogenation was performed in a motor-driven glass homogenizer with a Teflon pestle. The final mitochondrial pellet was re-isolated in 8.0 ml of 0.25 M sucrose, to give 250 mg equivalent of mitochondria/ml (particles from 250 mg of rat liver). All operations, before final incubation with substrate, were carried out at zero to 2°C.

[6] The abbreviations used are: ATP-Na_2 salt, adenosine triphosphoric acid disodium salt; DPN^+, diphosphopyridine nucleotide oxidized form; P_i, inorganic orthophosphate; P/O, ratio of micromoles of P_i esterified to microatoms of oxygen consumed; EDTA-NA_2, ethylenediaminotetraacetic acid disodium salt; TCA, trichloroacetic acid.

[7] Hexokinase, ATP-Na_2 salt and DPN^+ were purchased from Sigma Chemical Company, St. Louis; vitamin K_1 and dicumarol were purchased from Nutritional Biochemicals Corporation, Cleveland.

[8] Vitamin K_2 (20) was obtained through the courtesy of Professor O. Wiss of F. Hoffmann-LaRoche and Company, Lts., Basel, Switzerland.

TABLE 1

Oxidative phosphorylation system for liver mitochondria from rats

	Substrate	
	Glutamate	β-hydroxybutyrate
Each Warburg vessel contained		
Mitochondria	125 mg equivalent[1]	125 mg equivalent[1]
PO_4 buffer, pH 7.4	50 μmoles	50 μmoles
ATP	3 μmoles	3 μmoles
DPN	0.3 μmoles	2 μmoles
Glucose	150 μmoles	150 μmoles
Cytochrome c		0.03 μmoles
$MgCl_2$	15 μmoles	15 μmoles
EDTA-Na_2	3 μmoles	3 μmoles
Hexokinase	25 KM units	25 KM units
Substrate	20 μmoles	50 μmoles
H_2O to	2.5 ml	2.5 ml
Incubation in air at 30°C	20 min	30 min

[1] Particles from 125 mg rat liver.

Incubation procedure. The composition of the incubation media and the conditions of incubation given in table 1 are taken from Hatefi and Lester (19) for the substrates glutamate and β-hydroxybutyrate. During addition of the incubation medium components the vessels were kept in the ice bath; hexokinase and mitochondria were added last. Immediately after addition of the mitochondrial suspension, 0.2-ml aliquots of the reaction mixture were transferred rapidly to test tubes containing 4.8 ml of chilled 5% TCA, for zero-time analysis of P_i. After a thermal equilibration period of 10 minutes, the incubation was carried out in air, at 30°C, for 20 to 30 minutes depending on the substrate used. Oxygen consumption for the first 10 minutes was calculated by extrapolation. All experimental flasks were run in triplicate or in duplicate. At the end of the incubation period 0.5-ml aliquots of flask contents were transferred rapidly to test tubes containing 4.5 ml of chilled 5% TCA. The moles of phosphate esterified were calculated as the difference in inorganic phosphorus between the zero time and the corresponding incubated samples.

Mitochondria from vitamin K-deficient or dicumarol-treated rats were always tested simultaneously with mitochondria from a control animal. The results, relative to each group, were then pooled together to allow statistical treatment.

RESULTS

In preliminary experiments succinate was used as substrate as had been used by Martius and Nitz-Litzow (8), and by Beyer and Kennison (10). No differences were noted in confirmation of the data of Beyer and Kennison (10) and in contrast with the data of Martius and Nitz-Lotzow (8). In order to examine the whole pathway from DPNH to O_2 in which 3 moles of ATP are produced, glutamate and later β-hydroxybutyrate were used as substrates.

The results in table 2 show the plasma prothrombin times of normal rats and the mitochondrial P/O resulting from the oxidation of glutamate and β-hydroxybutyrate, respectively. Normally accepted P/O ratios were obtained for both substrates tested. The P/O ratios resulting from the oxidation of glutamate fall within the normal range of values expected for the oxidation of this substrate and are not statistically significantly different at the 10% level from those obtained with normal animals. Depressed P/O ratios, 1.63 and 1.89, were observed in only 2 out of 13 cases. On the other hand, the prothrombin times corresponding to these ratios were 90 and 86 sec, respectively, whereas prothrombin times as high as 192 and 203 sec were associated, in that order, with P/O ratios of 2.99 and 2.41.

Difference in the substrate used did not modify the response of mitochondria to vitamin K deficiency. The P/O ratios resulting from the oxidation of β-hydroxybutyrate were, again, not significantly different at the 5% level from those obtained with normal animals. Only one value, out of ten, was markedly low, 1.51, and corresponded to a prothrombin time of 227 sec. However, as in the previous group, even

TABLE 2

Oxidative phosphorylation by liver mitochondria

Substrate	No. of animals	Vitamin K status	Weight	Prothrombin time	ΔO_2	ΔP_i	P/O avg
			g	*sec*	*μatoms*	*μmoles*	
Glutamate	13	normal	77–300	14–19	4.2–17.3	10.8–39.9	2.46 ± 0.22[1]
Glutamate	12	deficient	165–266	40–203	6.1–16.1	15.1–37.9	2.30 ± 0.37
β-Hydroxybutyrate	10	normal	123–211	14–19	4.0– 8.2	10.9–21.1	2.68 ± 0.26
β-Hydroxybutyrate	10	deficient	98–197	32–> 360	3.5– 7.4	9.8–16.9	2.38 ± 0.42
Glutamate	13	dicumarol-fed[2]	141–348	191–> 360	3.1–16.9	7.8–34.2	2.27 ± 0.44

[1] SD.
[2] Days on treatment, 5 to 9.

TABLE 3

Effect of vitamin K in vitro on oxidative phosphorylation by liver mitochondria from rats fed a vitamin K-deficient diet and from rats fed dicumarol (0.25% added to a vitamin K-free diet)

Rat wt	Treatment	Prothrombin time	Substrate	Addition	ΔO_2	ΔP_i	P/O
g		*sec*			*μatoms*	*μmoles*	
197	Vitamin K-deficient diet	98	glutamate	none	15.1	30.3	2.01
				vitamin K_1[1,2]	13.1	26.3	2.01
				vitamin K_2[1,2]	14.8	25.3	1.71
327	Dicumarol-supplemented	> 360	glutamate	none	16.9	34.2	2.02
				vitamin K_1[3]	17.6	34.6	1.96
207	Dicumarol-supplemented	> 360	glutamate	none	14.3	30.4	2.13
				vitamin K_1[3]	15.3	31.0	2.03
205	Dicumarol-supplemented	> 360	β-hydroxy-butyrate	none	5.0	9.9	1.96
				vitamin K_1[3]	5.9	9.9	1.68

[1] Final concentration 2.5×10^{-5} M.
[2] Vitamin K_1 and K_2 were added in 0.05 ml of propylene glycol or 0.3% polyoxyethylene sorbitan monooleate (Tween 80). Addition of solvents only did not affect the system.
[3] Final concentration 1×10^{-5} M.

longer prothrombin times were associated with perfectly normal P/O ratios.

The administration of dicumarol to rats, at 0.25% in the diet, produced massive hemorrhages followed by death within a few days. The appearance of blood was markedly altered, both in color and density, probably as a consequence of the anemia produced by the severe hemorrhages. Nevertheless, as shown in table 2, the average P/O ratio, obtained with mitochondria from dicumarol-treated rats, was not significantly affected at the 10% level. As in the other groups occasional low values, 1.36 and 1.42, were observed. However, mitochondria from rats, whose blood was practically uncoagulable, had a normally efficient oxidative phosphorylation system.

The most convincing evidence presented in support of the hypothesis that vitamin K has a role in oxidative phosphorylation, is derived from those experiments in which in vitro addition of vitamin K to mitochondria with low oxidative phosphorylation activity following irradiation (3, 7) or following vitamin K deficiency (9), restored the activity to nearly maximal values. To test this hypothesis vitamin K_1 or K_2 were added in vitro to some of the mitochondrial preparations derived from vitamin K-deficient rats or from rats fed dicumarol. The vitamins were very finely emulsified in propylene glycol or in 0.3% polyoxyethylene sorbitan monooleate (Tween 80). The data in table 3 show that neither vitamin K_1 nor K_2 had any restorative activity on the P/O ratios of these mitochondria. On the contrary, the values obtained after addition of the vitamins were slightly lower. Preincubation of mitochondria with the vitamins gave similar results. Attempts to reverse the uncoupling effect of dicumarol in vitro, by simultaneous addition of vitamin K_1 or K_2 to the incubation medium (final concentration: 1×10^{-5} M to 7×10^{-9} M), were also unsuccessful (P/O ratios: 0.85 vs. 0.74).

Some experiments were run with liver mitochondria isolated from vitamin K-deficient chicks. Also in this case no impairment of oxidative phosphorylation was observed (P/O ratios: 1.67 vs. 1.75).

DISCUSSION

It is generally recognized that the primary function of vitamin K_1 in higher animals, is to stimulate the formation of prothrombin by the liver. It has been shown, by the fluorescent antibody technique (20), that as early as one to two hours after vitamin K_1 administration most of the parenchymal cells of the liver are actively synthesizing prothrombin, which is then released into the blood stream 3 to 4 hours afterwards. Conversely, vitamin K deficiency is accompanied by a sharp decrease of plasma prothrombin concentration, followed by death of animals from hemorrhage, unless vitamin K is given.

Despite these long-known facts, the molecular mechanism by which vitamin K influences prothrombin synthesis is still awaiting elucidation.

The hypothesis was put forward several years ago, by Martius (1) that vitamin K takes part in the respiratory chain, as an electron carrier between DPN and cytochrome c. According to this author (21) lack of vitamin K, by influencing the rate of phosphorylation, may decrease the energy available to the cell, thus affecting the formation of prothrombin and other proteins as well. Prothrombin would be more dramatically affected because of its very short half-life.

It appears, from the sum of evidence now available, that this postulated mechanism of action requires revision. The only author who has been able to observe low P/O ratios, in mitochondria isolated from vitamin K-deficient animals, is Martius (8).

Our results, on the contrary, show clearly that the efficiency of oxidative phosphorylation in mitochondria isolated from vitamin K-deficient animals is not impaired. A species difference cannot be claimed to explain the discrepancy since neither Beyer (10) nor we confirm Martius's data, using chicks as experimental animals.

The observation that liver mitochondria of 2 of the treated animals had lower P/O ratios than the majority of tested animals, prompted us to extend the number of observations and to prolong the experimental treatment almost to death of animals. Any effort to systematically obtain low P/O values by prolonged vitamin K deficiency or dicumarol treatment was, however, unsuccessful. It may be possible that the severe conditions of the animals, consequent to vitamin K deficiency or dicumarol treatment, produced, in the less resistant of them, some form of metabolic derangement or of structural alteration of the mitochondria which resulted in lower than average P/O ratios, independent of any participation of vitamin K in oxidative phosphorylation. This is confirmed by our observation that addition of vitamin K_1 or K_2 to mitochondria with low phosphorylative ability had no restorative effect.

The different effect of dicumarol in vitro and in vivo offers additional evidence against the hypothesis of participation of vitamin K in oxidative phosphorylation. In vitro dicumarol uncouples oxidative phosphorylation (22) at all 3 phosphorylating steps (23) and appears to bind mitochondrial enzymes very tightly. In fact, when added to irradiated mitochondria, restored to normal phosphorylative abilities by vitamin K_1 and cytochrome c, it can re-inhibit the system (3), apparently displacing the restoring substances from their site of action. On the other hand, vitamin K_1 even at high doses, was found to be unable to counteract the uncoupling effect of dicumarol in vitro, in agreement with the report of Green et al. (11). In vivo, dicumarol acts as an anticoagulant and its action is reversed by minimal doses of vitamin K; yet, animals treated with dicumarol showed no change in mitochondrial oxidative phosphorylation (cf Green et al. (11)).

Obviously, in the intact animal there must be a site for vitamin K action, other than oxidative phosphorylation, for which this vitamin K antagonist competes. It is possible, as Green et al. (11) suggest, that dicumarol forms in vitro a stable and non-dissociable compound with mitochondrial enzymes, by virtue of its known capacity of strongly binding proteins (24). If this is true, it can be assumed that dicumarol in vivo would be diluted among all other proteins for which it might have affinity, before it can reach an effective concentration in mitochondria. Its uncoupling effect in vitro would then be a nonspecific one.

From our results and those reported from other laboratories it appears to be legitimate to conclude that vitamin K is not involved in mitochondrial oxidative phosphorylation. We are of the opinion that the mechanism by which vitamin K influences prothrombin synthesis must be a specific and direct one and that an entirely different approach is needed to elucidate it.

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The Protein Quality of Waste-grown Green Algae

I. QUALITY OF PROTEIN IN MIXTURES OF ALGAE, NONFAT POWDERED MILK, AND CEREALS[1]

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ABSTRACT The protein efficiency ratio (PER) was determined, as a measure of protein quality, on thick gruels and on baked products containing waste-grown green algae, a mixture of *Scenedesmus quadricauda* and *Chlorella*, spp., at a ratio of 10:1. Algae-cereal-nonfat dry milk mixtures (the algae and cereals boiled for 30 minutes to make a thick gruel) and milk added to the cooked product, were submitted to a taste panel to determine which of the cereals and what proportions of cereals and nonfat dry milk to use to mask the strong algal flavor most effectively. The mixtures containing varying proportions of algae, oatmeal, cracked wheat, and nonfat dry milk, were judged to be most palatable and were selected for the rat growth experiments. The algae-cereal-milk mixtures tested ranged from 22 to 29 g of protein/100 g and compared well with nonfat dried milk in protein quality. Calculated amino acid patterns compared favorably with the FAO reference protein except for methionine, which was lower than recommended by the FAO, but more than in INCAP vegetable mixture 9. Oatmeal and wholewheat breads, muffins, and peanut butter cookies containing algae were found to be palatable but were lower in the amount of protein (from 9.8 to 15 g/100 g) than were the algae-cereal-milk mixtures. There was loss in protein quality in the cookies as a result of baking.

The shortage of protein foods in various areas of the world is of increasing concern. Because of overpopulation and a scarcity of land for production of sufficient protein at a cost within reach of the people, means of producing protein-rich foods in areas of shortage are being sought.

Green algae, because they contain a high percentage of protein, minerals, and vitamins, have been the subject of investigation in several laboratories as a possible source of food for animals and human beings. Oswald et al. (1) have developed a process called controlled photosynthesis by which algae can be grown in sewage at an estimated cost of from 3 to 5 cents/454 g. By means of this process, Oswald (2) states that cultures of the algae *Chlorella* yield an average of about 10.884 metric tons of protein/acre a year — more than tenfold the rate of soybeans which yield up to 0.907 metric ton of protein/acre a year. The prospect of interplanetary travel has also heightened interest in the possible use of these organisms as a partial food source during extended flights into space.

Morimura and Tamiya (3) incorporated algal powders into such foods as bread, ice cream, noodles, soups, and similar staples to increase their protein and vitamin content. They were able to increase the protein by 20% in bread and noodles and by 30% in ice cream. The resulting products were found to be palatable. These workers did not, however, determine the quality of the protein mixtures in the products studied nor the effect of preparation methods on protein utilization.

Studies have been published to support the conclusions that, although green algae contain a high percentage of protein, its quality is such that it cannot be used as the sole source of protein, but could be used effectively with casein (4). Tamura et al. (5), in rat-growth experiments, found that the protein quality of laboratory-grown *Chlorella* or *Scenedesmus* was inferior to that of casein. Nitrogen balance studies with men subjects showed the protein quality to be inferior to beef

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protein and the flavor of the algae intolerable. Decolorization of the algae with methyl alcohol improved the digestibility somewhat, but the flavor, although improved, remained unacceptable to the subjects.

Since cereal grains and legumes are the staple foods in most underdeveloped areas where protein malnutrition exists and the amount of milk powder formerly shipped overseas to supplement these foods has been drastically reduced, algae in combination with cereal grains and small amounts of milk in ratios to yield a satisfactory amino acid pattern is a possible solution to the protein shortage. Algae can also contribute generously to the intake of vitamins and minerals, commonly low in the diets eaten by people in those areas.

In the present study, varying mixtures of cereal products and algae, that could be boiled and then mixed with powdered skim milk, were submitted to a human taste panel for palatability tests. Those found to be acceptable were used as diets for rats, to determine the PER (protein efficiency ratio). The amino acid patterns in each of the mixtures were calculated and compared with the FAO provisional reference protein (6). The PER of the baked products in which the algae were substituted for part of the flour was measured; it was also determined on 2 lots of unbaked ingredients to learn the effect of subsequent baking temperatures on the quality of the protein.

EXPERIMENTAL

Two sets of experiments of 28 days each were carried out to determine the PER (grams of gain per gram of protein eaten). In each experiment, 21-day-old weanling rats of the Long-Evans strain were caged separately and continued to be fed stock diet containing 22% protein, until they attained weights between 50 and 60 g (usually in 2 to 3 days). Those failing to gain with this diet and adapt to life in a separate cage in 4 or 5 days were discarded. The rats selected for the experiments were divided into 8 groups, of 12 animals each, in which the sexes and litters were evenly distributed, and were fed the experimental diets. To equalize the protein intake in all of the groups, the same amount of food was offered to each rat, the amount being determined by the group of rats eating the smallest amount of food.

The diets used in these experiments had the following percentage composition: protein, 12 in experiment 1, and 10 in experiment 2, supplied by one of the following: algae, rolled oats, instant nonfat milk solids, and the boiled and baked mixtures described later; hydrogenated vegetable fat, taking into account the fat in the algae and cereals, to make 10; salt mixture (USP 14), 4; and powdered sugar to make 100.

A mixture containing sufficient cod liver oil and carotene to provide daily averages of 10 and 100 IU of vitamins D and A, respectively, and one milligram of mixed tocopherols, was fed 3 times a week. A solution of B-vitamins and menadione, also fed 3 times a week, provided daily: thiamine hydrochloride, 20 μg; riboflavin, 40 μg; niacinamide, 66 μg; pyridoxine hydrochloride, 20 μg; calcium pantothenate, 100 μg; inositol, 2.5 mg; biotin, 2 μg; *p*-aminobenzoic acid, 100 μg; folic acid, 20 μg; choline, 10 mg; vitamin B_{12}, 0.2 μg; and vitamin K, 49 μg.

A mixture of the green algae, *Scenedesmus quadricauda* and *Chlorella* spp., at a ratio of approximately 10 to 1, was used for the experiments. The algae[2] were grown in an open, outdoor pond, on sewage and organic wastes, by the low-cost production method developed by Oswald et al. (1). The organisms were separated from the water solution by centrifuge, which concentrated them to a paste. The paste was spread in a thin layer on a large sheet of aluminum suspended over infrared lights, heated until dry, and then pulverized to a fine powder in a Waring Blendor.

Experiment 1. To improve the flavor and protein quality, mixtures of algae and the following cereals were boiled for 30 minutes in sufficient water to produce a thick gruel: rolled oats; cracked wheat; peanut meal; soybean grits; potato meal; and rice, soya, and rye flours. The milk solids were added to the cooked mixtures,

[2] We are indebted to Dr. W. J. Oswald, Dr. C. G. Goleuke, and Henry Gee of the University of California Sanitary Engineering Research Laboratory, Richmond, California, for the algae used in these experiments.

and the resulting products were submitted to a taste panel for evaluation for color, texture, flavor, and odor. Those receiving the highest degree of acceptance by the taste panel were selected for protein quality tests.

Experiment 2. The diets fed were of the same general composition as those fed in experiment 1 with the following exceptions: (a) the percentage of protein in the diets was 10 instead of 12; and (b) in diet 4, the minerals and vitamins were omitted in order to test the adequacy of those supplied to the bread by the algae. The amount of sugar was adjusted to compensate for these changes.

The protein in the control diet was supplied by nonfat dry milk, and in the other diets by the following products in which algae replaced a portion of the flour in a standard recipe: baked oatmeal and baked whole wheat bread; muffins, baked and unbaked; and peanut butter cookies, baked and unbaked. The amount of algae incorporated into each recipe was the maximal amount that could be used and still result in a product acceptable from the standpoint of texture, color, and flavor. The baking times and temperatures were: bread, 25 to 30 minutes at 200°C; muffins, 20 to 25 minutes at 200°C; and cookies, 10 to 12 minutes at 190°C. The composition of 100 g, dry basis, of these products is given in table 3.

RESULTS

Experiment 1. The protein sources in the rat diets, the percentage of the protein supplied by algae, nonfat dry milk, oatmeal, and the mixtures of these foods with cracked wheat, and the PER of each are given in table 2. The taste panel's evaluation indicated that the cereals most effective for masking the algal flavor were oatmeal and cracked wheat. Peanut meal, soy grits, and soybean flour accentuated the undesirable flavor; other products tested had no effect on the strong algal flavor. Nonfat milk solids improved the palatability of the mixtures in proportion to increase in the amount of milk added.

When nonfat dry milk was fed as the sole source of protein in the diets, the PER, 2.64 ± 0.08, was significantly higher than that of either the uncooked or cooked algae or the cooked oatmeal. The cooked algae had a higher PER than did the uncooked, thus confirming the earlier observations of Cook (4) that boiling the algae for 30 minutes improved the digestibility and thus the quality of the protein for the rat. The protein quality of cooked oatmeal was significantly better than that of cooked algae, but significantly lower ($P < 0.01$) than that of nonfat dry milk.

The PER of the algae-cereal-milk mixtures used as the protein source in rat diets 5, 6, 7, and 8 were, respectively: 2.45 ±

TABLE 1

Comparison of the PER[1] of baked and unbaked algae-containing wholewheat bread, muffins, and peanut butter cookies with that of nonfat dry milk solids (12 rats/group fed 28 days)

Diet no.	Protein source	Amt protein[2] from algae	Protein intake	Total wt gain	PER[3]
		%	g	g	
1	Nonfat dry milk	0	26.6	76	2.87 ± 0.06
2	Oatmeal bread	14	26.7	51	1.91 ± 0.04
3	Wholewheat bread	9	27.0	44	1.69 ± 0.05
4	Wholewheat bread without minerals and vitamins added to diet	9	25.6	44	0.73 ± 0.06[4]
5	Muffins, unbaked	21	25.4	57	2.24 ± 0.05
6	Muffins, baked	21	26.9	61	2.28 ± 0.06
7	Peanut butter cookies, unbaked	27	26.5	54	2.05 ± 0.04
8	Peanut butter cookies, baked	27	25.6	43	1.66 ± 0.05[5]

[1] PER, grams of gain per gram of protein eaten.
[2] Level of protein in diet 10%.
[3] All results are expressed as averages ± SE of mean.
[4] Difference between this mean and diet 3 not significant.
[5] Difference between this mean and the unbaked cookies significant at $P < 0.01$.

TABLE 2

Average PER[1] of raw and cooked green algae alone and in combination with cereals and nonfat dry milk solids (12 rats/group fed 28 days)

Diet no.	Protein source		Total protein		Total wt gain	PER[3]
			% of[2]	Amt eaten		
				g	*g*	
1	Uncooked algae		100	29.3	47	1.61 ± 0.08
2	Cooked algae[4]		100	30.7	57	1.85 ± 0.05[5]
3	Nonfat dry milk		100	32.8	87	2.64 ± 0.08
4	Cooked oatmeal		100	33.8	80	2.35 ± 0.05
5	Algae	(16.7)[6]	36	34.2	86	2.45 ± 0.05[7]
	Oatmeal	(50.0)	30			
	Cracked wheat	(16.7)	9			
	Nonfat dry milk	(16.6)	25			
6	Algae	(33.3)	55	34.5	87	2.51 ± 0.07[8]
	Oatmeal	(22.3)	10			
	Cracked wheat	(22.2)	9			
	Nonfat dry milk	(22.2)	26			
7	Algae	(25)	44	33.8	87	2.55 ± 0.03[8]
	Oatmeal	(25)	12			
	Cracked wheat	(25)	12			
	Nonfat dry milk	(25)	32			
8	Algae	(16.7)	27	33.6	93	2.75 ± 0.07[8]
	Oatmeal	(16.6)	8			
	Cracked wheat	(16.7)	7			
	Nonfat dry milk	(50.0)	58			

[1] PER, grams of gain per gram of protein eaten.
[2] Level of protein in diet, 12%.
[3] All results are expressed as averages ± SE of mean.
[4] Boiled for 30 minutes.
[5] Difference between this mean and the raw algae significant at $P < 0.05$.
[6] Figures in parentheses represent grams, dry weight, of each food used in the mixture. Diet numbers are used as mixture numbers for discussion purposes.
[7] Difference between this mean and that of milk significant at $P < 0.05$.
[8] Difference between this mean and that of milk not significant.

0.05; 2.51 ± 0.07; 2.55 ± 0.03; and 2.75 ± 0.07 (table 2). The protein quality of mixture 5 was significantly better than that of cooked oatmeal or of cooked algae, but significantly lower ($P < 0.05$) than milk protein. The PER of mixtures 6, 7, and 8 were not significantly different from the PER of milk protein. Oatmeal provided the largest percentage of the non-algae protein (30%) in mixture 5, and milk (58%) in mixture 8. Mixture 8 ranked first in palatability, and 5, second. Mixtures 6 and 7, in which algae supplied 55 and 44% of the protein respectively, were acceptable and had a PER equal to that of milk; mixture number 5 was not far below and would probably be a satisfactory source of protein.

Experiment 2. The comparison of the PER of the nonfat skim milk with that of the baked breads, muffins, and cookies and also the unbaked muffin and cookie ingredients, and the percentage of the total protein supplied by algae in each of these products, are shown in table 1. The total protein and the percentage of the total protein supplied by each of the ingredients, including algae, is shown in table 3.

Nonfat dry milk had a PER significantly higher (2.87 ± 0.06) than those of all the baked or unbaked products tested. Oatmeal bread, containing 15.2% protein of which 14% was supplied by algae (tables 1 and 3), had a PER of 1.91 ± 0.04, which was significantly higher than either of the wholewheat breads that contained 12.2% protein, 9% of which was supplied by algae. This result was probably due primarily to the oatmeal content and to some degree, to the higher percentage of total and algal protein. Both breads contained some powdered milk, but the wholewheat had slightly more than the oatmeal. Table 2 shows the superiority of oat protein over that of algal protein.

The PER of baked and unbaked peanut butter cookies were 1.66 ± 0.05 and 2.05 ± 0.04, respectively. This decrease, a result of heat in baking, was highly significant ($P < 0.01$). The quality of the

TABLE 3

Ingredients in 100 g of the baked products

Ingredients	Oatmeal bread		Wholewheat bread		Muffins		Peanut butter cookies	
	g	*%*	*g*	*%*	*g*	*%*	*g*	*%*
Flour								
Wholewheat	73	(63)[1]	84	(76)	—	—	—	—
All-purpose	—	—	—	—	56	(44)	21	(16)
Nonfat drymilk	4	(10)	5	(12)	8	(21)	—	—
Algae	5	(14)	3	(9)	6	(21)	8	(27)
Oatmeal	11	(10)	—	—	—	—	—	—
Dried whole egg	—	—	—	—	4	(14)	3	(9)
Yeast	1	(3)	1	(3)	—	—	—	—
Peanut butter	—	—	—	—	—	—	25	(48)
Hydrogenated fat	2	—	3	—	11	—	—	—
Sugar	3	—	3	—	12	—	41[2]	—
Salt	1	—	1	—	1	—	—	—
Baking powder	—	—	—	—	2	—	2	—
Total protein	15.2		12.2		9.8		11.7	

[1] Values given in parentheses are the percentage of the total bread protein that is supplied by the ingredients.
[2] Brown sugar; white sugar was used in the breads and muffins.

muffin protein showed no significant difference in PER as a result of baking.

DISCUSSION

These experiments demonstrate that green algae, *Scenedesmus quadricauda* and *Chlorella spp.*, can be incorporated in mixtures of varying amounts of powdered skim milk and wheat and oat cereals that have a PER not significantly different from that of milk protein.

The essential amino acid content of algae-cereal-milk mixtures 5, 6, 7, and 8 (table 2) was compared (table 4) with the essential amino acids provided in INCAP vegetable mixture 9 (7), and with the FAO provisional reference protein (6). All of the mixtures, except 5, had a PER close to that of milk. In grams per gram of N, all of the mixtures had amounts of tryptophan ranging from 0.085 for mixture 6 to 0.098 for mixture 8; INCAP mixture 9 had less, 0.059 g. All the mixtures (5, 6, 7, and 8) were low in methionine according to the reference protein, and 2 of them, 5 and 6, had less than the INCAP mixture. The FAO standard does not include cystine, but our mixtures all contain considerably more of this amino acid and also of total sulfur amino acids than does the INCAP all-vegetable mixture 9. In all other amino acids our algae-cereal-milk mixtures were equal to, or in excess of, the standard FAO pattern and the INCAP mixture 9, except that the latter was higher in histidine. The tryptophan, lysine, and histidine in algae are approximately equal to those in nonfat dried milk, but the total sulfur amino acids are lower than in milk. The 2 cereals used in the mixtures, oats and wheat, are much lower in tryptophan, histidine, and sulfur amino acids than are milk and algae, and much lower in lysine. Oatmeal is a better source of these amino acids than wheat.

It is important, in the preparation of products that may be used for the relief or correction of protein malnutrition, to consider the effect of processing methods on the availability of the essential amino acids in the protein. Heat has been demonstrated to have either beneficial or damaging effects on protein utilization depending upon the degree of heat used, whether it is dry or moist heat and, upon the kind of food. Morgan (8) showed by experiments with rats, that the protein of cereals subjected to dry heat or toasting at approximately 120°C for 45 minutes was not well utilized for growth. Yet, when the cereals were cooked with water, no undesirable changes in the protein occurred. Boiling green algae for 30 minutes improved the PER and the digestibility; autoclaving the algae at 120°C for 30 minutes significantly decreased both the digestibility and the PER (4). It is possible that some of the gastrointestinal disturbances suffered by human subjects fed large amounts of green algae by Powell et al. (9) could have been due, to some extent, to the excessive heat treatment of the algae. As a precaution

TABLE 4

Essential amino acid content[1] of algae-cereal-milk mixtures and of algae

	Diet number											
	5		6		7		8		Algae	FAO[2] standard	INCAP[3] mixture 9
	g/100 g	*g/g N*	*g/100 g*	*g/g N*	*g/100 g*	*g/g N*	*g/100 g*	*g/g N*	*per 100 g*	*g/g N*	*g/g N*
Tryptophan	0.36	0.095	0.41	0.085	0.47	0.106	0.47	0.098	1.04	0.090	0.059
Threonine	0.83	0.219	1.29	0.269	1.19	0.269	1.22	0.253	2.24	0.180	0.198
Isoleucine	1.14	0.301	1.38	0.287	1.34	0.302	1.76	0.356	1.78	0.270	0.254
Leucine	1.84	0.486	2.40	0.499	2.24	0.505	2.66	0.552	3.53	0.306	0.473
Lysine	1.23	0.325	1.71	0.355	1.59	0.359	1.98	0.411	2.71	0.270	0.343
Methionine	0.39	0.103	0.52	0.108	0.50	0.113	0.63	0.131	0.73	0.270	0.111
Cystine	0.29	0.077	0.29	0.060	0.29	0.065	0.30	0.062	0.24	—	0.018
Total sulfur amino acids	0.68	0.179	0.81	0.168	0.79	0.178	0.93	0.193	0.97	—	0.129
Phenylalanine	1.11	0.293	1.36	0.282	1.28	0.289	1.43	0.297	1.99	—	—
Valine	1.38	0.365	1.77	0.368	1.76	0.397	1.91	0.397	2.67	0.270	0.259
Histidine	0.50	0.132	0.66	0.136	0.61	0.137	0.72	0.149	0.98	—	0.227
Nitrogen, %	3.78	—	4.81	—	4.43	—	4.81	—	7.51	—	4.40

[1] Values used for calculating the amino acids in the cereals and milk were obtained from Orr and Watt (11); those for the algae are unpublished values determined in this laboratory by the microbiological method.
[2] Amino acids in FAO provisional reference protein, Food and Agriculture Organization (6).
[3] INCAP mixture 9, Bressani et al. (7).

against possible intake of pathogens, the algae were autoclaved at 160°C for 2 hours.

Dry mixtures of the cereal grains, algae and powdered milk in the proportions in these diet mixtures, could be packaged commercially and prepared with ease in the home. The powdered milk, if kept separate from the other ingredients in the package, could be added after the algae and cereals were boiled a sufficient time to improve their digestibility thus avoiding damage to the milk protein by heat. Clegg and Dean (10), in nitrogen balance studies on children, showed that the protein value of peanut biscuits was reduced when the dried skim milk was cooked with the other ingredients.

Although the baked products prepared in this experiment were found acceptable by the taste panel, and can serve as a means of incorporation of algae into the diet, they are not so valuable a source of protein as the boiled mixtures tested in experiment 1 (tables 1, 2). The baked products had percentages of protein ranging from 9.8 to 15.2/100 g of product, as compared with 22.0 to 29.2 in the boiled mixtures. The protein quality in the latter would not vary in preparation, but that of the baked products could vary considerably, depending upon the baking time, the degree of heat, and the amount of sugar used.

The proposed mixtures containing algae may pose some problems in acceptance by the people. Algae, with its pronounced, lingering flavor, which is new to most people, would likely be rejected on that basis. It also imparts a green color to any product containing it and this may be objectionable. Waste-grown algae is very likely to lack aesthetic appeal — an obstacle that may be difficult to overcome. However, since the Japanese people have for years consumed products containing seaweeds with similar flavor, it is possible that other countries may learn to accept them.

The possible presence of toxic or interfering factors in algae needs further investigation. No gross symptoms indicating toxicity from the algae were observed in our 28-day study with rats. Powell et al. (9) reported that human subjects were able to adjust to 100 g of algae/day with-

out undue distress. Amounts above this caused severe gastrointestinal disturbances.

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Lack of Effect of Urease Injection on Growth Rate and Feed Efficiency of Chicks and Rats[1]

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ABSTRACT The effect of dietary urease and urease injections on growth rate and feed efficiency was studied, using 622 broiler-strain chicks and 51 male weanling rats. Urease injections totaling from 20.0 to 61.5 Sumner units were given at various periods of chick and rat growth. Dietary urease was fed at levels from 44 to 132 Sumner units per 454 g of chick diet. In no instance did the imposed treatment cause statistically significant differences in the measured growth responses or in feed efficiency.

Numerous reports have shown growth stimulation from dietary antibacterial agents. This is believed to be primarily due to their effect upon the bacterial flora of the intestine. Visek et al. (1) reported that addition of 100 ppm of 3 antimicrobial agents to a casein diet significantly decreased in vivo hydrolysis of C^{14}-labeled urea by rats and reduced production of urease by gastrointestinal bacteria. Their explanation was that antibacterial agents attack bacteria which produce urease, and, thus, less ammonia is produced.

Dang and Visek (2) and Visek and Dang (3)[4] have shown growth stimulation and improved feed efficiency in rats and chicks immunized with jackbean urease. Through the injection of pure urease, an immunity was developed against the urease produced naturally in the gut.

Billingham et al. (4) reported that the chick has a null period during which the newly hatched chicks do not produce antibodies. They considered a 2-week-old chicken to be immunologically mature. Thus, it would be purposeless to inject chicks before about 2 weeks of age.

The experiments reported here were conducted in an effort to confirm Visek's results of improved growth rate and feed efficiency through urease injections and urease administered orally.

EXPERIMENTAL PROCEDURE

The chick experiments were conducted with broiler-type chicks grown in typical battery brooders for the first 4 weeks and in unheated growing batteries during the succeeding 4-week growing period. Experimental rats were of the Sprague-Dawley strain. Chicks were randomly allotted to battery pens, and rats to individual cages, with treatments randomly assigned to pens within a replication. Animals were weighed individually at weekly intervals. Testing for statistical significance was by analysis of variance methods.[5] Urease preparations used in experiments 1 and 2 contained 400 Sumner units of activity/g,[6] and that used in experiments 3, 4 and 5, 3000 Sumner units/g.[7] Chicks were injected intramuscularly in the breast muscle in experiments 1, 2 and 3; subcutaneously, in experiment 4, rats were injected intraperitoneally.

Since no period differences were observed the growth and feed efficiency values are given as the average obtained for the entire period during which measurements were collected.

Experiment 1. Six groups of 12 straight-run chicks/group were used in experiment 1. Treatments consisted of a control

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[7] Obtained from Sigma Chemical Company, St. Louis.

(table 1); basal diet, plus an injection of saline solution equal in volume to the urease solution injected; urease, dissolved in saline injected either once a week or 3 times a week on alternate days (injections for respective weeks totaled 7.5, 12.0, 18.0 and 24.0 units of urease); and basal, plus urease in the feed at a level of 92 or 185 units of urease/kg. Urease was injected during the first 4 weeks, whereas dietary urease was given for the full 8-week period.

Experiment 2. Sixty male chicks 32 days of age were used in experiment 2, giving 15 birds/treatment. The treatments consisted of a control (table 1); 10 units of urease dissolved in saline injected/bird at 32 and again at 35 days of age; injection of 3 ml mineral oil; and an injection of 48 units urease suspended in 3 ml mineral oil at 32 days of age.

Experiment 3. Thirty pens of 10 one-day-old chicks were used in this experiment. Treatments involving males were triplicated, and treatments for females were duplicated. Treatments with males consisted of a control (table 1); dietary urease at levels of 44, 88 and 132 units/kg of diet fed throughout the entire experimental period; 88 units of urease/kg of diet for the initial 5 weeks and the basal ration for the remaining 3 weeks; urease injected 3 times a week; a single injection at 3 weeks of age; and 20 units of urease at 3 weeks of age followed by 30 units of urease at 3.5 weeks of age. A total of 50 units of urease dissolved in distilled water was given/chick in all the injection treatments.

The 3 treatments with female chicks were: control (table 1); 88 units of urease/kg of diet; and 50 units of urease injected in the 3-time/week sequence. In experiment 3, injections were made only during the first 5 weeks of experiment.

Experiment 4. One-hundred-and-ninety chicks were allotted to 20 pens with either 10 or 9 birds/pen. Treatments involved were control (ration 1, table 2); saline injected; urease injected (enzyme dissolved in 0.85% NaCl); urease injected and antibiotic in the diet; and antibiotic in the diet. The antibiotic treatment was given as a "positive" control and, urease plus antibiotic, to observe possible related effects. Injected birds received injections every other day for 4 weeks, and each urease-injected bird received a total of 52.5 Sumner units of urease.

The diet used in experiment 4 was similar to that reported by Dang and Visek (2) with differences being in the salt mixture and source of fat.

Experiment 5. Fifty-one male weanling rats were used in experiment 5. Treatments were: control (ration 2, table 2); urease injected (enzyme dissolved in 0.85% NaCl); and saline injected. Injected rats received injections every other

TABLE 1

Composition of diet

	%
Ground yellow corn	63.0
Solvent-extracted soybean meal (50% protein)	26.0
Animal fat	1.5
Fish solubles, condensed	3.0
Dehydrated alfalfa meal (20% protein)	2.0
Salt mix[1]	0.5
Oyster shell, finely ground	1.0
Dicalcium phosphate	2.0
Vitamin and antibiotic mix[2]	1.0

[1] Salt premixed with $MnSO_4$: 95% NaCl, 5% $MnSO_4$.
[2] Provided the following per kg of diet: vitamin A, 6,600 IU; vitamin D_3, 1,100 ICU; vitamin K, 2.2 mg; vitamin E, 11 IU; riboflavin, 8.8 mg; niacin, 52.8 mg; choline, 660 mg; vitamin B_{12}, 16.5 μg; Ca pantothenate, 8.8 mg; and penicillin, 11 mg.

TABLE 2

Composition of diets

	Ration 1	Ration 2
	%	%
Solvent-extracted soybean meal (50% protein)	40.0	31.4
Sucrose	41.8	50.4
Lard	—	8.0
Soybean oil	8.0	—
Cellulose[1]	4.0	4.0
Salt mix[2]	5.2	—
Salt mix (USP IV)	—	4.0
Vitamin mixture in sucrose[3]	1.0	2.2

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[2] Salt mixture provides per kg of diet: (in grams) dicalcium phosphate, 27.7; finely ground oyster shell, 13; iodized NaCl, 4; K_2HPO_4, 5; $MgSO_4$, 1.5; $MnSO_4$, 0.5; CCC trace mineral mix (Calcium Carbonate Co., Omaha, Nebraska), 0.5 (to provide Zn, 50 mg; Fe, 50 mg; Cu, 5 mg).
[3] Vitamin mixture provides per kg of diet: vitamin A acetate, 19,800 IU; vitamin D_3, 2,200 ICU; *dl*-α-tocopherol (250 IU/g), 110 mg; ascorbic acid, 990 mg; inositol, 110 mg; choline chloride, 165 mg; menadione sodium bisulfite, 16 mg; *p*-aminobenzoic acid, 110 mg; niacin, 110 mg; riboflavin, 22 mg; pyridoxine·HCl, 22 mg; thiamine·HCl, 22 mg; Ca pantothenate, 66 mg; biotin, 440 μg; folacin, 2 mg; and cobalamin, 30 μg.

day for 4 weeks and each urease-injected rat received a total of 30.0 Sumner units of urease.

The diet used in this experiment was similar to that reported by Dang and Visek (2) with the difference being in the source of fat.

RESULTS AND DISCUSSION

Experiment 1. Average gain and feed per unit gain is shown in table 3. Since straight-run birds were used, they were sexed by external characteristics at the end of the experiment, and the average gain per bird is shown by sex. However, the feed per unit gain data are for the straight-run pens. A low level of dietary urease tended to improve growth and feed per unit gain, but this was not a significant improvement. Other treatments failed to improve growth or feed per unit gain.

Experiment 2. Forty-eight units of urease, suspended in 3 ml mineral oil/bird, injected at 4.5 weeks of age caused an increase in average gain per bird over the control (table 4). However, injection of 3 ml mineral oil alone caused a greater increase in gain than the urease suspension in mineral oil. Therefore, this increased gain may not have been due to urease. Two injections of 10 units urease each/bird at 4.5 and 5 weeks of age resulted in a lower gain than the control. There were no significant differences among treatment groups in either weight gains or feed conversion.

Experiment 3. The summary of gains and feed efficiency are presented in table 5. A slight decrease in body weight gains and a slightly increased feed requirement per unit of gain were observed for birds receiving the urease in the feed. The massive injection at 3 weeks of age was the only treatment showing a greater gain than the basal group. However, the differences in gains were not significant within sex. There were no significant differences in feed conversion among treatments in females. However, some differences were noted in the feed per gain data of male groups. The authors must point out that greater differences were observed between replications than between treatments. Since the differences in the treatments follow no logical pattern, the authors conclude that these differences are meaningless.

TABLE 3

Weight gains (g) and feed per unit gain of chicks from 4 days to 8.5 weeks (exp. 1)

Sex	Treatments					
	Basal	Saline injection	92[1]	185[1]	3[2]	1[2]
♂	1388(7)[3]	1284(6)	1480(7)	1365(5)	1204(3)	1267(4)
♀	1161(5)	1141(6)	1205(6)	1089(6)	1098(8)	1154(8)
	Feed/unit gain					
	2.74	2.88	2.51	2.81	2.70	2.73

[1] Units urease fed per kg of feed.
[2] Injections per week (total of 61.5 units/bird).
[3] Numbers in parentheses indicate the number of birds of that sex in the treatment group.

TABLE 4

Weight gains (g) and feed per unit gain of chicks from 4.5 weeks to 8.5 weeks (exp. 2)

Treatments[1]			
Basal	20 Units urease	Mineral oil injection	48 Units urease
Gain			
596	586	642	621
Feed/unit gain			
3.04	3.10	3.00	2.95

[1] Twenty units dissolved in saline and 48 units dissolved in mineral oil.

Experiment 4. Weight gain and feed conversion differences (table 6) among treatment groups did not approach statistical significance. Males grew faster than females; however, this is to be expected on the basis of the known sex difference in rate of gain in broiler strains. The data give some indication that males may respond differently than females to urease injections, but no significant differences were observed when the data were analyzed independent of sex. Results were

TABLE 5

Weight gains (g) and feed per unit gain of chicks from one day to 8 weeks (exp. 3)

Replication	Treatment							
	Level of urease fed[1]					Number of injections[2]		
	Basal	44	88	132	88[3]	12	1	2
				Gains				
1 ♂	1209	1286	1234	1147	1268	1182	1216	1182
2 ♂	1201	1250	1118	1240	1170	1240	1407	1330
3 ♂	1354	1210	1289	1272	1171	1176	1362	1184
1 ♀	1038		1121			1008		
2 ♀	1183		973			1150		
Avg of ♂	1255	1249	1211	1220	1203	1196	1328	1220
Avg of ♀	1110		1047			1079		
				Feed/unit gain				
1 ♂	2.55	2.51	2.50	2.66	2.30	2.44	2.55	2.44
2 ♂	2.44	2.33	2.63	2.54	2.41	2.50	2.21	2.34
3 ♂	2.32	2.38	2.31	2.33	2.31	2.41	2.29	2.56
1 ♀	2.38		2.54			2.71		
2 ♀	2.62		2.80			2.50		
Avg of ♂	2.44	2.41	2.48	2.51	2.34	2.45	2.35	2.45
Avg of ♀	2.50		2.67			2.63		

[1] Units urease fed per 454 g of feed.
[2] Total of 50 units/bird.
[3] Eighty-eight units urease per 454 g of feed was fed until the birds were 5 weeks of age and then they were fed basal diet until the end of the experiment.

TABLE 6

Weight gains (g) and feed per unit gain of chicks from one day to 8 weeks (exp. 4)

Replication	Treatment				
	Basal	Urease injection[1]	Saline injection	Antibiotic[2]	Urease + antibiotic
			Gains		
1 ♂	1053	1276	1099	1285	1180
♀	1099	981	903	1022	981
2 ♂	1199	1180	1330	1171	1289
♀	989	890	985	999	985
Avg of ♂	1126	1226	1217	1226	1235
Avg of ♀	1044	935	944	1008	980
Avg of ♂+♀	1085	1081	1081	1117	1108
			Feed/unit gain		
1 ♂	2.30	2.33	2.32	2.20	2.21
♀	2.24	2.28	2.48	2.24	2.39
2 ♂	2.30	2.23	2.30	2.44	2.31
♀	1.94	2.71	2.40	2.33	2.35
Avg of ♂	2.30	2.28	2.31	2.32	2.26
Avg of ♀	2.09	2.50	2.44	2.29	2.37
Avg of ♂+♀	2.20	2.39	2.39	2.30	2.32

[1] Each bird received a total of 52.5 units.
[2] Antibiotic in ration at level of 8.8 mg of penicillin and 22.0 mg of chlortetracycline/kg of complete feed.

TABLE 7

Weight gains (g) and feed per unit gain of rats from zero to 8 weeks (exp. 5)

Treatment		
Control	Saline injection	Urease injection[1]
	Gains[2]	
158.00	161.00	162.00
	Feed/unit gain[2]	
3.85	3.66	3.59

[1] Each rat received a total of 30.0 units.
[2] Each group represents 17 rats.

somewhat erratic between replications, and indicate the importance of replications of experimental units.

The slight improvement in gain which results when antibiotics were included in the diet did not approach statistical significance. Since a response could be expected to dietary antibiotics if harmful bacteria were present, it appears possible to postulate that such bacteria were not present.

Experiment 5. Rat weight gains and feed conversion differences among treatment groups (table 7) did not approach statistical significance. The results of these experiments are in disagreement with those of Dang and Visek (2) and Visek and Dang (3).[8] Differences in source of injectable urease may explain the discrepancy. Dang and Visek (2) and Visek and Dang (3)[9] used a recrystallized urease preparation, in contrast with the highly concentrated, although not crystalline, preparation of urease used in these experiments.

Another explanation of lack of agreement could be differences in flora or environmental conditions between laboratories.

Because no differences in gain and feed efficiency were observed that could be ascribed to the urease treatments, no chemical or bacteriological studies were conducted on blood and gastrointestinal contents.

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[9] See footnote 8.

Effect of Alternate Depletion — Repletion on the Laboratory Rat

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ABSTRACT Rats alternately-fed large and small quantities of laboratory chow gained significantly more weight and survived longer than did control animals receiving the same total quantity of diet in equal daily increments. Tibial measurements and epididymal fat pad weights indicate that the greater weight gain is due to greater fat deposition, although neither group of animals was obese.

It has been amply demonstrated that obesity is associated with a decreased survival time in man (1). That this association may be one of cause and effect is indicated by the observation that weight reduction in an overweight group of human adults increased their mean survival time (2). It has generally been conceded that regularity in dietary habits is beneficial to man and therefore the value of a regimen of alternate weight reduction and repletion has been questioned, although many individuals find such a regimen a more practical means of weight control than one of steady restriction.

That the mode of eating is of profound importance in experimental animals has been demonstrated by the experiments of Cohn and Joseph (3). These investigators observed that rats given all their food in 2 meals per day had a much different body composition and biochemical make-up from those allowed to feed ad libitum, although both groups of animals consumed approximately equal quantities of food. Further, Cox et al. (4) have reported that the feeding of monkeys only once daily enhances the production of atherosclerosis. Similarly, Cohn et al. (5) observed that atheromatous plaques developed more rapidly and regressed more slowly in chickens given their food in meals than in those fed ad libitum.

Reported herein are observations made in the course of maintaining rats with an alternately depleting-repleting diet from weaning to death. Included also are some results obtained by similar treatment of animals over a shorter period of time.

EXPERIMENTAL

Forty male weanling rats of the Holtzman strain weighing approximately 50 g each were divided into 2 groups equal in weight and number. They were housed individually on screen and given free access to water. All animals received only commercial laboratory[1] chow throughout the experiment. One group, hereinafter referred to as the control group, was given a specific amount of food daily, the quantity of which was increased stepwise as the animals increased in size until on the 40th day of the experiment it had reached 15 g, after which it was continued at this level for the duration of the experiment. The second group referred to as the variably-fed group received for 5 days considerably less of the diet than the controls, for 2 days the same amount, for 5 days considerably more and for 2 days again the same amount. This cycle was repeated for the duration of the experiment. Thus, after the 40th day the cycle was as follows: 10 g for 5 days, 15 g for 2 days, 20 g for 5 days, 15 g for 2 days. All rats were weighed at each change in the cycle. In all cases the food was introduced into the cage once each day. By the 107th day of the experiment 4 of the variably-fed group and one of the control animals had died. No further deaths occurred until the 427th day. It was therefore concluded that the 5 deaths prior to 107 days were not due to the treatment regimen. Since 3 of the variably-fed group and 2 of the

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

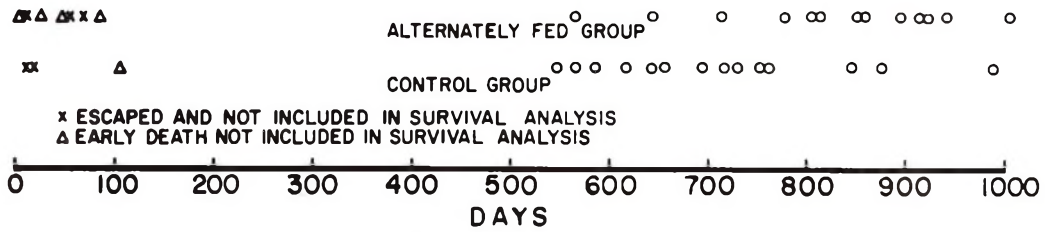

Fig. 1 Survival time of individual variably-fed and control rats.

controls escaped during the course of the experiment, the final evaluation is based upon data obtained with 13 variably-fed and 17 control animals.

In a later experiment involving 30 animals/group subjected to the same regimen, blood pressure determinations were made after 165 days by an indirect means using the foot. Systolic pressures were recorded from a photoelectric tensometer.[2] After 192 days the rats were bled from the heart under pentobarbital sodium[3] anesthesia and killed. Body length and rear tibial bone length were measured and the epididymal fat pads were weighed. Portions of the livers and epididymal fat pads were preserved in dry ice. Livers, prostates, testes, thymuses, adrenals, thyroids and pituitaries were weighed and sections were made for histological examination.

Plasma cholesterol was determined by the method of Abell et al. (6). The glucose-6-phosphate dehydrogenase content of the livers and fat pads was determined by a modification of the method of Glock and McLean (7).

RESULTS

In the first experiment after 60 days the variably-fed rats began to gain weight more rapidly than the control animals and a significant difference in weight was maintained until about the 750th day of the experiment at which time two-thirds of the controls had died. At 403 days when both groups had received the same total amount of food, and at which time no deaths had yet occurred due to treatment regimen, the average weights were 302 ± 6.8 and 254 ± 7.2 g, respectively, and this difference was highly significant ($P < 0.001$).

A second and more important difference between the 2 groups was that the variably-fed rats survived an average of 148 days longer than their controls, the mean survival time being 843 ± 35 and 695 ± 35 days, respectively. Survival times are shown in figure 1. Again this difference is highly significant ($P < 0.01$).

Since 4 variably-fed animals and only one control died early in the experiment, it might be that the 3 weakest animals were thus eliminated from the variably-fed group resulting in an erroneous apparent difference in survival times of the 2 groups. To avoid this criticism, the 3 shortest-lived animals were eliminated from the control group and the statistical evaluation was repeated. On this basis the difference in mean survival time was 110 days ($P = 0.03$).

The data obtained in the second experiment are recorded in table 1. They indicate that the highly significant greater weight gain of the variably-fed rats cannot be accounted for by greater skeletal growth as evidenced by lack of difference in tibial length, but by greater accumulation of fat as shown by the significantly greater weights of the epididymal fat pads. It has been previously demonstrated that the weight of this tissue is proportional to total body fat (8). Histological examination of the tissues revealed no differences between the 2 groups of animals.

DISCUSSION

The apparent greater deposition of fat in the variably-fed animals is probably explainable on the basis of the observations of Tepperman and Tepperman (9) that rats fed a high carbohydrate diet after a 48-hour fast have a greatly increased rate of lipogenesis. Cohn and Joseph (3) have also reported that the fat content of the carcasses of "meal" eating animals is greater than that of "nibbers" when food

[2] Metro Industries, Long Island City 6, New York.
[3] Nembutal, Abbott Laboratories, North Chicago, Illinois.

TABLE 1

Comparative data obtained from variably fed and control rats[1]

	Weight gain	Tibia length	Fat bodies	Liver	Prostate	Testes	Thymus
	g	*cm*	*g*	*g*	*mg*	*g*	*mg*
Variably-fed	291[2] ± 3.9[3]	4.02 ± 0.05	4.34[4] ± 0.17	11.3 ± 0.2	919 ± 62	3.44 ± 0.09	201 ± 24
Controls	271 ± 2.9	4.02 ± 0.06	3.63 ± 0.20	10.5 ± 0.2	810 ± 45	3.55 ± 0.06	197 ± 14

	Adrenal	Thyroid	Pituitary	Plasma cholesterol	Glucose-6-phosphate dehydrogenase		Systolic blood pressure
					Liver	Fat body	
	mg	*mg*	*mg*	*mg/100 ml*	*units/g tissue*		*mg/Hg*
Variably-fed	45.3 ± 1.2[3]	17.1 ± 0.9	12.1 ± 0.6	50.7 ± 4.0	1.1 ± 0.05	0.08 ± 0.04	105 ± 4.1
Controls	47.4 ± 1.2	16.2 ± 1.0	10.9 ± 0.5	49.5 ± 1.8	1.2 ± 0.1	0.03 ± 0.01	110 ± 3.2

[1] Duration of experiment, 192 days.
[2] Body weight gain of variably-fed animals is significantly greater than that of control animals ($P < 0.001$).
[3] SE of mean.
[4] Weight of fat pads of variably-fed animals is significantly greater than that of control animals ($P = 0.01$).

intake is identical. During the portion of the cycle when the variably-fed rats were receiving 10 g of diet daily they were on a one meal per day routine usually consuming their allotment in less than one hour, although somewhat unexpectedly, the rats of the control group often required no more than 3 hours to consume their daily allowance. At first thought, it was unexpected that no increase in glucose-6-phosphate dehydrogenase was observed in the livers of the variably-fed rats. It must be remembered, however, that the rate of excess fat accumulation is quite slow.

The most unexpected observation arising from this study is that the animals with the greater apparent deposition of fat had the greater survival times. This is contrary to the generally accepted concept of the effect of obesity on longevity, although it must be kept in mind that both groups were restricted in their food intake and neither could be considered to be obese.

It is possible, or even probable, that fat deposition under the conditions of these experiments is coincidental and unrelated to time of survival. One possibility is that the variably-fed animals are placed under recurring stress which in some manner enhances survival.

It is not known whether the control animals with lower body fat resulting from a food intake identical with that of the variably-fed animals are expending the extra energy as radiated heat or in the form of muscular activity. Since the daily accumulation of fat is quite small and therefore the amount of energy involved is correspondingly small, it would be difficult to determine this experimentally.

ACKNOWLEDGMENT

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Effects of Cholesterol and Cholic Acid Supplements on Rats Fed Low Iodine Diets[1]

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ABSTRACT The addition of cholesterol and cholic acid to a purified diet low in iodine fed to young female rats was found to increase the degree of thyroid enlargement caused by the iodine deficiency. The supplement accentuated the loss of stainable colloid and the degree of hyperplasia. The I^{131} uptake at 24 hours did not appear to be affected by the cholesterol-cholic acid supplementation, but the loss of I^{131} from the thyroid in the subsequent 5 days was increased. From data obtained with diets containing small amounts of additional iodate it appears that the iodine requirement was increased by the cholesterol-cholic acid supplements.

The importance of thyroid function in the regulation of cholesterol metabolism is well known. Hypothyroidism in man and animals is associated with hypercholesterolemia, whereas thyroxine administration lowers the serum cholesterol level in both the normal and hypothyroid states. The evidence that cholesterol, bile acids and dietary fat [4,5] might partially overcome the toxic effects of excessive thyroxine suggested that these materials might have some influence upon thyroid function or the metabolism of thyroxine. In some exploratory studies with rats a number of materials were added to low iodine diets similar to those described below. Since animals were killed at varying intervals, relatively few were available for comparison at any one time. Nevertheless, animals that received the low iodine diet containing 15% coconut oil during a 5-month period had thyroids with a mean weight of 26.2 mg, whereas the animals receiving this diet supplemented with cholesterol and cholic acid had thyroid glands with a mean weight of 44.0 mg, and a minimal degree of hyperplasia was apparent histologically. Iodine supplementation of the diet without cholesterol or cholic acid produced animals with mean thyroid weights of 18 mg. More definitive experiments are presented in this paper. These demonstrate that the addition of cholesterol and cholic acid to the low iodine diet caused distinct thyroid enlargement, epithelial hyperplasia, increased vascularity, and depletion of colloid as compared with those without such supplementation

EXPERIMENTAL

The low iodine diets had the following percentage composition: soy protein,[6] 20; glucose, 60.6; salt mixture, (Jones and Foster (1) salt mix with the potassium iodide omitted), 4; choline chloride, 0.2; methionine, 0.1; vitamin A-D-E mixture,[7] 0.1; water-soluble vitamin mixture,[8] 0.5; and fat, 15.0. In the first experiment the diets contained 0.1% percomorph oil and 0.2% α-tocopherol instead of the A-D-E mixture. The dietary oils used were coconut oil, olive oil, and safflower oil. When cholesterol and cholic acid were included in the diet, they were added at 1.0 and

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[5] Page, A. C., Jr., F. R. Koninszy, D. Wolf, P. Aldrich and K. Folkers 1956 Factors in liver reversing thyroid stress in rats. Federation Proc., 15: 568 (abstract).

[6] Promine R, Central Soya, Chicago.

[7] Contained one gram of vitamin A acetate, one gram of α-tocopherol and 5 mg of calciferol dissolved in 50 g of corn oil.

[8] Contained 200 mg thiamine; 400 mg riboflavin; 200 mg pyridoxine·HCl; 1.25 g Ca pantothenate; 2.0 g niacinamide; 50 mg folic acid; 5 mg menadione; 10 mg biotin; and 1.5 mg cobalamin in 250 g of glucose.

0.3%, respectively. Iodine analysis[9] of the soy protein, glucose, and salt mixture indicated that they contained 0.31, 0.40 and 0.18 ppm iodine, respectively. The dietary fats did not contain measurable amounts of iodine and are presumed to contain less than 0.05 ppm. Although the iodine content of the diet appears to be relatively high, the results demonstrate that it is sufficiently low to produce significant thyroid enlargement.

The animals used were young female rats from the Charles River Laboratories. They were housed in group cages containing 5 to 7 animals and fed the diets and tap water ad libitum. Before being killed, they were given an intraperitoneal injection of I^{131}, all animals in an experiment receiving the same dose. At intervals thereafter the radioactivity in the neck region over the thyroid was determined. In the first experiment this was made in a manner similar to that described by Fish et al. (2). The animals were anesthetized with pentobarbital sodium,[10] placed in a plastic funnel, and the probe of the scintillation counter placed directly on the funnel over the thyroid. In later studies, the animals were not anesthetized, but simply held with the neck firmly against the probe. Two determinations were always made, and if these did not show reasonable agreement, a third reading was taken. In the early studies a "background" count was made by placing the probe over the lower abdomen, and this value subtracted from the neck count. This measure did not appear to improve the accuracy of the neck count appreciably. The neck count is relatively inaccurate shortly after the injection of I^{131} because of the rather large amount of activity in tissues other than the thyroid, and it is difficult to select an appropriate area to evaluate the body background count.

Immediately after the last neck count the animals were killed with ether and the trachea, including the thyroid, was removed and placed in a tube containing 5 ml of 10% formalin. The tubes were counted in the well of the scintillation counter. The formalin fixed thyroids were

[9] Performed by the Wisconsin Alumni Research Laboratories, Madison, Wisconsin.

[10] Nembutal, Abbott Laboratories, North Chicago, Illinois.

TABLE 1

Effect of dietary fats and cholesterol-cholic acid supplementation

Diet	Mean body wt	Mean serum cholesterol	Mean thyroid weights		Mean neck count
			Total	Per 100 g body wt	
Experiment A (2 months)	*g*	*mg/100 ml*	*mg*	*mg*	*% at 5 days*
Safflower oil	272	89	22.6 ± 7.1[1]	8.42 ± 3.1[1]	27.5 ± 3.7[1]
Safflower oil + cholesterol + cholic acid	299	211	19.5 ± 4.9	6.46 ± 1.1	27.9 ± 6.6
Olive oil	321	73	24.3 ± 6.5	7.69 ± 2.5	31.8 ± 9.61
Olive oil + cholesterol + cholic acid	281	437	21.8 ± 3.3	7.79 ± 1.5	26.2 ± 7.9
Coconut oil	300	79	23.9 ± 5.2	8.11 ± 2.1	23.6 ± 8.0
Coconut oil + cholesterol + cholic acid	297	234	30.1 ± 8.0	10.15 ± 2.9	13.7 ± 4.7
Experiment B (3 months)					
Safflower oil	355	84	26.7 ± 5.0[2]	7.56 ± 1.7[2]	30.3 ± 9.5[2]
Safflower oil + cholesterol + cholic acid	377	187	45.6 ± 11.5	12.02 ± 2.9	15.8 ± 6.4
Olive oil	363	91	25.1 ± 4.5	6.95 ± 1.6	28.5 ± 7.8
Olive oil + cholesterol + cholic acid	351	552	33.5 ± 4.8	9.45 ± 3.0	23.4 ± 6.7
Coconut oil	404	97	27.3 ± 11.2[2]	6.78 ± 0.8[2]	27.2 ± 9.3[2]
Coconut oil + cholesterol + cholic acid	340	379	42.1 ± 2.8	12.50 ± 3.2	15.3 ± 10.9

[1] SD.

[2] Mean values are significantly different ($P < 0.05$).

dissected from the surrounding tissue, and weighed on a torsion balance. Paraffin-embedded sections, cut at a thickness of 5 μ, were stained with alcian blue-hematoxylin-eosin for histological examinations.

RESULTS

The design of the first experiment is apparent from table 1. These animals weighed approximately 80 g when starting to be fed the diet, which contained 0.1% percomorph oil instead of the A-D-E mixture. The 3 oils were fed at the dietary level of 15% with and without cholesterol-cholic acid supplementation. One-half of the animals were killed after 2 months and the remainder after another month on the diet. The cholesterol-cholic acid supplementation did not affect the body weight appreciably. The serum cholesterol values are consistent with a previous report (3), the olive oil producing considerably higher levels than coconut oil and safflower oil producing lower levels than the other 2 oils. The thyroid weights were not greatly different after 2 months. Those receiving cholesterol and cholic acid in the coconut oil diet had the largest mean weights and after 5 days, the lowest neck count. The latter are expressed as a percentage of the mean count at 24 hours, all groups having substantially the same count at 24 hours.

After 3 months, however, the thyroids of all groups receiving cholesterol and cholic acid were enlarged, significantly so, for the groups on the safflower and coconut oil diets when compared with the unsupplemented groups. This enlargement was associated with a lower neck count at 5 days. Although the correlation was not very good, there was a tendency for the animals with the largest thyroids to have the least I^{131} retention at 5 days. Since no difference in the neck counts of the various groups was apparent after 24 hours, the output of I^{131} from the enlarged thyroids appears to have been elevated.

In the second experiment the diet was made with coconut oil, 15%. Groups with and without the cholesterol-cholic acid supplement which received iodine supplementation were also included. The latter supplement provided 5 mg of potassium iodate/kg of diet. Thirteen animals which received each diet were divided into 2 cages containing 6 and 7 animals each. The average weight of the animals when given the experimental diets was 50 g and they continued to be fed the diets for 3 months before being killed. All animals received the same dose of I^{131}, approximately 3 μc. Neck counts were determined on 7 animals from each group after one, three and five days. The thyroids were removed on the fifth day and the radioactivity measured. Following fixation, the thyroids were dissected from other tissue and weighed.

The mean body weights, thyroid weights, thyroid I^{131} activity 5 days after injection and histologic gradings are shown in table 2. The thyroid glands of the animals receiving the basal diet were larger than those of rats that received the iodine supplement ($P < 0.001$). The animals receiving the cholesterol-cholic acid supplement without iodine were larger than

TABLE 2

Effects of cholesterol-cholic acid and iodine supplementation

	Supplement[1]	I^{131} at 5 days[2]	Mean body wt	Mean thyroid wt	Mean relative thyroid wt	Mean % of colloid	Mean epithelial cell height[3]	Mean degree vascularity[3]
		count × 10^{-3}	*g*	*mg*	*mg/100 g*			
1	None	113[4]	260	21.1[4]	8.1	3.5	1.2	2.8
2	Iodate	10.2	255	13.9	5.5	32.0	1.4	2.7
3	Cholesterol-cholic acid	54.4[4]	263	33.7[4]	12.8	1.1	4.0	4.3
4	Cholesterol-cholic acid-iodate	7.9	254	13.2	5.2	25.0	2.1	2.5

[1] Basal diet contained 15% coconut oil.
[2] Well counts determined on excised thyroid glands.
[3] Arbitrary counts, scale 1 to 5, visual grading.
[4] Cholesterol-cholic acid supplementation produced significant thyroid enlargement (1 vs. 3) $P < 0.001$ and significantly lower I^{131} in the thyroids, $P < 0.001$. Cholesterol-cholic acid supplementation had no significant effect on these determinants in the presence of the iodine supplement although histologically differences can be noted between the thyroid epithelial cell heights of groups 2 and 4.

those receiving the basal diet alone ($P < 0.001$). The cholesterol-cholic acid supplement had no apparent effect on thyroid size or I^{131} retention in the presence of the iodine supplement. The thyroid enlargement due to cholesterol-cholic acid feeding was associated with a lower retention of I^{131} in the glands 5 days after administration ($P < 0.001$). As shown in figure 1 this appears to be due to a higher rate of output rather than a low uptake since the neck counts at 24 hours were not significantly different. The slightly lower value at this time in the group with the cholesterol-cholic acid supplement is due largely to one animal with an abnormally low count.

The histologic preparations of the thyroids were examined objectively, the specimens being randomized and their identities unknown at the time. The following details were graded visually: percentage of follicles containing colloid apparent at low powers of magnification, intensity of colloid staining, epithelial cell height and degree of vascularity. The last 3 categories were designated on an arbitrary scale of 1 to 5 plus. This range of values represented the complete spectrum from minimum to maximum (for each characteristic being analyzed) present in the total sample. Examples of the various degrees of magnitude were selected after several rapid examinations of the entire population and were then used as reference standards for the definitive grading. In the second experiment (table 2) these criteria indicated that the degree of iodine deficiency achieved by the basal diet was sufficient to produce clear-cut evidence of colloid depletion consistent with the increased I^{131} uptake of these glands. There was a distinct decrease in both the amount of stainable colloid as well as in the intensity of colloid staining in the unsupplemented animals and with virtually no overlap when compared with those receiving added iodine (figs. 2 and 3). No differences in epithelial cell height or vascularity were apparent despite the distinct ($P = 0.001$), though modest, increase in thyroid weights of the unsupplemented rats.

The addition of cholesterol and cholic acid to the basal diet produced a further, almost total, loss of stainable colloid and

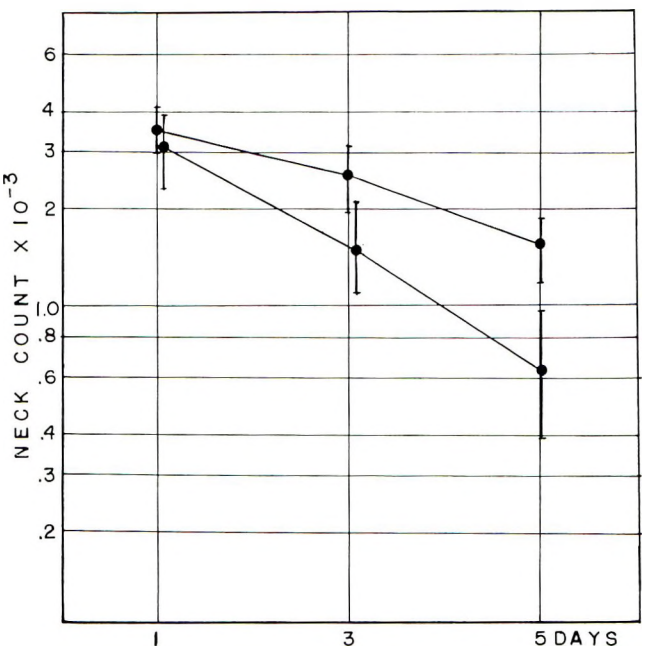

Fig. 1 Basal diet contained 15% coconut oil. Neck counts at 1, 3, and 5 days after I^{131} administration. Upper curve represents animals fed the iodine-deficient diet; lower curve the same diet supplemented with cholesterol and cholic acid. Bars indicate standard deviations.

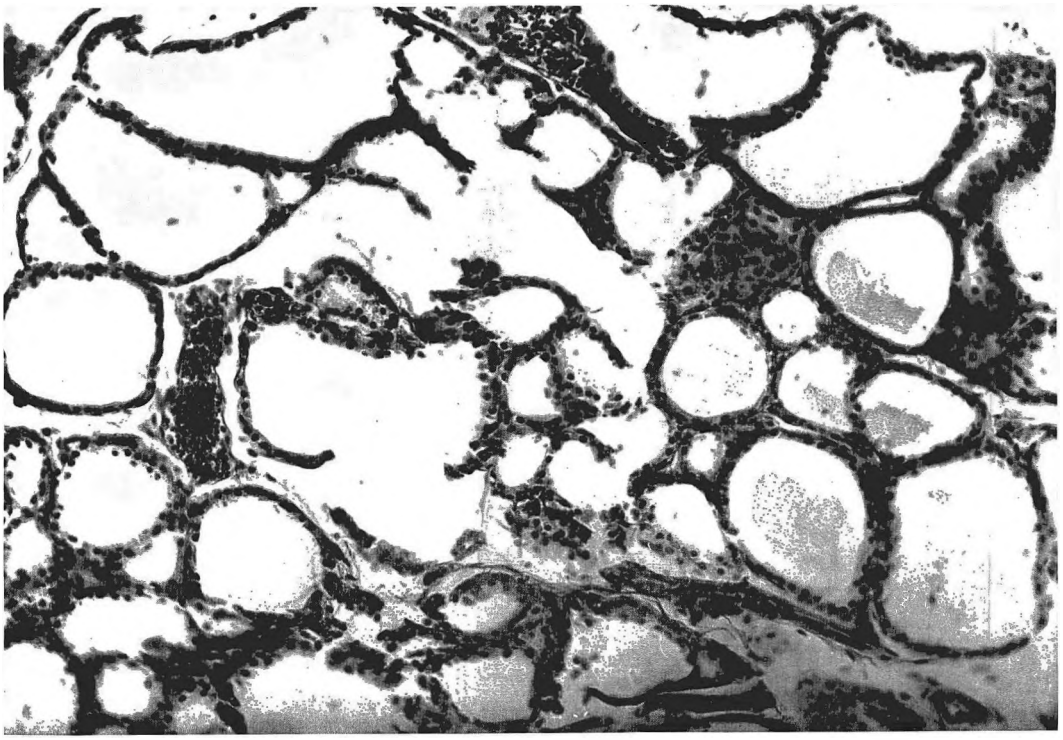

Fig. 2 Thyroid gland of rat maintained with basal diet, low in iodine. Basal diet here and in following figures contained 15% coconut oil. There is loss of colloid and a reduction in the intensity of staining of that colloid remaining when compared with glands from iodine-supplemented animals as in figure 3. In this animal, which is representative of its group, roughly 5% of the follicles contain colloid apparent at low powers of magnification. Alcian blue, H & E. × 160.

definite evidence of hyperplasia in all animals as judged by increases in epithelial cell heights and vascularity (fig. 4). Cell height estimates showed no overlapping of values and those of degree of vascularity, virtually none when compared with values for the unsupplemented group. These distinctive histologic changes are reflected in the highly significant changes ($P = 0.001$) in thyroid weight and iodine retention.

Although the addition of cholesterol and cholic acid to the iodine-supplemented animals produced no apparent effect on gland weight or iodine retention, there were histologic variations from the control group suggesting the possibility of early hyperplastic changes. Thus, the intensity of colloid staining was appreciably lessened in the cholesterol-supplemented group and in one-half of these animals there were scattered foci of follicles with heightened epithelium. In one rat this increase in cell height was generalized (fig. 5).

As to the histological findings in the first experiment, in general, there was a direct correlation between thyroid weight and evidence of hyperplasia. Thus, at 2 months some degree of hyperplasia was present, although no group differences could be detected. At 3 months, however, hyperplastic changes were more pronounced and differences were apparent in those groups receiving safflower and coconut oils. Among these animals, those receiving the cholesterol-cholic acid supplements showed more extensive hyperplasia than the unsupplemented groups. These differences were quite well defined with but little overlapping of values and were reproducible on repeated gradings. In the case of olive oil, however, no consistent differences could be observed between the supplemented animals and their controls.

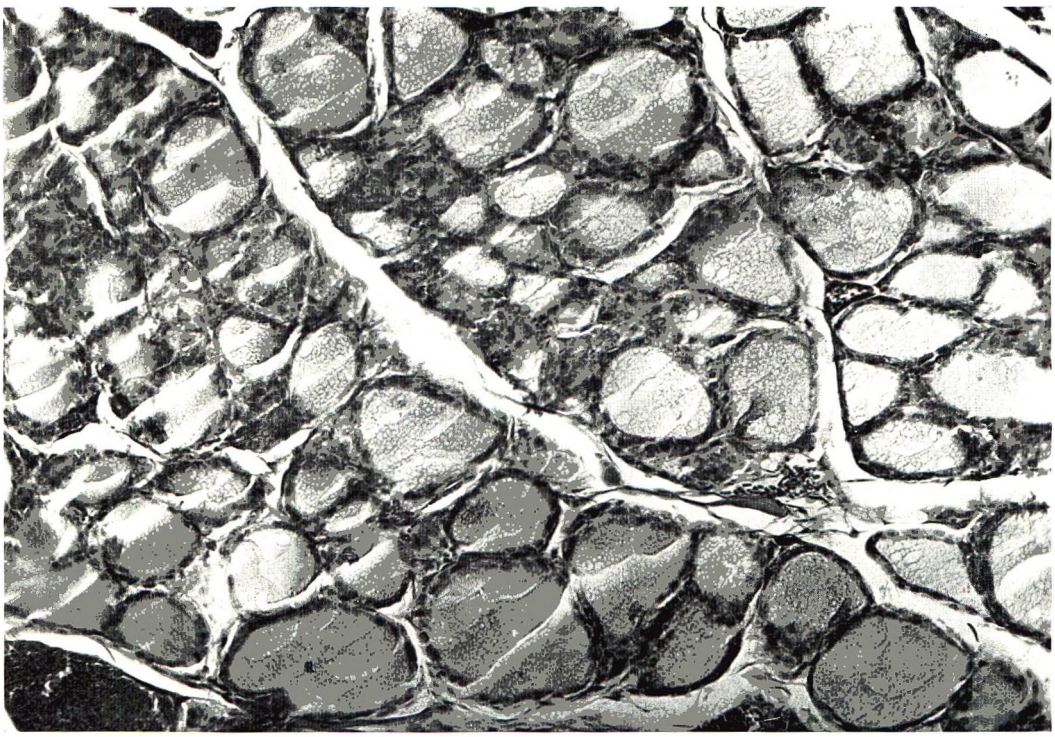

Fig. 3 Thyroid gland of rat maintained with basal diet supplemented with iodine. Approximately 30% of the follicles contain a moderately intensely staining colloid. The range of epithelial cell heights noted in control animals is apparent. The follicles below showing a flat epithelium are from the periphery of the gland, whereas those above are from the interior of the gland. These central follicles are somewhat smaller and the epithelium slightly more colloidal. Alcian blue, H & E. × 160.

DISCUSSION

It is clear from the results presented that the diet used was sufficiently low in iodine to produce a moderate degree of thyroid enlargement and histological change during a 2- or 3-month period. The further addition of cholesterol and cholic acid caused considerably more thyroid enlargement and accentuated the histological changes. The addition of a rather low level of potassium iodate to the diet, 5 mg/kg, supplying 3 mg iodine/kg of diet, prevented the appearance of the abnormalities observed with the basal diet and largely abolished those noted with the cholesterol-cholic acid-supplemented diet, histologic evidence alone indicating some degree of hyperplasia in the latter instance. It thus appears that the addition of these supplements probably increased the iodine requirement of the animals that received these diets. Although higher levels of iodine were not fed with the cholesterol-cholic acid supplements in these experiments, other data[11] show that with 24 mg of iodine/kg diet, as supplied by our usual dietary level of 4% salts IV (4), cholesterol-cholic acid supplementation is completely without effect upon the female rat thyroid. It is likely therefore, that the level of iodate supplied in the present experiment was adequate for the animals receiving the basal diet, but probably slightly inadequate for those which received cholesterol and cholic acid supplements.

Since the completion of the above experiments, certain distinct sex-oriented differences in the morphology of the rat thyroid have been recognized.[12] Thus, in the Charles River strain (CD) of white rat, maintained with a control synthetic diet based on 20% casein and containing 24

[11] Unpublished data, S. B. Andrus and L. C. Fillios.
[12] See footnote 11.

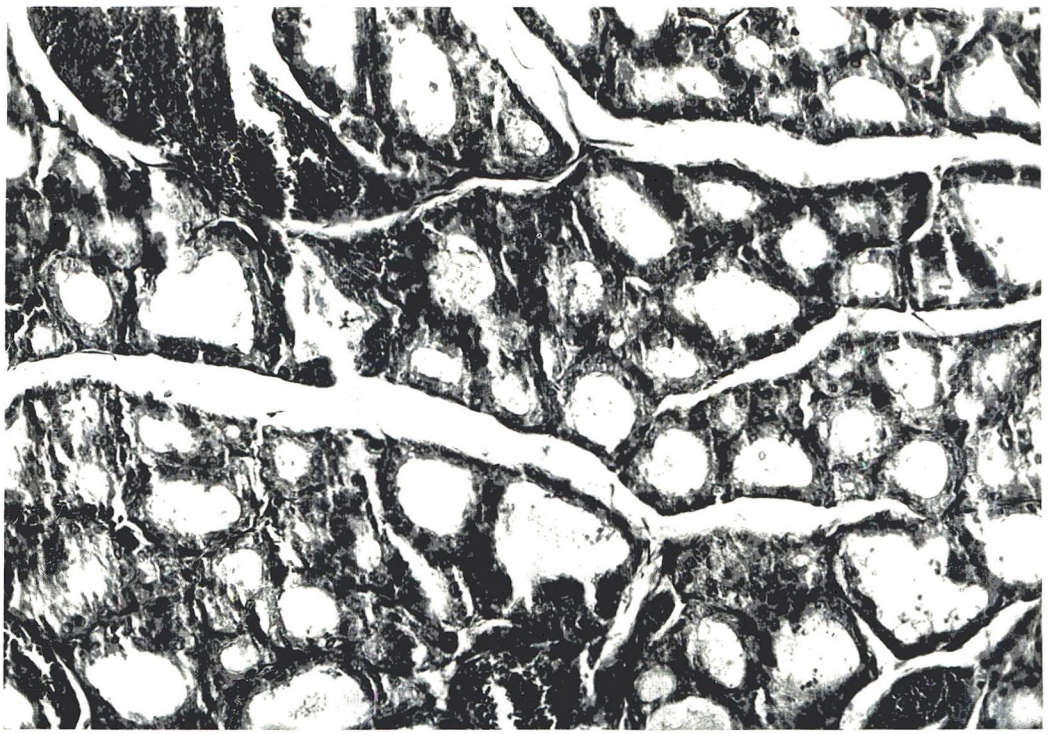

Fig. 4 Thyroid gland from a rat receiving cholesterol and cholic acid but no iodine supplements. There is frank hyperplasia with a uniform increase in epithelial cell height and distinctly increased vascularity. The follicles appear consistently smaller than heretofore, and contain even less colloid (estimated at 1%) than the glands from animals receiving only the basal diet as in figure 2. Alcian blue, H & E. × 160.

mg of iodine/kg diet, the follicular epithelial cells of the male thyroid are higher and more active in appearance than in the female and the amount of colloid storage distinctly less. This differential has been noted in rats maintained with different diets with a varied fat content and appears to be independent of the iodine content of the diet. Thus, while iodine deficiency produces hyperplasia in both male and female rats, the changes are distinctly more pronounced in the former. Similarly, it has been noted that when cholesterol and cholic acid are added to the control synthetic diet containing 24 mg of iodine/kg diet, histologic evidence of hyperplasia (without significant increases in thyroid gland weight) are present in the males and absent in the females. It is unfortunate that in the present study only one sex was used.

Since the completion of this study we have become aware of the report of Bernick and Patek (5) in which hyperplastic changes are observed in male rats when a natural diet[13] is supplemented with 1% cholesterol and 5% cottonseed oil. In the absence of thyroid weights and other quantitative data and in view of the obvious dietary differences between that experiment and the present one, it is difficult to compare the results of the 2 experiments. The thyroid changes reported by the above authors occurred unusually rapidly (as early as 2 weeks) and were accompanied by increased numbers of thyrotropic cells in the anterior hypophysis.

In the first experiment the animals with the most severe hypercholesterolemia, those that received the diet containing olive oil, showed less thyroid enlargement than those receiving either safflower oil or coconut oil. These differences were not statistically significant with the number of ani-

[13] Allied Mills, Inc., Chicago.

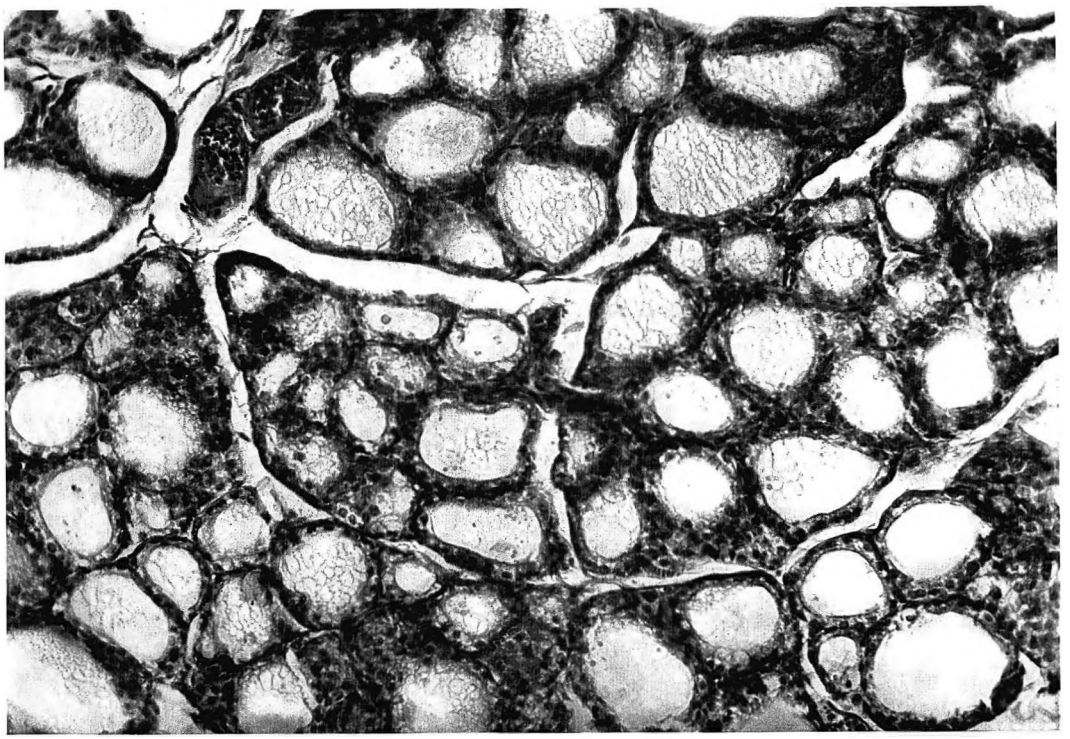

Fig. 5 Thyroid gland from a rat receiving supplements of cholesterol, cholic acid, and iodine. The height of the epithelial cells appears somewhat increased over that noted in iodine-supplemented control animals, figure 3, and the colloid less intensely stained. In this particular animal the epithelial change is uniform throughout the glands, whereas in one-half of the group such changes were present focally. Alcian blue, H & E. × 160.

mals available. The evidence does not, therefore, demonstrate an effect of the dietary oil, although this requires further study. If there is any relationship between the degree of thyroid enlargement and the serum cholesterol level, it would appear to be quite clear that the diets producing the highest serum cholesterol level do not produce the most thyroid change.

As stated above, the measure of the I^{131} uptake of the thyroid obtained by placing the probe over the neck is relatively less accurate shortly after I^{131} administration because of the activity in other tissues. None of our studies to date indicate a significant difference in I^{131} uptake due to cholesterol-cholic acid supplementation. This conclusion should probably be accepted with some reservation in view of the method used. On the other hand, either "neck counts" or the total thyroid count obtained by placing the thyroid in the well of the scintillation counter clearly demonstrate a lower I^{131} retention 5 days after I^{131} administration. Thus, it appears that cholesterol-cholic acid supplementation accelerates the removal of iodine-containing materials from the thyroid gland. It is possible that this may be mediated by an increased excretion of thyroxine from the body and thus explain the apparent increased iodine need with the cholesterol-cholic acid supplemented diet. Such an action would be consistent with the effects of these materials on thyroxine toxicity (see Westerfeld (6)), which may be due to an increased rate of thyroxine excretion from the body.

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Nutrition and Biochemistry of Survival During Newcastle Disease Virus Infection

I. LIVER NUCLEIC ACID, PROTEIN AND LIPID PATTERNS IN CHICKS[1]

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ABSTRACT Liver size and content of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein and lipids were observed in 580 White Leghorn cockerel chicks one to 7 weeks of age. Comparisons were made of the effects of age and Newcastle disease virus (NDV) infection at 2 levels of virulence. Biochemical changes per gram of liver tissue in infected birds were related to the stage of the disease cycle as follows: Incubation of the virus: Increased liver weight and DNA. RNA was depressed, with no apparent change in protein and lipids. Active involvement of virus: Reduced liver weight. DNA and RNA increased, with no apparent effect of the infection on protein and lipids. Initiation of recovery of the host (19 days post inoculation): No differences between treatments for DNA, RNA, protein and lipids, indicating that metabolic processes of the infected bird had normalized. Total quantities of liver DNA, RNA, protein and lipids were significantly less in the NDV-infected birds as compared with controls. This phenomenon was hypothesized to be a function of body size. Calculations made of a phase of protein metabolism termed "protein efficiency of cellular RNA" were postulated as being part of the defense mechanism. Serum HI antibody titers were highest in birds with the greatest degree of involvement.

Previous reports from our laboratories have shown that diet may influence the course of a Newcastle disease virus (NDV) infection in susceptible immature cockerels. Chicks deficient in vitamin A had a higher rate and greater terminal mortality than birds with adequate reserves of this dietary essential (1). Greater growth depression was observed in infected chicks given balanced rations than in birds fed diets imbalanced by a deficiency of lysine (2).

Sanslone and Squibb (3) demonstrated that with the virulence of an NDV inoculum adjusted to yield 50% mortality, nitrogen retention was increased during the incubation stage of the virus and decreased in the active involvement stage. However, when an immunizing level of inoculum was used, nitrogen retention increased following inoculation and stayed at a higher level than controls until the 11th day post-inoculation. The increase in nitrogen retention was interpreted as having a possible relationship to defense mechanisms. A similar increase in nitrogen retention was observed by Panda et al. (4) during the 14th week of a coccidiosis infection in chickens.

The experiments reported here were designed to obtain preliminary base line data on levels of liver nucleic acids, protein and lipids in normal chicks during the first 6 weeks of age and in 4- to 6-week-old-birds subjected to an NDV infection at 2 levels of virulence.

METHODS AND RESULTS

In each experiment White Leghorn cockerels of known breeding were maintained in all-wire batteries in rooms air conditioned to 22°C. Feed and water were supplied ad libitum. The same reference basal diet was provided all chicks from day of hatch until the end of the trials. This diet, which produces normal growth, contained: (in per cent) ground wheat, 63.185; soybean oil meal (50%), 30; fish meal (60%), 2.5; salt mixture, including minor elements (1), 4.2; DL-methionine, 0.1; ribo-

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flavin concentrate,[2] 0.005; vitamin D_3, 0.01 (3000 ICU/g); and vitamin A, 6000 IU/kg of diet.

Procedures for chemical analyses were the same for all experiments. Liver samples were stored in a freezer under conditions previously determined to be satisfactory for the preservation of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), protein and lipids. The analyses were not begun until samples for an entire experiment were collected. Due to large numbers, and to equalize possible experimental error, samples of an experiment were analyzed in lots each of which contained an equal representation of the different treatments and sampling periods.

The livers were homogenized under refrigeration in cold distilled water. The RNA and DNA were determined by modifications of the Schmidt-Thannhauser procedure as suggested by Hutchison and Munro (5); liver protein by the Biuret technique which was standardized by the Kjeldahl procedure; and lipids by chloroform, petroleum- and diethyl-ether extractions of the homogenate, the solvent extracts taken to dryness and weighed.

For a high level of virulence (50% mortality) the chicks were inoculated intramuscularly with a 10^{-1} concentration of an "H" strain of NDV, and with a 10^{-7} concentration when an immunizing level was desired. This particular strain of NDV has been described and characterized as to its effect on dietary intake, efficiency of feed utilization, water consumption, growth, mortality, body temperatures, and other metabolic phenomena (1, 3, 6).

Antibody titers were determined on the sera of the NDV-infected chicks by the hemagglutination-inhibition (HI) test according to the method outlined by Cunningham (7).

All data were statistically treated using analysis of variance, with separation of sums of squares for individual degrees of freedom and orthogonal comparisons carried out according to Snedecor (8).

Experiment 1. The object of this trial was to establish patterns of liver nucleic acids, protein and lipids in normal control chicks fed the reference basal diet from day of hatch to 6 weeks of age. At weekly intervals 10 chicks were selected at random from a group of 70, weighed and killed; the livers were removed, weighed and analyzed for the previously described components.

As shown in table 1, there was a highly significant linear ($< 1\%$) increase in body and liver weights of the growing chicks. Liver size as a function of body weight decreased from 4.5 to 2.8% by the third week of age. From 3 to 6 weeks of age the slope was less abrupt, dropping from 2.8 to 2.4%. Liver lipids were highest (61 mg/g of liver) in the 1- and 2-week-old chicks and thereafter ranged from 35 to 58 mg/g of liver. Liver DNA was highest from 1 to 3 weeks of age and irregular, but tended to level off in the 4- to 6-week-old bird. Liver RNA and protein were lowest during the first 2 weeks, increasing significantly ($< 1\%$) from the 3rd to 6th week. Liver RNA in terms of DNA was lowest during

[2] BY-24, Commercial Solvents Corporation, New York.

TABLE 1

Effect of age on size, lipid and nucleic acid content of the livers of White Leghorn cockerel chicks

Age	Body wt[1]	Liver wt[1]	Liver as % of body wt	DNA	RNA[2]	Protein[2]	Lipids	RNA/DNA	Lipids/DNA	Protein efficiency[3]
weeks	*g*	*g*		*mg/g liver*		*mg/g liver*			*mg/g liver*	
1	71	3.2	4.5	1.92	9.0	160	60.9	4.69	31.7	34.1
2	129	5.5	4.3	1.59	9.2	161	60.8	5.79	38.2	27.8
3	200	5.5	2.8	2.01	10.5	174	54.7	5.22	27.2	33.3
4	320	8.6	2.7	1.60	11.4	185	34.5	7.13	21.6	25.9
5	432	10.8	2.5	1.67	11.2	195	58.1	6.71	34.8	29.1
6	556	13.4	2.4	1.70	10.4	190	45.1	6.12	26.5	31.0

[1] Linear component highly significant $< 1\%$.

[2] Significant rise ($< 1\%$) 3 to 6 weeks.

[3] Protein efficiency of cellular RNA = $\frac{\text{liver protein}}{\text{RNA/DNA}}$.

the first 3 weeks. Protein efficiency of cellular RNA[3] was highest and irregular during the first 3 weeks, dropping to 25.9 on the 4th week and then increasing linearly with age to the 6th week. Liver lipids/DNA showed no definite trend.

Experiment 2. This experiment, replicated on different dates, was designed to study the effect of a virulent NDV infection (10^{-1} concentration) on liver size, moisture content, nucleic acids, protein and lipids of 4- to 6-week-old cockerels. For each replicate a total of 200 chicks was reared with the basal diet to 4 weeks of age and then assigned to 2 treatment groups: 1) noninfected controls which remained in the same room, and 2) chicks to be inoculated; the latter were transferred to an isolated disease room. Twenty birds were sampled at random from the treatment groups of each replicate at 2, 5 and 19 days following inoculation, intervals calculated to correspond to the midpoints of the incubation and active involvement periods of the virus, and the end of the initiation of recovery of the chick (1). After serum samples were obtained the birds were killed, the livers removed and treated as previously described.

The effects of this level of NDV infection on liver constituents are summarized here under specific periods of the NDV cycle. Since statistical treatment of the data indicated no significant differences between replicates the values were averaged.

Incubation of the NDV (2 days post-inoculation). Liver weight of infected birds increased significantly ($< 2\%$) over that of the control chicks (fig. 1). The weight increase was other than water since determination of the moisture content indicated the control livers contained 70.3% moisture and the NDV-infected livers 69.3% moisture. Serum HI antibody titers were less than 80 units. Liver DNA of the infected chicks (table 2) increased significantly ($< 5\%$) over that of the controls both on a per gram of tissue and total liver basis ($< 1\%$). The RNA per gram of liver decreased significantly ($< 1\%$) in

[3] Defined, for the results reported herein, by the following formula:

$$\text{Protein efficiency} = \frac{\text{liver protein}}{\text{RNA/DNA}}$$

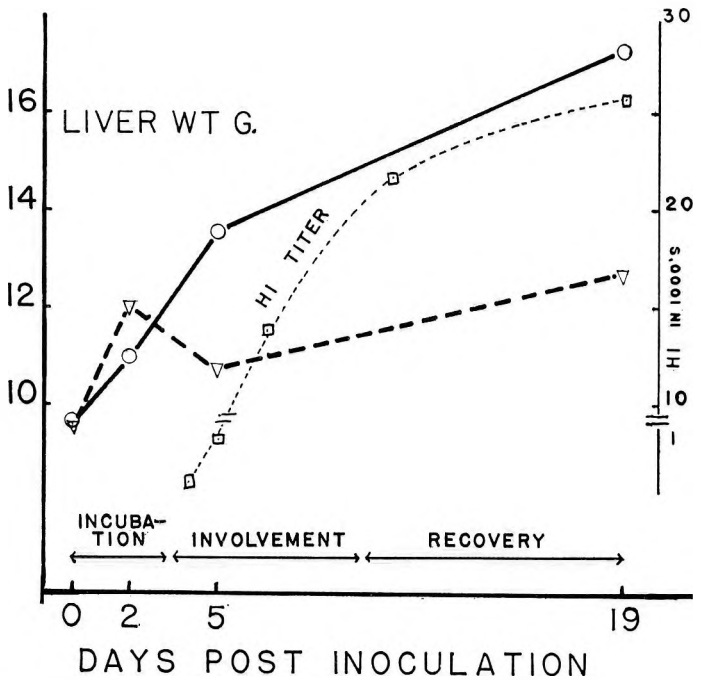

Fig. 1 Liver weights and antibody titers of chicks infected with 10^{-1} concentration of Newcastle disease virus. Solid line indicates noninfected chicks; dotted line NDV-infected.

TABLE 2

Effect of virulent level of Newcastle disease virus (NDV) infection on the liver nucleic acids, protein and lipids of 4 to 6 week old chicks

Liver components[1]	Days post-inoculation					
	2		5		19	
	C[2]	NDV[2]	C	NDV	C	NDV
	Milligrams per gram liver					
DNA	1.76	2.02*	2.05	2.45**	2.32	2.33
RNA	15.0	13.4**	14.4	15.1*	14.1	13.8
Protein	200	193	197	209	212	220
Lipids	34.6	36.1	33.3	34.9	33.6	32.8
RNA/DNA	8.52	6.63	7.02	6.16	6.08	5.92
Lipids/DNA	19.7	17.9	16.2	14.2	14.5	14.1
Protein efficiency[3]	23.5	29.1	28.1	33.9	34.9	37.2
	Milligrams per total liver					
DNA	19.2	24.3**	28.1	25.5*	39.8	29.4**
RNA	165	161	195	160**	244	175**
Protein	2207	2296	2665	2219**	3672	2775**
Lipids	388	434	454	363**	583	413**

[1] Each analysis represents 35 to 40 chicks.

[2] C = control; NDV = infected.

[3] Protein efficiency of cellular RNA = $\frac{\text{liver protein}}{\text{RNA/DNA}}$.

** Significant at 1% level between controls and infected.

* Significant at 5% level between controls and infected.

the infected birds; on the basis of total RNA no differences were noted between groups. Liver protein and lipids were not affected by the infection. Ratios of RNA and lipids to DNA decreased in the infected birds. Protein efficiency of cellular RNA, however, increased.

Active involvement stage of the virus (5 days post-inoculation). There was a significant ($< 1\%$) loss in liver weight (fig. 1) in the infected chicks compared with controls, a loss which was not due to moisture. Serum HI antibody titer rose to approximately 20,000 units. The DNA per gram of liver increased significantly ($< 1\%$) over that of the controls (table 2); on a total liver basis DNA decreased significantly ($< 5\%$). There was a significant ($< 5\%$) increase of RNA per gram of tissue in the infected chicks; total liver RNA, however, decreased significantly ($< 1\%$). Liver protein and lipids were not affected on the basis of per gram of tissue, but total quantities decreased significantly ($< 1\%$). Ratios of RNA and lipids to DNA were lowered by the NDV; on the other hand, protein efficiency of cellular RNA increased.

Initiation of recovery of the host (19 days post-inoculation). Liver weight of the infected birds increased in relation to body size. Serum HI antibody titers (fig. 1) increased from 20,000 to 25,000 units. Liver DNA (table 2) per gram of tissue in the infected chicks was the same as the controls. Total liver DNA, however, was significantly ($< 1\%$) less in the infected birds. There were no differences between treatments in RNA per gram of tissue but total RNA was significantly ($< 1\%$) less in the infected chicks. Protein and lipids per gram of liver were essentially the same for control and infected birds but total quantities were significantly ($< 1\%$) reduced in the infected chicks. Ratios of RNA and lipids to DNA were similar between treatments but protein efficiency of cellular RNA was elevated.

Experiment 3. This trial was designed to provide data on the effect of an NDV infection of low virulence (10^{-7}, immunizing) on liver size, nucleic acids, protein and lipids. One hundred and fifty cockerels were reared to 4 weeks of age with the basal ration and then divided into 2 treatment groups: 1) controls, and 2) those to be infected. The management of the birds was the same as in experiment 2 but the NDV inoculum was set at 10^{-7}, an immunizing dosage. At 4, 7 and 11 days post-inoculation, 22 birds were selected randomly from each group. These inter-

TABLE 3

Effect of a Newcastle disease virus (NDV) immunization of chicks on liver nucleic acids, protein, lipids and antibody titers

Liver components[1]	Days post-inoculation					
	4		7		11	
	C[2]	NDV[2]	C	NDV	C	NDV
Liver weight, g	11.0	10.6	11.9	11.4	12.5	9.9**
DNA, mg/g liver	1.90	1.98	1.87	2.02	1.95	2.76**
RNA, mg/g liver	14.0	13.6	14.1	13.2*	14.7	15.2
Protein, mg/g liver	207	209	207	196	225	221
Lipids, mg/g liver	38.4	38.0	38.8	—	33.8	34.7
Protein efficiency[3]	28.1	30.4	27.5	30.0	29.8	40.1
Hemagglutination-inhibition, units		80		160		14,100

[1] Each analysis represents 20 to 22 chicks.

[2] C = control; NDV = infected.

[3] Protein efficiency of cellular RNA = $\frac{\text{liver protein}}{\text{RNA/DNA}}$.

** Significant at 1% level between controls and infected.

* Significant at 5% level between controls and infected.

vals coincided with the end of the incubation and active involvement stages of the virus, and the midpoint of initiation of recovery of the host. Serum samples were collected, the birds killed, the livers removed, weighed and analyzed.

As shown in table 3, liver weight was not significantly ($< 1\%$) decreased in the infected chicks until 11 days after inoculation. In the controls, DNA followed the pattern noted in experiments 1 and 2, whereas in the infected birds there was a gradual linear increase of DNA and at 11 days post-inoculation there was a highly significant ($< 1\%$) difference between treatments. The RNA decreased at 7 days ($< 5\%$) post-inoculation, but increased slightly above the controls at 11 days. Protein and lipids showed no differences between treatments. The protein efficiency of cellular RNA of the infected birds was again increased over that of the controls during all stages of the disease cycle. On the 7th day post-inoculation serum HI antibody titers averaged 160 units and increased to 14,100 units by the 11th day.

DISCUSSION

In experiment 1, determining the effect of age on liver weight, nucleic acids, protein and lipids in normal cockerel chicks provided a pattern for comparing changes in levels of these liver components during stress of an NDV infection. The observations on the relation of liver size of normal chicks to body weight are in accord with those of Marion and Edwards (9). For the liver components analyzed, greatest variation occurred during the first 4 weeks of age, a time when the chick apparently is normalizing its metabolic processes. From 4 to 6 weeks of age, levels of liver DNA, RNA, protein and lipids did not change significantly. However, protein efficiency of cellular RNA increased linearly. Similar results for this age period were observed in the control chicks of experiments 2 and 3.

Earlier observations from our laboratories indicated that the biochemistry of an NDV infection must be assessed not only in terms of the stage of the disease cycle but also the extent of the virulence of the infection. It is recognized that in attempting to correlate biochemical changes in tissue to the animal's ability to survive an infection, differences from the norm must not be considered a reflection in toto of the defense mechanism; such changes also may be interactions both independent of and caused by the infection per se. For example, an infection may limit diet intake, which in turn affects metabolism. Or a biochemical change may be the direct result of abnormal metabolism due to an accumulated toxicity. In the trials reported here, 2 levels of virulence were used to overcome the confounding effect of a reduction in diet intake due to NDV. Moreover, the infection was studied over an ex-

tended period to allow the possibility of interactions.

With a virulent infection, liver size was increased during incubation and then depressed in the active involvement stage of the virus. Although the increase in liver size cannot be attributed to diet intake or moisture content, it coincided with an increase in nitrogen retention, a phenomenon believed to be part of the defense mechanism (3). With an immunizing level of NDV there was no difference in liver size between treatments until the 11th day post inoculation; livers of infected birds were then significantly smaller than controls. Again, this phenomenon correlated with an increased nitrogen retention in chicks given an immunizing level of NVD (3).

The high level of infection increased liver DNA at 2 and 5 days post-inoculation, whereas with the immunizing dosage a significant increase of DNA was not observed until 11 days after inoculation. These observations were to be expected if DNA is a cellular constant (10), since histological examination[4] of the liver tissue of the trials reported herein indicated an increased number of liver cells.

With a virulent infection RNA decreased during incubation and increased during the active involvement period. A low level of virulence produced a similar depression and elevation of RNA, but here the effect was delayed for several days. Diet was not involved in these changes since feed intake is not affected during the course of a low level of infection or during the incubation stage of the virus in a virulent infection (1, 3). It may be postulated that these changes, therefore, are a part of the animal's defense mechanism. Viral RNA, until shown otherwise, must be considered as a probable contributor to any elevation of this liver component. Liver protein ($N \times 6.25$) and lipids were not significantly affected at either level of virulence.

Within the periods observed, antibody HI titers showed a relationship to the degree of virulence of the infection, since lower titers were obtained with the immunizing level of virus. However, the height of the HI titer increase apparently is independent of the various liver constituents studied and of mortality (11).

When liver RNA and lipids of the chicks subjected to a virulent infection (experiment 2) were calculated in terms of DNA per gram of tissue (table 2), all were reduced during the incubation and active involvement stages of the disease but were the same as the noninfected controls 19 days after inoculation.

The depression of total liver DNA, RNA, protein and lipids noted in the NDV-infected birds in experiment 2 could be interpreted as running counter to defense mechanisms. However, when protein efficiency of cellular RNA was calculated, there was little doubt that the NDV-infected cell was making protein in excess of growth requirement. Furthermore, 2 distinct phenomena were also present 19 days after inoculation which would support this belief. First, with a high level of virulence there were no differences, on a per gram of tissue basis, between control and infected birds in the liver components studied. These observations indicate that at this time the metabolism of the bird surviving the infection had normalized. Second, there was the possibility that the highly significant decrease of total liver DNA, RNA, protein and lipids was a function of body size. For example, NDV-infected birds, after passing through the active involvement stage of the virus, will recover with a slope of growth greater than that of noninfected controls (2). Such birds, if not paralyzed, soon become indistinguishable from noninfected controls.[5] This phenomenon is an apparent attempt on the part of the bird to re-attain its genetic potential for size. If the results of experiment 2, therefore, are re-calculated in light of this dimension, then total quantities of liver DNA, RNA, protein and lipids were in excess during the active involvement stage of the virus and almost equal to that of the control birds 19 days after inoculation.

ACKNOWLEDGMENTS

The chemical analyses were performed by Marie Utzinger, and Harry Veros was responsible for the antibody titers and care and management of the birds.

[4] Performed by Dr. Henry Siegel, Office of the Chief Medical Examiner, City of New York.
[5] Unpublished data.

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Effect of Diet on the Uptake of Carbon-14-labeled Acetate, Glucose and Leucine into Lipids of the Chick[1,2]

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ABSTRACT Two trials were conducted in which chicks were fed to 2 weeks of age the following diets: (1) low protein, low fat; (2) high protein, low fat; (3) low protein plus 8% of corn oil; (4) high protein plus 8% of corn oil. These trials were conducted to compare the synthesis of lipids in the liver from acetate, glucose and leucine, and to study the effect of dietary protein and fat on the incorporation of acetate and leucine into lipids of the liver, plasma and adipose tissue. Increasing the level of dietary protein and adding corn oil to the diet each decreased the uptake of C^{14}-labeled acetate and glucose into liver lipids. The uptake of L-leucine-2-C^{14} activity into liver lipids was decreased by adding corn oil to the diet, with the greatest decrease occurring at a low level of diet protein. This apparent "sparing" of protein was related to the growth response of chicks to corn oil at different protein levels. It was concluded from these studies that a high level of protein and fat is necessary in the diet of chicks to maintain a low level of liver and plasma lipids, and to obtain a rapid transport and deposition of lipids in storage sites in the body of the chick.

The effect of dietary protein on the synthesis of lipids in chicks has been studied by Nishida et al. (1) and others (2). These workers concluded that the rate of incorporation of labeled acetate into fatty acids and possibly cholesterol of the liver was accelerated by feeding a low protein diet. The latter workers also showed that the presence of corn oil in the diet decreased the uptake of acetate into liver lipids, and that the effect of both dietary protein and fat on lipid metabolism was more pronounced during the very early growth period of chicks. It was also concluded that a high level of dietary protein and fat was necessary for maximal growth rate and for regulating the level and synthesis of liver lipids in chicks.

The present study was designed to investigate the influence of dietary protein and fat levels on: 1) the synthesis of lipids from acetate, glucose and leucine in the chick's liver, and 2) the incorporation of acetate and leucine into lipids of the liver, plasma and adipose tissue. This was accomplished by raising chicks to 2 weeks of age with diets containing different protein and fat levels, and then dosing them with C^{14}-labeled acetate, glucose or L-leucine.

EXPERIMENTAL

Two trials were conducted in this study with a total of 192 female Athens-Canadian Randombred chicks. All chicks were maintained from one day to two weeks of age in heated battery brooders and given feed and water ad libitum. In each trial 2 groups of 12 chicks were fed one of the 4 following diets: 1) low protein, low fat; 2) high protein, low fat; 3) low protein plus 8% of corn oil; 4) high protein plus 8% of corn oil. These diets were composed of isolated soybean protein, glucose monohydrate, cellulose, amino acids, vitamins and minerals. The diets were of the same composition as those reported by Marion and Edwards (2) except that the level of corn oil was increased from 5 to 8% in the present study. The low and high protein diets used in the previous study contained by analysis 14.4 and 24.1% protein (N × 6.25), respectively.

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The experimental diets were formulated to contain the same level of metabolizable energy for chicks.

In the first trial, all chicks were weighed at 2 weeks. Twelve chicks from each diet treatment having body weights nearest the mean body weight for that treatment were distributed randomly into 3 lots of 4 each. Chicks appearing to be in poor health were not included in these lots. Chicks were not fasted prior to dosing since it has been shown by Feigenbaum and Fisher[4] that feed restriction will greatly influence fatty acid metabolism in the chick's liver. Each lot of 4 chicks from each diet treatment was injected intraperitoneally with either 12 μc of sodium acetate-1-C^{14}, specific activity 10.0 mc/mmole; 5 μc of D-glucose-U-C^{14}, specific activity 31.0 mc/mmole; or 5 μc of L-leucine-2-C^{14}, specific activity 1.11 mc/mmole.[5] Thirty minutes later the chicks were killed and the livers excised, weighed, frozen in liquid air and stored at −10°C until analyzed. Analysis consisted of thawing, homogenizing, freeze-drying and extracting the livers under reflux for 24 hours with 30 to 60° bp petroleum ether. The extracted lipids were fractionated into major lipid fractions by the method of Hirsch and Ahrens (3). Aliquots of each fraction were counted in a low-background beta counter and corrections made for self absorption.

Trial 2 was conducted in the same manner as the first trial except that 6 chicks from each dietary treatment were injected intraperitoneally with either acetate-1-C^{14} or L-leucine-2-C^{14}. Also, blood samples were obtained in heparinized syringes by heart puncture, and adipose tissue samples were excised from the abdominal cavity; this adipose tissue was in the posterior ventral area next to the skin. The samples of plasma (obtained by centrifuging blood samples) adipose tissue and liver samples were analyzed in the same manner as the liver samples in trial 1 except that the radioactivity was determined only on the total lipids extracted from each of these sites.

In both trials, statistical significance was determined for most measurements by the analysis of variance and the multiple range test of Duncan (4).

RESULTS

Body weights were increased and feed conversion was improved by raising the protein level and by adding corn oil to the diet in both trials (tables 1 and 2). Also, the weight, dry matter and lipid content of the livers were lower when either high protein or corn oil diets were fed.

The fractionation of liver lipids into the major lipid classes by silicic acid chromatography showed clearly that either the addition of corn oil to the diet or an increase in dietary protein significantly lowered the levels of free cholesterol, cholesterol esters and triglycerides in the liver (table 1). However, the influence of added protein and corn oil on these lipid components appeared to be additive in reducing the triglyceride fraction.

The effect of diet on the incorporation of labeled acetate, glucose and leucine into the lipid fractions of chick livers is shown for trial 1 in table 3. Added dietary protein and fat appeared to decrease the uptake of labeled acetate into the lipid fractions that contain fatty acids, whereas no consistent differences in the uptake of this compound into cholesterol were noted. Apparently, the same effect of diet on the incorporation of labeled glucose into lipid fractions did not occur since glucose uptake into both fractions containing the cholesterol and fatty acids appeared to be depressed by added protein and fat. In general, the effect of the dietary variables on leucine uptake into liver lipids was similar to that on acetate uptake. However, the addition of corn oil to the diet resulted in a much greater reduction of leucine activity in liver lipids at the low rather than the high level of dietary protein.

The incorporation of labeled carbon from acetate into the lipids of the liver, plasma and adipose tissue, and leucine uptake into the lipids of the liver and plasma are shown for trial 2 in table 4. When expressed as a percentage of intraperitoneal dose, the total uptake of acetate and leucine activity into liver lipids showed the same trend due to diet treat-

[4] Feigenbaum, A. S., and H. Fisher 1961 Fatty acid metabolism in the liver following starvation. Federation Proc., 20: 366 (abstract).

[5] The C^{14}-labeled compounds were obtained from the California Corporation for Biochemical Research, Los Angeles.

TABLE 1

Effect of diet on body weights and liver measurements (trial 1)

Protein, % Corn oil, %	14.4 0	24.1 0	14.4 8	24.1 8
14-Day body weight, g[1]	121[a]	147[b]	137[c]	161[d]
Feed consumed/wt gained	2.02	1.78	1.82	1.59
Liver wt, % of body wt	4.94[a]	4.02[b]	3.35[b]	3.57[b]
Liver dry matter, %	32.4[a]	30.2[b]	29.8[b]	29.4[b]
Liver lipids, % of dry matter	24.8[a]	13.1[b]	12.8[b]	10.0[b]
Liver lipid fractions[2]				
Cholesterol	29[a]	14[b]	20[b]	18[b]
Cholesterol esters	39[a]	24[b]	17[b]	19[b]
Triglycerides	203[a]	86[b]	64[c]	45[d]
Diglycerides	20	10	6	3
Monoglycerides	16	11	7	7
Phospholipids	48[a]	40[a]	26[b]	43[a]

[1] Values for each measurement having different superscript letters are significantly different ($P < 0.05$).
[2] Lipid fractions expressed in milligrams per liver.

TABLE 2

Effect of diet on body weights, and liver and plasma measurements (trial 2)

Protein, % Corn oil, %	14.4 0	24.1 0	14.4 8	24.1 8
14-Day body wt, g[1]	122[a]	152[b]	143[b]	178[c]
Feed consumed/wt gained	2.36	1.70	2.03	1.50
Liver wt, % of body wt	3.49[a]	3.00[b]	2.52[c]	2.46[c]
Liver dry matter, %	31.8[a]	30.1[b]	29.4[c]	29.1[d]
Liver lipids, % of dry matter	26.0[a]	15.1[b]	14.6[bc]	12.2[c]
Plasma lipids, mg/100 ml	160[a]	121[a]	411[b]	84[a]

[1] Values for each measurement having different superscript letters are significantly different ($P < 0.05$).

TABLE 3

Influence of diet on the uptake of acetate-1-C^{14}, glucose-U-C^{14} and leucine-2-C^{14} into liver lipid fractions (trial 1)

Protein, % Corn oil, %	14.4 0	24.1 0	14.4 8	24.1 8
C^{14} of acetate in[1,2]				
Cholesterol	0.55	0.62	0.78	0.41
Cholesterol esters	1.68	0.78	1.25	0.62
Glycerides	8.91[a]	5.51[ab]	7.48[ab]	1.93[b]
Phospholipids	2.23[a]	0.99[ab]	0.04[b]	0.04[b]
Total	13.37[a]	7.90[ab]	9.55[ab]	2.99[b]
C^{14} of glucose in[1,2]				
Cholesterol	1.43[a]	0.88[ab]	0.26[b]	0.11[b]
Cholesterol esters	0.97[ab]	1.28[a]	0.53[ab]	0.17[b]
Glycerides	13.89[a]	9.18[ab]	2.42[ab]	0.95[b]
Phospholipids	1.78	1.42	0.87	0.81
Total	18.07[a]	12.76[ab]	4.08[b]	2.04[b]
C^{14} of leucine in[2,3]				
Cholesterol	0.9	1.2	0.5	2.1
Cholesterol esters	1.4	5.0	0.6	1.8
Glycerides	24.1[a]	20.4[a]	5.7[b]	4.3[b]
Phospholipids	25.6[a]	12.6[b]	6.6[b]	10.2[b]
Total	52.0[a]	39.2[ab]	13.4[b]	18.4[b]

[1] Expressed as a percentage of intraperitoneal dose observed in each fraction 30 minutes after dosing.
[2] Values for each measurement having different superscript letters are significantly different ($P < 0.05$).
[3] Expressed as a per cent $\times 10^{-2}$ of intraperitoneal dose observed in each fraction 30 minutes after dosing.

TABLE 4

Influence of diet on the uptake of acetate-1-C^{14} and leucine-2-C^{14} into lipids of the liver, plasma and adipose tissue (trial 2)

Protein, % Corn oil, %	14.4 0	24.1 0	14.4 8	24.1 8
C^{14} of acetate in[1]				
Liver, % of ip dose	16.61[a]	13.40[ab]	8.40[ab]	5.20[b]
Liver, count/min/mg fat	81.7	152.2	174.9	116.2
Plasma, count/min/100 ml	12,405[a]	13,185[a]	25,868[b]	2,261[a]
Plasma, count/min/mg fat	65.4	87.8	68.6	34.9
Adipose, count/min/g lipid	1,385	8,107	3,665	12,796
C^{14} of leucine in[1]				
Liver, % $\times 10^2$ of ip dose	41.6	27.4	25.4	36.3
Liver, count/min/g fat	413	539	517	861
Plasma, count/min/100 ml	127	127	138	37
Plasma, count/min/g fat	781[a]	1,465[b]	432[a]	359[a]

[1] Values for each measurement having different superscript letters are significantly different ($P < 0.05$).

ment as noted in trial 1. However, treatment differences in leucine uptake were not statistically significant. When expressed in counts per minute per 100 ml of plasma, acetate uptake into plasma lipids of chicks fed the low protein, corn oil diet was significantly higher than that observed with the other diets. This same trend was also noted with leucine-labeling in the plasma. The specific activity values for acetate and leucine in the liver, plasma and adipose tissue were calculated and are presented in table 4 for comparative purposes. These values show no significant differences due to dietary treatment except for the significantly higher leucine uptake in the plasma of chicks that received the high protein, low fat diet.

DISCUSSION

The increased body weight gains, improved feed conversion, and the lower weight, dry matter and lipid content of livers due to high protein or corn oil feeding in the present study have been reported previously (5) when similar dietary variables were used. The larger number of observations taken on each of these measurements during the study reported here would account for statistical significance being noted in some instances where no statistically significant differences were detected in our previous study.

The differences noted in the uptake of acetate and glucose activity into liver lipid fractions demonstrate that the metabolism of labeled acetate cannot be considered as completely indicative of the amount of carbohydrates that are metabolized to lipids in the chick's liver. The most pronounced difference in glucose and acetate distribution into the lipid fractions due to diet treatment occurred in the phospholipid fraction. The reduction of activity in the phospholipid fraction due to feeding corn oil was not as pronounced with glucose as with labeled acetate. Possibly this was due to glucose serving as a source of glycerol in phospholipids, a role that acetate might not readily assume.

The lack of effect of dietary protein on the incorporation of acetate into liver cholesterol does not agree with previous observations in our laboratory (2) nor with the results of Nishida et al. (1). A low protein level in the previous studies tended to accelerate the uptake of acetate into liver cholesterol. However, a direct comparison cannot be made between the studies since the age of the chicks at the time of dosing with acetate was different from each of these studies.

When leucine is catabolized, the number 2 carbon atom appears in the carboxyl group of acetyl coenzyme A. Acetyl coenzyme A is known to be the basic component for fatty acid synthesis in animals. Therefore, the conversion of the C^{14} of leucine-2-C^{14} to fat is an *indicator* of the fate of protein in the liver. The feeding of corn oil appeared to reduce the conversion of protein, as indicated by leucine-2-C^{14} metabolism, to fat, and this reduction was greater at the low level of dietary protein.

Therefore, when corn oil was added to the low protein diet, more protein was available for the formation and maintenance of body tissues. This would explain the reason for the previous observation (6, 7) that the growth response of 3- or 4-week-old chicks to high levels of added corn oil was greater at a low rather than a high level of protein.

Havel and Goldfien (8) have presented evidence indicating that the triglycerides of the plasma of dogs are either synthesized by the liver from lipid precursors or formed from free fatty acids that were transported from adipose tissue. Presumably the fatty acids from adipose tissue are from exogenous and endogenous sources (see review (9)). The circulating neutral fat in the plasma is available for storage in adipose tissue or for oxidation in numerous tissues. If we accept this general scheme for the synthesis, transport and deposition of neutral fat, certain speculations can be made when the data of both trials of the present study are considered together.

The accumulation of lipids in the liver and the higher uptake of acetate into liver lipids due to feeding low protein or low fat diets to chicks are probably due to either: 1) an increased hepatic synthesis of fatty acids and possibly cholesterol; 2) an impaired transfer of lipids from the liver; 3) an interference with the removal of plasma lipids to other tissues; or 4) any combination of these processes.

A deficiency of protein in dogs due to lipid nephrosis has been shown by Marsh and Drabkin (10) to result in an increased hepatic synthesis of β-lipoprotein and a "striking" hyperlipemia. A high removal rate of lipids from the liver would explain why the high level of plasma lipids and the high acetate uptake into plasma lipids were noted when the low protein, corn oil diet was fed in trial 2. Apparently the high removal rate of lipids from the liver into the plasma was accompanied by a high rate of lipid deposition in adipose tissue only when high protein diets were fed. There appeared to be a relationship between dietary protein and dietary fat in that the lowest level of liver and plasma lipids, the lowest uptake of acetate into liver and plasma lipids, and the highest acetate incorporation into adipose tissue were noted with the high protein, corn oil diet. Also, the specific activity data for acetate in the liver, plasma and adipose tissue support the hypothesis that high protein and corn oil feeding act mainly by accelerating the transfer and deposition of lipids rather than suppressing the synthesis of lipids in the liver.

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Interrelations among Magnesium, Vitamin B_6, Sulfur and Phosphorus in the Formation of Kidney Stones in the Rat[1]

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ABSTRACT Dietary levels of magnesium, sulfur and phosphorus have been shown to affect markedly the excretion of a variety of urinary constituents and to influence the deposition of renal calcium oxalate in vitamin B_6-deficient rats. Diets high in magnesium or low in sulfur-protected rats against stone formation. The protective effect of high magnesium diets was interfered with when the diets were also low in phosphorus even though there was a decrease in the hyperoxaluria associated with vitamin B_6 deficiency. Apatite nephrocalcinosis was observed in rats receiving high phosphorus diets containing vitamin B_6 and 0.04% of magnesium. In vitamin B_6-deficient rats receiving diets high in phosphorus and containing 0.4% magnesium, renal calculi composed of calcium oxalate and calcium phosphate were formed.

Reports from this laboratory have shown that vitamin B_6 deficiency is associated with hyperoxaluria and formation of renal calcium oxalate calculi (1,2). Protection against stone formation without affecting the urinary oxalate level was achieved by increasing the level of dietary magnesium from 40 to 400 mg/100 g of diet (3). This protection was thought to be partially due to increased citric acid excretion obtained by high dietary magnesium. Studies reported here of the effect of high levels of dietary magnesium on the excretion of various urinary metabolites other than oxalates and citrates in vitamin B_6-deficient and control rats were conducted. The observation that high levels of dietary magnesium resulted in significant decreases in urinary levels of inorganic sulfates and phosphates resulted in a study of the effect of different levels of dietary sulfur and phosphorus on the production of renal stones and on the excretion of various urinary metabolites in vitamin B_6-deficient rats.

EXPERIMENTAL AND RESULTS

Male weanling Charles River CD rats were used. The basal diet contained: (in per cent) sucrose, 72.7; casein, 15; glycine, 3; corn oil, 4; cod liver oil, 1; choline, 0.3; and salts IV (4), 4. This diet contained 0.04% of magnesium. The diets were supplemented with: (in milligrams) thiamine, 4; riboflavin, 8; niacin, 40; Ca pantothenate, 20; folic acid, 1; menadione, 1; biotin, 0.2; and vitamin B_{12} 0.1 per kilogram of diet. When used, 4 mg of pyridoxine·HCl were added per kilogram of diet. Diets containing 0.4% magnesium were prepared by adding 3.6 g of magnesium in the form of magnesium oxide per kilogram of the basal diet. Varying the levels of dietary sulfur or phosphorus was achieved by manipulating salts IV and will be described in connection with the relevant experiments.

After the animals had been fed the diets for 3 weeks, 3 successive 48-hour urine collections were made by placing 3 or 6 rats in metabolic cages which provide continuous access to water and food. A 1.2 × 1.2-cm wire mesh was placed on the top of the food to minimize diet spillage. Toluene was used as a urine preservative.

The urine was analyzed for oxalic acid (5), citric acid (6), mucoprotein (7), inorganic sulfates (8), inorganic phosphates (9), magnesium (10), and calcium (11). To avoid magnesium interference, calcium in the urine of rats fed the high levels of magnesium was precipitated as the oxalate. The washed precipitate was ashed as

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described by Sobel and Sobel (11). The ash was dissolved in water with the HCl and the calcium determined by the complexometric method of Yarbro and Golby (12). At the end of feeding the experimental diets for 6 weeks, the urinary tracts of the rats were examined *in situ* under a dissecting microscope for the presence of any abnormality, particularly crystalline deposits.

Effect of dietary magnesium and vitamin B_6 on the composition of rat urine. The data shown in table 1 demonstrate that in rats receiving vitamin B_6 the feeding of high levels of magnesium resulted in a significant increase in citrate and magnesium excretion and a significant decrease in the urinary excretion of inorganic sulfates and phosphates. There were no statistically significant effects on the urinary pH or the excretion of oxalic acid, mucoprotein and calcium.

In vitamin B_6-deficient animals, high levels of dietary magnesium resulted in a significant increase in citrate, magnesium and calcium excretion. Oxalic acid excretion and pH were not significantly affected. There were significant decreases in urinary mucoprotein, inorganic sulfates, and inorganic phosphates.

These data also show that irrespective of the dietary magnesium level used, vitamin B_6 deficiency resulted in a significant increase in oxalate excretion and a decrease in urinary calcium, citrate and mucoproteins. Urinary tract examinations after the diets had been consumed for 6 weeks revealed stones only in vitamin B_6-deficient animals fed 40 mg of magnesium/100 g of diet. All of these animals had stones. Weight changes observed were similar to those previously reported (3).

Effect of dietary sulfur and vitamin B_6 on the composition of rat urine. Two levels of dietary sulfur were used. The low sulfur diet was the basal diet with the magnesium sulfate of salts IV replaced by magnesium carbonate to supply the same amount of magnesium (0.04%). This diet contained 89 mg of sulfur/100 g from casein. One hundred grams of the normal basal diet contained 145 mg of sulfur, 89 mg of sulfur from the casein and the rest from inorganic sulfates. Four groups of animals, 6 in the vitamin B_6-deficient groups and 3 in the control groups, were fed these diets.

The data in table 2 demonstrate that in rats receiving vitamin B_6 the low levels of dietary sulfur resulted in a significant de-

TABLE 1

Urinary metabolites in rats receiving two levels of magnesium and vitamin B_6

	With vitamin B_6		Significance of difference	Without vitamin B_6		Significance of difference
	0.04% Mg	0.4% Mg		0.04% Mg	0.4% Mg	
	mg/100-g rat/48 hr[1]			*mg/100-g rat/48 hr*[1]		
Oxalic acid	0.7 ± 0.03[2]	0.6 ± 0.04		5.3 ± 0.4	5.3 ± 0.4	
Citric acid	58.3 ± 6.7	94.4 ± 5.7	$P < 0.002$	8.1 ± 1.2	62.2 ± 4.7	$P < 0.001$
Mucoprotein	18.7 ± 4.5	13.4 ± 1.6		10.9 ± 0.2	7.4 ± 0.9	$P < 0.05$
Inorganic sulfate as S	8.5 ± 0.1	4.2 ± 0.2	$P < 0.001$	9.1 ± 0.9	6.5 ± 0.2	$P < 0.05$
Inorganic phosphate as P	13.9 ± 0.7	6.3 ± 0.3	$P < 0.001$	15.4 ± 1.7	7.7 ± 0.7	$P < 0.02$
Magnesium	3.0 ± 0.1	17.8 ± 1.9	$P < 0.01$	2.9 ± 0.4	17.1 ± 1.2	$P < 0.01$
Calcium	2.6 ± 0.5	4.6 ± 0.6		0.5 ± 0.0	1.18 ± 0.1	$P < 0.02$
pH	5.7 ± 0.2	5.9 ± 0.2		6.2 ± 0.1	6.2 ± 0.1	
Avg wt gain, g/6 weeks	208 ± 8	166 ± 3		9 ± 3	29 ± 4	

[1] Each value is the mean of determinations made on 3 successive 48-hour collections made of the pooled urine of 9 rats.
[2] SE.

TABLE 2

Effect of dietary sulfur on excretion of various urinary metabolites in rats and on formation of renal stones

Diet	Oxalic acid	Citric acid	Inorganic sulfate as S	Inorganic phosphate as P	Mg	Ca	pH	Animals with renal stones
	mg/100-g rat/48 hr[1]			*mg/100-g rat/48 hr*[1]				
With vitamin B_6, low S	0.3 ± 0.03**[2,3]	14.2 ± 3.9*	0.0***	9.0 ± 0.8**	1.2 ± 0.1**	0.5 ± 0.1***	6.0 ± 0.1	0/3
With vitamin B_6, normal S	0.7 ± 0.1	36.3 ± 5.3	8.8 ± 0.2	15.7 ± 0.7	3.2 ± 0.5	2.9 ± 0.1	6.0 ± 0.2	0/3
Without vitamin B_6, low S	2.3 ± 0.2*	12.4 ± 0.8	0.14 ± 0.1***	7.8 ± 0.1**	0.4 ± 0.05**	0.2 ± 0.02	7.0 ± 0.2	1/6
Without vitamin B_6, normal S	3.7 ± 0.3	9.3 ± 2.3	4.8 ± 0.3	10.5 ± 0.5	1.7 ± 0.1	0.1 ± 0.02	6.4 ± 0.0	4/6

[1] Each value is the mean of determinations made on 3 successive pooled 48-hour urine collections.

[2] SE.

[3] Asterisks indicate statistical significance when compared with the normal sulfur diet containing same level of vitamin B_6.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

crease in the excretion of all the urinary metabolites measured. This may have been in part the result of lower food consumption by the animals receiving the low sulfur diet. However, food consumption was not different in the vitamin B_6-deficient animals fed the 2 levels of sulfur, and in these rats, those fed the low sulfur diet excreted significantly less urinary oxalate, sulfate, phosphate, and magnesium than the others.

In vitamin B_6-deficient animals fed the normal level of sulfur, renal calcium oxalate monohydrate stones were detected in 4 of 6 animals, whereas in vitamin B_6-deficient animals fed the low level of sulfur, stones were found only in one of 6 animals. This protective effect was confirmed in another experiment in which 2 groups of 12 animals were fed vitamin B_6-deficient diets with low or normal sulfur levels. When the animals were killed after 6 weeks, renal stones were found in 50% of the animals fed the low sulfur diet and in 90% of the animals fed the normal sulfur diet. Moreover, in the animals fed the low sulfur diet, the average size of stones was 0.18 mm^3, whereas the average size of the stones from rats fed the normal sulfur diet was 0.59 mm^3.

Effect of dietary phosphorus, magnesium and vitamin B_6 on the excretion of urinary metabolites. Three levels of dietary phosphorus were used. The normal phosphorus diet was the basal diet which contains 398 mg of phosphorus/100 g diet, 115 g from the casein, the rest from salts IV. The low phosphorus diet contained 200 mg of phosphorus/100 g, one-half the phosphorus content of the normal phosphorus diet. This was achieved by replacing the dibasic potassium phosphate in salts IV by potassium carbonate to supply the equivalent amount of potassium ions. In addition, 1.2 g of monobasic ammonium phosphate were added per kilogram of diet to bring the phosphorus level to 200 mg/100 g of diet. The high phosphorus diet, containing 800 mg of phosphorus/100 g was prepared by adding 15 g of monobasic ammonium phosphate per kilogram of the regular phosphorus diet. All of these diets contained 0.55% calcium. Twelve groups of animals were used in this study. The animals were

TABLE 3

Effect of various levels of phosphorus on urinary metabolites in rats receiving 0.04% magnesium

Diet	Oxalic acid	Citric acid	Inorganic sulfate as S	Inorganic phosphate as P	Mg	Ca	pH
	mg/100-g rat/48 hr[1]			*mg/100-g rat/48 hr*[1]			
With vitamin B_6, low P	0.2 ± 0.1[2]	55.1 ± 10.4	5.8 ± 0.3** [3]	0.3 ± 0.0**	2.9 ± 0.7	18.4 ± 1.0***	5.6 ± 0.4
With vitamin B_6, normal P	0.4 ± 0.1	44.9 ± 4.8	11.5 ± 0.9	20.5 ± 2.5	1.4 ± 0.3	3.9 ± 0.7	6.3 ± 0.2
With vitamin B_6, high P	0.5 ± 0.1	10.9 ± 3.9**	11.7 ± 0.1	70.6 ± 1.6***	0.9 ± 0.1	0.9 ± 0.2*	5.2 ± 0.4
Without vitamin B_6, low P	0.8 ± 0.1***	42.5 ± 4.4**	6.9 ± 0.1	0.8 ± 0.3***	2.1 ± 0.5	1.9 ± 0.4*	6.0 ± 0.2
Without vitamin B_6, normal P	3.7 ± 0.2	7.5 ± 1.8	6.2 ± 0.3	10.9 ± 0.8	1.2 ± 0.1	0.14 ± 0.4	6.1 ± 0.1
Without vitamin B_6, high P	3.2 ± 0.2	6.0 ± 2.7	7.4 ± 0.1*	37.8 ± 3.3**	1.0 ± 0.2	0.1 ± 0.04	5.6 ± 0.2

[1] Each value is the mean of determinations made on 3 successive pooled 48-hour urine collections.
[2] SE.
[3] Asterisks indicate statistical significance when compared with normal phosphorus diet containing same level of vitamin B_6.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

TABLE 4

Effect of various levels of phosphorus on urinary metabolites in rats receiving 0.4% magnesium

Diet	Oxalic acid	Citric acid	Inorganic sulfate as S	Inorganic phosphate as P	Mg	Ca	pH
	mg/100-g rat/48 hr[1]			*mg/100-g rat/48 hr*[1]			
With vitamin B_6, low P	0.7 ± 0.1* [2,3]	180.6 ± 7.4*	4.3 ± 0.3	0.3 ± 0.1**	23.1 ± 2.7	30.5 ± 1.7***	7.5 ± 0.2**
With vitamin B_6, normal P	0.4 ± 0.1	127.3 ± 11.9	4.3 ± 0.3	6.8 ± 0.8	15.6 ± 1.7	3.4 ± 0.3	5.8 ± 0.0
With vitamin B_6, high P	0.5 ± 0.04	55.6 ± 5.4**	6.3 ± 0.5*	42.5 ± 2.0***	8.6 ± 1.1*	2.4 ± 0.4	5.3 ± 0.00***
Without vitamin B_6, low P	0.7 ± 0.3**	80.5 ± 8.4	4.5 ± 0.3	0.2 ± 0.0	13.7 ± 0.7	7.9 ± 1.8*	7.9 ± 0.0*
Without vitamin B_6, normal P	3.9 ± 0.2	66.2 ± 4.5	3.9 ± 0.3	2.4 ± 0.8	12.7 ± 1.7	0.6 ± 0.2	6.6 ± 0.3
Without vitamin B_6, high P	2.7 ± 0.5	29.9 ± 7.9*	3.2 ± 0.5	19.5 ± 5.0*	6.2 ± 2.2	0.4 ± 0.1	5.3 ± 0.1*

[1] Each value is the mean of determinations made on 3 successive pooled 48-hour urine collections.
[2] SE.
[3] Asterisks indicate statistical significance when compared with the normal phosphorus diet containing same level of vitamin B_6.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

fed diets containing 0.04 or 0.4% of magnesium and one of the 3 levels of phosphorus. The vitamin B_6-deficient groups contained 10 animals and the groups receiving vitamin B_6 contained 6 rats.

The excretion of various metabolites by rats in these groups is summarized in tables 3 and 4. These data show that as dietary phosphorus is decreased, there is a decrease in urinary phosphates and an increase in urinary citrates and calcium unrelated to the vitamin B_6 or magnesium content of the diets used in these experiments. In vitamin B_6-deficient rats receiving both levels of magnesium, a marked decrease in oxalate excretion was observed when the low phosphorus diets were fed. In rats receiving vitamin B_6 and either level of magnesium, urinary sulfate decreased as dietary phosphorus was decreased. Animals receiving the high magnesium diets with or without vitamin B_6 showed a decrease in urinary pH and magnesium excretion as dietary phosphorus was increased.

Table 5 summarizes the incidence of stone formation in the vitamin B_6-deficient rats receiving various levels of magnesium and phosphorus. Renal oxalate stones composed of calcium oxalate monohydrate were produced in vitamin B_6-deficient rats fed diets containing 0.4% magnesium and all levels of phosphorus. The effect of high dietary magnesium in protecting against renal stone formation in vitamin B_6-deficient rats was interfered with when low or high levels of dietary phosphorus were fed. In rats fed the low phosphorus diet renal stones composed of a mixture of calcium oxalate monohydrate and dihydrate were formed. In those fed the high phosphorus diet, stones composed of a mixture of calcium oxalate monohydrate and apatite were detected. All rats receiving diets containing vitamin B_6, 0.04% magnesium and 0.8% phosphorus showed marked nephrocalcinosis due to intratubular deposition of apatite. The latter gave a red color when fresh sections of the kidneys were stained with alizarin red. Relatively slight nephrocalcinosis was observed in only one rat of the animals fed diets containing vitamin B_6, 0.04% magnesium and 0.4% phosphorus. High levels of dietary magnesium protected against the deposition of calcium phosphate in the kidneys of rats fed diets containing vitamin B_6 and either the normal or the high levels of phosphorus; and calcium phosphate deposits did not occur in vitamin B_6-deficient rats fed diets containing 0.04% magnesium and any level of phosphorus.

DISCUSSION

In previous studies, high magnesium diets appeared remarkably effective in preventing renal calcium oxalate deposition in both vitamin B_6-deficient rats (3) and in rats receiving vitamin B_6 but fed ethylene glycol in their drinking water (13). In the present study, it was observed that rats eating diets high in magnesium showed a significant decrease in their excretion of inorganic sulfates and phosphates. The feeding of low sulfur diets resulted in partial protection against the formation of oxalate stones in rats receiving vitamin B_6-deficient diets containing 0.04% of magnesium. This protection was associated with a significant decrease in

TABLE 5

Incidence of stones in vitamin B_6-deficient rats fed various levels of phosphorus and magnesium

Diet	Low P		Normal P		High P	
	0.04% Mg	0.4% Mg	0.04% Mg	0.4% Mg	0.04% Mg	0.4% Mg
No. of animals with stones	9/10	8/10	8/10	0/10	8/10	3/10
No. of stones	26[1]	14[2]	16[1]	0	32[1]	4[3]
Size of stones, mm[3]	2.1	2.5	1.7	0	0.9	0.7
No. of stones in kidneys	17	6	16	0	16	3
No. of stones in ureters	6	3	0	0	14	0
No. of stones in bladders	3	5	0	0	2	1

[1] Calcium oxalate monohydrate.
[2] Mixture of calcium oxalate monohydrate and dihydrate.
[3] Mixture of calcium oxalate monohydrate and apatite.

urinary excretion of oxalic acid, inorganic sulfates and inorganic phosphates. The observation by Miller et al. (14), using samples of artificial urines which vary in their solvent characteristics from normal urine, that the solubility of oxalic acid is increased in the presence of inorganic sulfate and phosphate suggests that the protective effect of low sulfur diets was not directly related to the sulfate or phosphate content of the urine.

Calcium oxalate monohydrate renal stones were produced in vitamin B_6-deficient rats fed diets containing 0.04% magnesium and 0.2, 0.4, or 0.8% of phosphorus. Unexpectedly, renal calculi were also found in vitamin B_6-deficient animals receiving 0.4% magnesium and either low or high levels of phosphorus in their diets. In vitamin B_6-deficient rats fed low phosphorus, high magnesium diets, the production of calculi was accompanied by marked decreases in oxalate and phosphate excretion and increases in urinary calcium and citrate. These stones were not similar to the citrate stones produced by Schneider and Steenbock (15) and Sager and Spargo (16) in rats fed very low phosphorus diets (0.04%) of approximately normal calcium composition (0.57%). No citric acid was found on chemical analysis of these stones. Through the use of polarization optics (17), these stones were identified as mixtures of calcium oxalate monohydrate and dihydrate. This is the first time that calcium oxalate dihydrate-containing calculi have been observed in this laboratory in vitamin B_6-deficient rats. The occurrence of any renal stones was unexpected because of the high magnesium diets fed and because low phosphorus intake independent of dietary magnesium level resulted in a significant decrease in oxalate excretion and an increase in citrate excretion. The formation of stones in these animals might be attributed partially to the increased excretion of calcium. However, the observation that their controls receiving vitamin B_6 excreted even more calcium indicates that other factors must be involved in the mechanism of stone formation in these animals.

Further evidence that low dietary phosphorus can result in urinary tract lithiasis has been reported by Coburn and Packett (18). These authors observed that low phosphorus (0.16%) normal calcium (0.56%) diets produced a high incidence of mild urolithiasis in rats after periods up to 40 weeks. Few data were reported on the composition of these uroliths. Three of the rats had bladder stones which were analyzed and these were composed predominantly of calcium oxalate. One contained 90% calcium oxalate dihydrate and 10% calcium citrate.

High levels of dietary phosphorus partially interfered with the action of high levels of dietary magnesium in preventing formation of oxalate stones in vitamin B_6-deficient rats. The calculi formed under these dietary regimens were composed of a mixture of calcium oxalate monohydrate and apatite. Furthermore, high levels of dietary phosphorus resulted in marked apatite nephrocalcinosis in rats receiving vitamin B_6 and 0.04% magnesium. As in a previous study (3), vitamin B_6 deficiency protected against apatite nephrocalcinosis.

There have been many studies in which variations in dietary levels of calcium, phosphorus and magnesium have been shown to alter bone calcification or to result in soft tissue calcification. Cramer's (19) original description of nephrocalcinosis produced by magnesium deficiency has been confirmed in many species. Magnesium deficiency has also been shown to result in calcification of soft tissues other than the kidneys in calves (20), guinea pigs (21) and cotton rats (22). When guinea pigs, which are particularly prone to soft tissue calcification, are fed diets low in magnesium, the amount of dietary phosphorus is a major factor in determining the extent of the calcification (21). The feeding of high phosphorus diets to guinea pigs decreases magnesium absorption regardless of the dietary magnesium level (23). It appears that the magnesium requirements of rats used in this study were increased when the high phosphorus diets were fed and that this resulted in the renal deposition of calcium phosphate salts in control animals fed 0.04% of magnesium and in vitamin B_6-deficient rats fed 0.4% magnesium.

These studies show that a variety of dietary factors can affect the deposition of calcium oxalate and calcium phosphate

in the urinary tracts of rats and that the mechanism for formation is complex and not solely dependent upon the urinary concentration of calcium, oxalate and phosphate. There is considerable evidence (14) that the solvent characteristics of urine, which can be affected by a variety of its components, can be of primary importance in determining whether calcium salt precipitation in the urinary tract will occur. In the present studies, variations in the dietary levels of magnesium, sulfur, phosphorus and vitamin B_6 had a pronounced effect on the renal deposition of calcium oxalate and calcium phosphate in rats, accompanied by a variety of changes in urine composition. It is apparent that, as in so many other nutrition studies, investigators of the etiology of nephrocalcinosis must consider not only the absolute levels of individual nutrients fed, but also the interrelationships among them.

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Effect of Linolenic Acid Upon the Metabolism of Linoleic Acid[1]

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ABSTRACT Weanling rats were fed a fat-free diet supplemented with highly purified linoleate and linolenate combined in several ratios. The fatty acid composition of lipids of liver, heart and adipose tissue was analyzed by gas chromatography. Increasing amounts of dietary linolenate suppressed the levels of arachidonate in tissue lipids, thus showing that the conversion of linoleate to arachidonate is inhibited by dietary linolenate.

Recent investigations in this laboratory have shown that dietary linolenate, as well as linoleate, decreases the concentration of 5,8,11-eicosatrienoic acid in liver lipids of the rat. Moreover, a slight decrease in the concentrations of arachidonate and docosapentaenoate was observed when increasing amounts of linolenate were fed to rats supplied with a fat-free diet (1). These experiments led to our investigation of the interrelationship of linoleate and linolenate when fed together at different levels.

Greenberg et al. (2) have described a "sparking" effect in which the growth-promoting activity of linolenate was enhanced by minimal doses of linoleate. Privett et al. (3) noted a similar effect by grading dermatitis of fat-deficient rats. They also observed that dietary linolenate depressed the content of tetraenoic acid in rats. Klenk and Oette (4) reported that the content of arachidonate in liver phospholipids of rats supplemented with linolenate was less than in those fed a fat-free diet. Marco et al. have reported (5) that the linoleate-arachidonate ratio in mitochondria from chicken liver and cerebellum was increased by feeding linolenate-containing oils. During the course of our present investigation Machlin reported (6) that linseed oil fed to chickens doubles the amount of linoleic acid deposited in the liver. The arachidonate content, however, is decreased significantly. Thus, he proposed a theory that linolenic acid inhibits the conversion of linoleic acid to arachidonic acid.

All of these observations indicate that linolenate has an influence upon the metabolism of linoleate. It is our purpose in the present investigation to study this interaction systematically by feeding highly purified linoleate and linolenate in several ratios. Intake levels chosen are in the range which enables one to study the changes in fatty acid composition of tissue lipids at the onset of essential fatty acid (EFA) deficiency. Thus, the results of this experiment can be compared with those of the previous one, when linoleate and linolenate were fed singly (1).

EXPERIMENTAL

One hundred eight weanling, 24-day-old, male rats of the Sprague-Dawley strain were maintained with a basic fat-free diet as described previously (1). The major constituents of the diet were vitamin-free casein, sucrose, cellulose and the required salts and vitamins. The daily intake of basic diet was recorded for each animal. The rats were divided into 18 groups of 6 animals each. Linoleate and linolenate were fed in daily oral doses by microsyringe as indicated in table 1. Three levels of linoleate were combined with 6 levels of linolenate.

The ethyl linoleate was prepared according to the methods used by the The Hormel Foundation, Austin, Minnesota. It contained 2.3% oleate, but no linolenate could be detected by gas-liquid chromatography (GLC). The ethyl linolenate (The Hormel Foundation) was free from oleate

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TABLE 1

Fatty acid composition of liver lipids (% of total methyl esters)

Group no.	Dietary fatty acid		14:0	16:0	16:1	18:0	18:1	18:2
	18:2	18:3						
	% of calories							
1	0.08	0.05	0.79 ± 0.22[2]	21.76 ± 3.09	9.19 ± 1.30	13.75 ± 1.49	37.98 ± 2.68	1.33 ± 0.31
2	0.07	0.09	0.77 ± 0.32	22.43 ± 2.72	10.44 ± 3.18	12.57 ± 3.06	38.05 ± 3.54	1.53 ± 0.33
3	0.09	0.21	0.99 ± 0.27	25.22 ± 2.05	11.09 ± 2.47	11.15 ± 3.12	37.75 ± 4.52	1.34 ± 0.20
4	0.09	0.37	0.70 ± 0.16	21.28 ± 0.66	9.81 ± 0.99	14.42 ± 0.96	33.53 ± 2.35	1.53 ± 0.20
5	0.07	0.72	0.79 ± 0.39	22.78 ± 1.75	10.51 ± 2.46	12.47 ± 2.17	35.63 ± 4.22	1.38 ± 0.32
6	0.08	1.83	0.85 ± 0.35	26.06 ± 2.07	11.36 ± 2.16	11.20 ± 1.59	31.08 ± 2.05	0.88 ± 0.10
7	0.34	0.05	0.68 ± 0.09	26.83 ± 2.77	8.12 ± 0.98	14.00 ± 2.04	32.68 ± 2.80	2.64 ± 0.81
8	0.34	0.11	0.83 ± 0.21	22.30 ± 1.39	9.22 ± 1.75	15.18 ± 2.46	31.86 ± 2.06	2.61 ± 0.69
9	0.27	0.16	0.76 ± 0.12	24.64 ± 2.14	10.01 ± 1.26	13.80 ± 1.22	32.82 ± 1.62	2.04 ± 0.41
10	0.28	0.33	0.71 ± 0.09	25.01 ± 2.32	8.07 ± 0.79	13.73 ± 2.01	33.10 ± 1.70	2.04 ± 1.07
11	0.30	0.68	0.68 ± 0.19	25.58 ± 2.04	10.68 ± 1.35	13.41 ± 0.89	31.97 ± 0.79	1.95 ± 0.27
12	0.30	1.80	0.84 ± 0.15	21.81 ± 2.40	10.92 ± 0.66	13.21 ± 1.83	30.02 ± 1.34	2.39 ± 0.43
13	0.73	0.06	0.78 ± 0.20	24.07 ± 1.33	8.22 ± 2.62	13.85 ± 1.76	31.68 ± 3.39	3.21 ± 0.34
14	0.58	0.09	0.68 ± 0.09	26.83 ± 2.77	8.12 ± 0.98	14.00 ± 2.04	32.68 ± 2.80	2.64 ± 0.81
15	0.59	0.18	0.82 ± 0.10	22.58 ± 2.01	8.42 ± 1.02	14.50 ± 1.13	31.59 ± 1.46	3.40 ± 1.05
16	0.62	0.37	0.74 ± 0.08	24.79 ± 3.39	9.38 ± 0.66	13.56 ± 1.57	30.47 ± 2.37	3.05 ± 0.67
17	0.64	0.75	0.72 ± 0.18	23.81 ± 1.46	9.38 ± 1.71	13.22 ± 1.57	30.52 ± 2.05	3.69 ± 0.81
18	0.67	1.92	0.87 ± 0.21	21.91 ± 1.34	9.86 ± 1.29	13.56 ± 1.87	29.01 ± 2.73	5.40 ± 1.92

[1] See Experimental section of text for explanation.
[2] SD.

and contained traces of linoleate. At the end of the experiment, the intake of fatty acids for each animal was calculated in percentage of calories. One per cent of calories of linoleate and linolenate is equivalent to approximately 40 mg/day/animal.

After 87 days the animals were killed by ether anesthesia. The livers, hearts and epididymal fat were quickly removed and kept in saline solution at −20°C until analyzed.

The tissues were homogenized and the lipids extracted with chloroform:methanol (2:1) according to Folch (7). The lipids were transesterified by refluxing with 30 volumes of a 5% solution of HCl in methanol. The methyl esters were analyzed by GLC using a Barber-Coleman Model 10 apparatus with argon ionization detector.

The column was 210 cm by 5 mm i.d., packed with 20% ethylene glycol succinate (EGS) coated on Gaschrom P, 80–100 mesh.[3] The flowrate was 60 ml argon/min, and the temperatures were at the inlet heater 270°C, detector cell 250°C. The column was held at 180°C for methyl esters with retention times shorter than that of 18:3 and at 200°C for long-chain esters.

The individual esters were identified by carbon number and by internal standards. Isolation of the composite 20:3 fraction by preparative GLC followed by ozonolysis showed that 5,8,11-eicosatrienoate was the major component, and that the 8,11,14- and 7,10,13-eicosatrienoate isomers were present. The 8,11,14-isomer varied depending on the amounts of dietary linoleate. Hereafter, 5,8,11-eicosatrienoate will be referred to as 20:3ω9 and 8,11,14-eicosatrienoate will be designated 20:3ω6. This notation is used to denote relationship between polyunsaturated fatty acids (PUFA) which exist in families, the terminal structures of which are the same. Thus, acids designated ω3 are related to linolenate, ω6 to linoleate and ω9 to oleate. The calculated carbon numbers on EGS columns for 20:3ω9 and 20:3ω6 were found to be 21.85 and 22.14, respectively. These isomers probably correspond to the 2 reported by Morin et al. (8). 4,7,10,13,16-Docosapentaenoate (22:5ω6) has been identified by isolation via GLC and ozonolysis[4] and has a calculated carbon number of 25.22 on EGS columns. Quantitation was carried out by triangulation. The fatty acid composition of tissue lipids is

[3] Applied Science Laboratories, State College, Pennsylvania.

[4] Rahm, J. J. 1963 The effects of dietary fatty acids upon lipids of subcellular particles. Ph.D. Thesis, University of Minnesota (June).

TABLE 1 (*Continued*)

Fatty acid composition of liver lipids (% of total methyl esters)

18:3	20:3ω9[1]	20:3ω6[1]	20:4	20:5	22:5ω6[1]	22:5	22:6
0.60 ± 0.26	6.39 ± 1.76	0.96 ± 0.23	3.36 ± 0.71	0.46 ± 0.09	0.63 ± 0.23	0.13 ± 0.07	2.14 ± 0.76
0.37 ± 0.06	5.51 ± 2.17	0.48 ± 0.09	3.38 ± 1.26	0.54 ± 0.16	0.46 ± 0.17	0.24 ± 0.15	3.26 ± 1.30
0.35 ± 0.07	3.42 ± 1.42	0.72 ± 0.18	2.28 ± 0.39	0.71 ± 0.12	0.23 ± 0.02	0.36 ± 0.11	4.32 ± 1.00
0.66 ± 0.09	4.26 ± 0.90	0.74 ± 0.15	2.49 ± 0.15	1.88 ± 0.44	0.20 ± 0.03	0.68 ± 0.26	7.85 ± 1.16
0.83 ± 0.40	1.83 ± 0.71	0.28 ± 0.15	1.74 ± 0.46	2.43 ± 0.62	< 0.1	1.07 ± 0.47	8.25 ± 1.27
1.25 ± 0.34	0.93 ± 0.30	0.35 ± 0.31	1.14 ± 0.32	4.76 ± 0.94	< 0.1	2.28 ± 0.42	7.87 ± 1.52
0.31 ± 0.07	3.31 ± 0.93	0.92 ± 0.19	6.94 ± 1.62	0.37 ± 0.08	1.16 ± 0.31	0.18 ± 0.07	1.87 ± 0.41
0.35 ± 0.05	5.13 ± 1.14	1.30 ± 0.36	6.33 ± 0.78	0.51 ± 0.07	0.88 ± 0.15	0.27 ± 0.13	3.25 ± 0.75
0.40 ± 0.05	4.23 ± 0.70	0.59 ± 0.17	5.12 ± 1.04	0.76 ± 0.15	0.64 ± 0.19	0.29 ± 0.13	3.91 ± 0.85
0.44 ± 0.17	3.29 ± 0.64	0.79 ± 0.25	4.30 ± 1.10	1.15 ± 0.34	0.28 ± 0.11	0.53 ± 0.18	6.31 ± 1.60
0.45 ± 0.09	2.21 ± 0.57	0.56 ± 0.11	2.79 ± 0.33	1.90 ± 0.37	0.11 ± 0.01	0.78 ± 0.18	6.91 ± 0.63
1.51 ± 0.51	1.03 ± 0.19	0.42 ± 0.08	2.22 ± 0.37	5.00 ± 0.68	< 0.1	2.47 ± 0.61	8.18 ± 0.84
0.41 ± 0.09	3.00 ± 1.03	1.13 ± 0.29	8.48 ± 1.83	0.75 ± 0.17	1.83 ± 0.23	0.20 ± 0.09	2.40 ± 0.51
0.31 ± 0.07	3.31 ± 0.93	0.92 ± 0.19	6.94 ± 1.62	0.37 ± 0.08	1.16 ± 0.31	0.18 ± 0.07	1.87 ± 0.41
0.44 ± 0.12	3.33 ± 0.79	1.09 ± 0.18	7.70 ± 0.99	0.56 ± 0.05	0.95 ± 0.21	0.37 ± 0.16	4.26 ± 0.95
0.51 ± 0.09	2.59 ± 0.59	0.62 ± 0.10	6.00 ± 0.97	1.03 ± 0.21	0.41 ± 0.10	0.63 ± 0.17	6.08 ± 1.25
0.66 ± 0.20	1.51 ± 0.44	0.89 ± 0.17	4.45 ± 0.67	1.84 ± 0.17	0.18 ± 0.05	1.19 ± 0.36	7.95 ± 1.06
1.83 ± 0.98	0.58 ± 0.29	0.51 ± 0.15	3.20 ± 0.37	3.64 ± 0.54	0.10 ± 0.03	2.13 ± 0.40	7.41 ± 1.25

reported as area per cent, a procedure justified in cases where changes in concentration, but not the absolute composition, are to be measured.

Liver lipids were analyzed for each animal. The results reported are averages for 6 animals with their standard deviations. Hearts, as well as epididymal fat, were pooled and one GLC analysis was performed per group.

RESULTS

The animals showed approximately equal growth with all linoleate-linolenate supplementations. The average weight gain was 240 g during the 3-month period of the experiment. No severe fat deficiency symptoms could be observed, although mild dermatitis was detectable, especially in the groups (1–6) with the lowest linoleate supplement (0.08% of calories). The fatty acid composition of the liver lipids is shown in table 1.

No consistent changes can be observed in the concentrations of the saturated fatty acids — myristic (14:0), palmitic (16:0) and stearic (18:0) — throughout the experiment.

Increasing levels of dietary linoleate lead to the following changes in the fatty acid composition of the liver lipids:

1) The levels of palmitoleic (16:1) and oleic (18:1) acids are decreased slightly.

2) The concentrations of the fatty acids of the linoleate family — 18:2, 20:4 and 22:5ω6 — are increased significantly.

3) The level of 5,8,11-eicosatrienoic acid (20:3ω9) is decreased.

4) No significant changes can be observed in the concentrations of the fatty acids of the linolenate family — 18:3, 20:5, 22:5 and 22:6.

Increasing amounts of dietary linolenate influence the fatty acid composition of the liver lipids at all 3 levels of linoleate intake as follows:

1) The concentrations of the fatty acids of the linolenate family — 18:3, 20:5, 22:5 and 22:6 — are increased significantly.

2) The levels of 20:3 and the fatty acids of the linoleate family — 20:4 and 22:5ω6 — are lowered significantly.

3) No pronounced influence upon the concentration of the monoenoic and dienoic acids of the liver lipids can be observed.

In table 2 the fatty acid composition of heart lipids is shown. The data represent analyses of the pooled hearts of 6 animals for each group. In general, the same changes are observed as described for liver lipids. At all 3 levels of linoleate intake,

TABLE 2

Fatty acid composition of heart lipids (% of total methyl esters)

Group	Dietary fatty acid		14:0	16:a1[1]	16:0	16:1	18:0	18:1	18:2	18:3	20:3ω9[2]	20:3ω6[2]	20:4	20:5	22:5ω6[2]	22:5	22:6
	18:2	18:3															
	% of calories																
1	0.08	0.05	0.58	1.15	17.32	6.66	18.43	33.74	3.55	< 0.1	10.60	0.58	5.74	< 0.1	0.39	0.14	1.11
2	0.07	0.09	0.77	1.10	18.08	6.80	18.95	32.63	3.91	< 0.1	6.75	0.49	7.34	0.1	0.42	0.36	2.32
3	0.09	0.21	0.89	1.12	20.17	7.75	17.17	33.20	4.40	< 0.1	6.01	0.52	5.28	0.21	0.20	0.43	2.64
4	0.09	0.37	0.83	1.20	20.09	7.22	17.17	34.57	4.08	0.48	4.05	0.37	4.67	0.66	< 0.1	0.65	3.96
5	0.07	0.72	0.82	1.28	18.83	6.59	18.16	33.28	4.23	1.24	2.21	0.45	4.52	1.66	< 0.1	1.22	5.54
6	0.08	1.83	1.04	1.23	21.80	9.93	15.37	34.79	3.33	2.57	0.95	0.21	3.00	—	—	—	—
7	0.34	0.05	0.74	1.50	17.24	5.25	18.74	29.47	8.27	< 0.1	4.13	1.01	11.21	< 0.1	0.92	0.17	1.36
8	0.34	0.11	0.75	1.45	18.34	5.56	18.72	29.05	8.05	< 0.1	3.88	0.68	10.45	< 0.1	0.77	0.26	2.05
9	0.27	0.16	1.05	1.02	21.50	7.73	15.69	33.37	6.15	< 0.1	3.47	0.56	7.02	< 0.1	0.41	0.26	1.82
10	0.28	0.33	0.67	1.54	16.85	5.46	19.70	29.50	8.25	< 0.1	3.60	0.74	8.09	0.30	0.25	0.82	4.23
11	0.30	0.68	0.93	1.06	19.96	7.89	15.32	34.13	7.44	< 0.1	1.84	0.48	5.18	0.94	< 0.1	0.85	4.00
12	0.30	1.80	0.81	1.18	18.08	5.19	18.01	26.96	9.85	1.51	1.39	0.74	6.09	1.53	< 0.1	2.64	6.02
13	0.73	0.06	1.39	0.94	21.31	8.46	13.57	32.45	8.17	< 0.1	1.74	0.50	9.35	< 0.1	0.93	0.23	0.96
14	0.58	0.09	0.52	1.67	17.56	4.30	19.39	27.35	10.64	< 0.1	2.48	0.73	12.68	< 0.1	1.09	0.08	1.49
15	0.59	0.18	0.71	1.27	18.06	4.62	18.01	29.96	9.53	< 0.1	1.92	0.53	12.01	< 0.1	0.59	0.26	2.54
16	0.62	0.37	0.63	1.40	17.71	5.14	17.35	30.66	10.70	< 0.1	1.49	0.53	10.14	0.09	0.31	0.48	3.36
17	0.64	0.75	1.00	1.45	18.93	6.20	15.82	31.11	11.15	< 0.1	0.95	0.56	7.37	0.48	0.09	0.91	3.97
18	0.67	1.92	0.70	1.44	16.94	4.71	17.36	25.27	16.99	0.78	0.56	0.49	6.15	1.49	< 0.1	2.05	5.10

[1] 16:a1 identified as the C-16 saturated aldehyde.

[2] See Experimental section of text for explanation.

TABLE 3

Fatty acid composition of the lipids of depot fat (% of total methyl esters)

Group	Dietary fatty acid		14:0	16:0	16:1	18:0	18:1	18:2	18:3
	18:2	18:3							
	% of calories								
1	0.08	0.05	1.55	30.54	17.82	1.71	48.06	0.32	< 0.1
2	0.07	0.09	1.70	34.27	18.36	1.51	43.75	0.42	< 0.1
3	0.09	0.21	1.85	31.04	21.00	1.33	44.41	0.37	< 0.1
4	0.09	0.37	1.91	27.56	19.86	1.54	48.46	0.35	0.32
5	0.07	0.72	2.33	37.42	26.86	1.79	30.29	0.56	0.75
6	0.08	1.83	1.38	38.91	19.69	1.31	37.06	0.39	1.26
7	0.34	0.05	2.06	26.17	18.46	2.07	50.60	0.66	< 0.1
8	0.34	0.11	1.87	27.83	18.34	2.03	49.33	0.61	< 0.1
9	0.27	0.16	1.99	32.50	19.96	1.28	43.65	0.62	< 0.1
10	0.28	0.33	1.83	31.92	18.80	1.39	45.40	0.67	< 0.1
11	0.30	0.68	1.82	29.10	20.92	1.75	45.29	0.62	0.49
12	0.30	1.80	1.61	36.01	21.53	1.34	37.25	0.74	1.50
13	0.73	0.06	1.80	31.26	19.56	1.72	44.66	0.98	< 0.1
14	0.58	0.09	1.67	31.44	20.57	1.48	44.10	0.75	< 0.1
15	0.59	0.18	1.79	28.82	18.06	1.95	48.05	0.86	0.48
16	0.62	0.37	1.79	30.60	19.70	1.62	44.98	1.01	0.31
17	0.64	0.75	1.87	31.89	20.70	1.50	42.15	1.26	0.63
18	0.67	1.92	2.70	31.24	20.01	1.77	40.46	1.76	2.06

the decrease in concentration of 20:4 and 22:5ω6 with increasing amounts of dietary linolenate is several fold. At constant linolenate intake the concentrations of 18:3 and 20:5 appear to decrease with increased linoleate intake.

The analysis of depot fat (table 3) shows that different amounts of dietary linoleate and linolenate are reflected in the 18:2 and 18:3 concentrations of the lipids. The saturated and monoenoic fatty acids are not significantly influenced. The concentration of arachidonic acid was too low to be measured.

DISCUSSION

The effect of dietary linolenate upon the concentration of arachidonate and 22:5ω6 in lipids of rat liver as described recently (1) could be confirmed and extended by the results of the present experiment. The percentage of arachidonate in liver lipids of rats fed 1.8% of calories as linolenate is less than one-half the level observed in livers of rats fed 0.05% of calories. This is true for all 3 levels of linoleate fed (fig. 1). The same effects were observed in heart tissue, although the changes were not quite as consistent as in liver lipids.

The increase in linoleate content at higher levels of dietary linolenate as observed by Machlin in chickens (6) is not so pronounced in lipids of rat tissue at the low levels of linoleate we fed. At the highest intake level (0.6% of calories linoleate) an increase of linoleate content can be noted, however, especially in heart lipids.

The ratio of linoleate to arachidonate (precursor to metabolic product) increases sharply at all linoleate intake levels with increasing amounts of dietary linolenate (table 4). These changes are most pronounced at the highest linoleate level fed.

Concurrent with the decrease of 20:4, the synthesis of 22:5ω6 is suppressed by increasing amounts of dietary linolenate. This is to be expected because this docosapentaenoate is synthesized from linoleate via arachidonate (1).

From our observations it might be concluded that the conversion of linoleate to arachidonate in the rat is partially inhibited by dietary linolenate. If no external dienoic or trienoic fatty acids are available, oleic acid is converted into 20:3ω9 (9). If linoleate or linolenate are present in sufficient amounts, they will occupy the enzymes responsible for dehydrogenation and chain lengthening and are converted into the polyunsaturated fatty acids (PUFA) of their corresponding

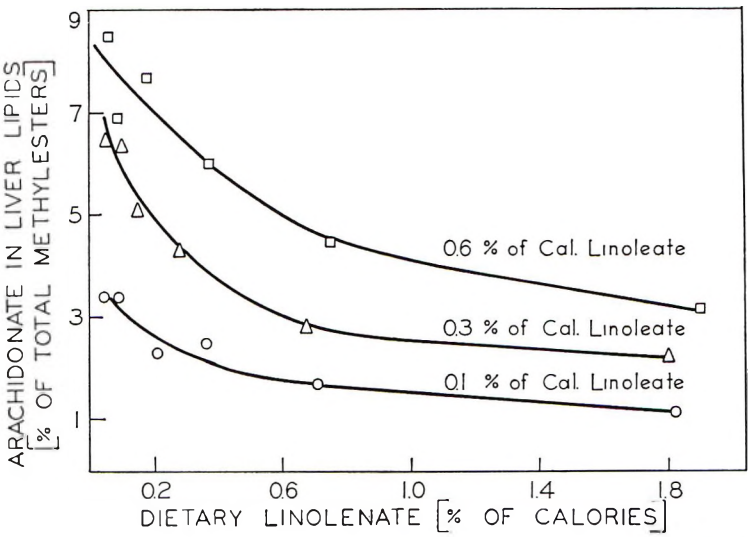

Fig. 1 The effect of varying levels of dietary linolenate upon the biosynthesis of arachidonate in liver lipids.

TABLE 4

Effects of dose level of dietary linolenate upon the linoleate-arachidonate ratio in liver and heart lipids

Dietary fatty acid		Linoleate-arachidonate ratio	
18:2	18:3	In liver lipids	In heart lipids
% of calories			
0.08	0.05	0.39	0.62
0.07	0.09	0.45	0.53
0.09	0.21	0.59	0.83
0.09	0.37	0.61	0.87
0.07	0.72	0.79	0.94
0.08	1.83	0.77	1.11
0.34	0.05	0.37	0.74
0.34	0.11	0.41	0.77
0.29	0.16	0.39	0.88
0.28	0.33	0.47	1.02
0.30	0.68	0.69	1.44
0.30	1.80	1.08	1.62
0.73	0.06	0.38	0.87
0.58	0.09	0.38	0.84
0.59	0.18	0.44	0.79
0.62	0.37	0.51	1.06
0.64	0.75	0.83	1.51
0.67	1.92	1.46	2.76

families (1). In case linoleate and linolenate are fed together, especially if the linolenate concentration exceeds that of linoleate in the diet, linolenate is converted preferably, and the linoleate-arachidonate conversion is inhibited.

This concept of substrate competition leads to the assumption that the same or similar metabolic pathways are responsible for all conversions of oleic, linoleic and linolenic acids to the PUFA of their respective families. The degree of unsaturation between the 9-position and the terminal methyl group of the substrate fatty acid determines the affinity to the enzymes involved. The triene, linolenate, blocks the conversion of the diene, linoleate. Either linoleate or linolenate blocks the conversion of the monoene, oleate. Thus, the affinities for the enzyme sites are linolenate > linoleate > oleate. A similar mechanism may be applicable for the conversion of 20-carbon to 22-carbon PUFA. The biosynthesis of the PUFA of the linolenate type, 22:5 and 22:6, may inhibit the conversion of arachidonate to 22:5ω6, or the inhibition could be due to simple lack of 20:4 substrate.

Recently, we have reported that the synthesis of 20:3ω9 from oleic acid is inhibited by feeding linolenate to fat-deficient rats. This effect is confirmed in the present experiment. At all 3 levels of linoleate fed, additional amounts of linolenate in the diet decrease the 20:3ω9 content of liver and heart lipids even farther. Thus, the abilities of linolenate and linoleate to lower the content of eicosatrienoate in tissue lipids are additive. This may be the

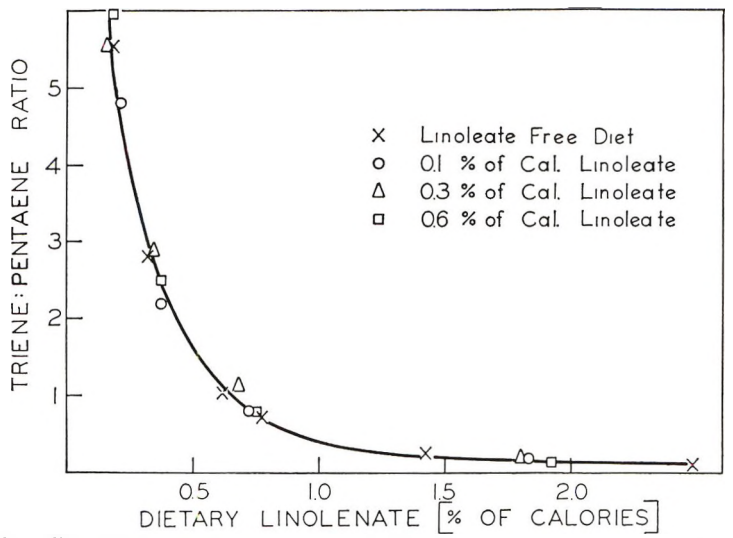

Fig. 2 The effect of increasing amounts of dietary linoleate upon the triene-penetaene ratio at different levels of linoleate intake.

biochemical basis for the "sparking" effect reported by Greenberg et al. (2).

The ratio of triene-to-tetraene was proposed to determine the fat deficiency status of rats (10), swine (10, 11) and other species. Experiments with purified fatty acids which were fed singly to rats supplied with fat-free diets supported the usefulness of the triene-tetraene ratio as a parameter for describing linoleate metabolism (1). It was shown, however, that the proposed ratio could not be used in cases where linolenate is the only dietary fatty acid. The present experiments show that the triene-tetraene ratio is not meaningful either when the linolenate concentration exceeds the linoleate content of the diet. Since most normal diets, especially human diets, contain much more linoleate than linolenate, the triene-tetraene ratio still has practical value.

The ratio of eicosatrienoic (20:3ω9) to eicosapentaenoic acid (22:5ω3) proposed to describe the fat status of the rat with respect to linolenate metabolism (1) has been found to be useful in the whole range of linoleate intake studied in the present experiment. Figure 2 shows the effect of dietary linolenate upon the triene-pentaene ratio at 4 different levels of linoleate intake. The triene-pentaene ratio for linoleate-free diets is taken from an earlier publication (1). All points relating the triene-pentaene ratio to dietary linolenate lie on the curve derived for linoleate-free diets. Thus, the triene-pentaene ratio is independent of dietary linoleate within the range studied here.

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Dietary Fat and the Structure and Properties of Rat Erythrocytes[1]

I. EFFECT OF DIETARY FAT ON THE ERYTHROCYTE LIPIDS

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ABSTRACT The lipid composition of rat erythrocyte stroma was studied when corn oil and lard were fed with or without vitamin E supplements. Substitution of lard for corn oil did not influence the relative concentrations of the neutral lipid, or choline- and noncholine-containing phospholipids of the stroma lipids. Gas-liquid chromatography indicated that the noncholine-containing phospholipids were rich in arachidonic acid. Hexadecanal, octadecanal and octadecenal also occurred, presumably in the form of phosphatide plasmalogens. The choline-containing lipids contained more saturated fatty acids, primarily palmitic acid. More linoleic acid occurred in this fraction than in the noncholine phospholipids, whereas the longer-chain-length polyenoic acids were less abundant. Aldehydes were present in lower concentrations, indicating that a smaller fraction of choline phosphatides occurred as plasmalogens. The corn oil diet, rich in linoleic acid, resulted in the occurrence of higher concentrations of this acid and of docosaenoic acids, whereas oleic acid was present in higher concentrations in the phospholipid fatty acids when lard was used as the dietary fat. Arachidonic acid was independent of this dietary variation as were the aldehydes. Vitamin E deficiency appeared to exert no influence on the fatty acid composition of the phospholipids.

The changes that occurred in the lipids of erythrocytes in response to dietary factors have been studied by Witting et al. (1) and Monsen et al. (2). The former reported on the variation in the mixed fatty acid composition of the lipids from rats fed fats of differing degrees of unsaturation; the latter extended this work by fractionating the lipids of the erythrocytes into neutral and phospholipid fractions prior to fatty acid analysis. In the present study, the lipids extracted from the erythrocytes of rats that had been fed corn oil or lard were separated into neutral lipids, noncholine-containing phospholipids, and choline-containing phospholipids by chromatography on a silicic acid column and the mixed fatty acid composition of the phospholipid fractions determined.

MATERIALS AND METHODS

Four groups of weanling male albino rats were fed for 6 months a diet consisting of glucose, 68%; casein, 18%; salt mixture (Wesson salts W),[2] 4%; fat, 10% and water-soluble vitamins (3). The fat-soluble vitamins were administered in stripped corn oil by dropper once each week. The first group was fed 10% corn oil + vitamin E; the second, 10% corn oil, no vitamin E; the third, 10% lard + vitamin E; and the fourth, 10% lard, no vitamin E. The corn oil and lard (table 1) had been stripped of tocopherols by vacuum distillation.[3] The animals were fed ad libitum and weighed weekly.

At the end of 6 months, 5 animals were chosen at random from each dietary group. Blood samples were taken from the tail veins of these rats and subjected to the dialuric acid hemolysis test for vitamin E deficiency proposed by Friedman et al. (4). Since this test confirmed the vitamin deficiency only in the groups receiving no tocopherol supplements, all of the animals were anesthetized with pentobarbital sodium[4] and the blood was withdrawn from

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[1] Supported in part by a grant (H-1819) from the National Institutes of Health, U. S. Public Health Service.

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

[3] The stripped fats were donated by Distillation Products Industries, Rochester, New York.

[4] Nembutal, Abbott Laboratories, North Chicago, Illinois.

TABLE 1
Fatty acid composition[1] of the dietary fats

Identity[2]	Corn oil	Lard
10:0	—	trace[3]
12:0	—	0.1
14:0	0.6	1.4
14:1	—	trace
16:0	11.7	27.9
16:1	0.2	2.4
17:0	—	0.3
18:0	3.8	13.4
18:1	29.3	42.8
18:2	52.3	10.2
20:0	0.3	trace
20:1	1.7	1.1
20:2	—	0.4
20:4	—	trace

[1] Expressed as a percentage of the total acids detected.
[2] Number before the colon represents the carbon chain length, that after it represents the number of double bonds.
[3] Trace, less than 0.1% detected.

the abdominal aorta into sodium citrate solution. The blood from 15 animals was combined into a single sample and 3 such samples were obtained for each dietary group. After washing 3 times with isotonic saline and removal of the platelets and white cells, the erythrocytes were hemolyzed and the stroma isolated by the carbon dioxide precipitation procedure of Parpart (5).

The stroma were lyophilized and the lipids extracted with 2:1 chloroform-methanol (3 extractions). The combined extracts were washed with 0.2 volumes of distilled water to remove protein (6). The extracts were then dried over sodium sulfate, filtered, evaporated under reduced pressure, and weighed. Phosphorus (7) and cholesterol (8) were determined and the lipids were chromatographed on silicic acid columns (9). The eluting solvents used were chloroform, 20% methanol in chloroform, 2% water and 20% methanol in chloroform, and 4% water in methanol. The compositions of the fractions were determined by thin-layer chromatography and the lipids obtained in the final 2 eluants were combined into one fraction on the basis of these results. The 3 fractions finally obtained were classified as neutral lipids (A), noncholine-containing phospholipids (B), and choline-containing phospholipids (C). Phosphorus and cholesterol analyses were performed on selected fractions.

The methyl esters were prepared by interesterification of the phospholipid fractions with methanolic hydrogen chloride (10); their composition was determined by gas-liquid chromatography. The gas chromatograph was equipped with a 152-cm stainless steel column, 0.32-cm internal diameter, packed with 83% by weight of 80 to 100 mesh chromasorb W impregnated with 17% by weight of polyethylene glycol succinate.[5] The column temperature was 185 to 190°C, and the flow rate of the nitrogen carrier gas was 55 ml/minute. The instrument was equipped with a hydrogen flame ionization detector.

The peaks obtained on the gas chromatogram were identified by comparison of their retention times with those of known standards under identical operating conditions. A curve also was constructed relating the carbon number of the parent acids to the logarithm of the retention time of the saturated methyl esters, and the unknown components were assigned empirical carbon numbers (11) by comparison of their retention times with this curve. These carbon numbers are recorded in tables 2 and 3. Saponification was used to separate the methyl esters of the fatty acids from the alkali-stable dimethyl acetals of the fatty aldehydes (12). Hydrogenation (13) was used to ascertain which peaks represented unsaturated compounds. The increases in the amounts of the various saturated methyl esters after hydrogenation aided in the assignment of chain lengths to the unknown unsaturated compounds. The area of each peak was found by triangulation and was expressed as a percentage of the total area of the peaks on the chromatogram.

RESULTS

The primary effect of the dietary changes was noted in the fatty acids of the phospholipid fractions. No dietary influence was observed in the total extractable lipid, cholesterol, lipid phosphorus or the distribution of the lipid and lipid phosphorus between the 3 fractions obtained by silicic acid chromatography. The chloroform-methanol extract represented 25% of the dry weight of the stroma. Despite the vigorous washing procedures used in

[5] This packing was purchased from Applied Science Laboratories, State College, Pennsylvania.

TABLE 2

Fatty acid composition[1] of the noncholine-containing phospholipids

Carbon no.	Identity[2]	Group 1	Group 2	Group 3	Group 4
		Corn oil, + vitamin E	Corn oil, no vitamin E	Lard + vitamin E	Lard, no vitamin E
14.0	14:0	0.1 ± 0.1[3]	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2
15.1	16:0 al	5.8 ± 0.8	6.4 ± 1.0	6.5 ± 1.0	6.2 ± 0.7
16.0	16:0	6.5 ± 0.7	7.5 ± 0.9	8.0 ± 1.5	7.0 ± 0.8
16.7	16:1	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.3	0.6 ± 0.2
17.1	18:0 al	5.4 ± 1.4	5.5 ± 1.6	4.8 ± 0.9	5.7 ± 1.3
17.8	18:1 al	5.0 ± 1.0	4.9 ± 1.1	5.6 ± 1.0	6.4 ± 1.3
18.0	18:0	7.5 ± 0.8	8.6 ± 0.8	6.9 ± 0.6	6.4 ± 1.1
18.6	18:1	9.9 ± 0.8	9.9 ± 0.8	11.4 ± 1.0	10.6 ± 1.3
19.6	18:2	5.3 ± 0.6	4.8 ± 0.7	2.1 ± 0.5	1.6 ± 0.8
20.6	20:1	0.3 ± 0.0	0.7 ± 0.3	0.3 ± 0.0	0.5 ± 0.1
20.9		0.3 ± 0.1	0.8 ± 0.3	0.5 ± 0.2	0.5 ± 0.1
21.5	20:2	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.2 ± 0.0
22.0	22:0	0.1 ± 0.1	0.2 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
22.2	20:3	0.3 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	0.9 ± 0.3
22.8	20:4	33.5 ± 1.9	31.2 ± 1.5	35.6 ± 2.9	35.0 ± 2.2
24.0	24:0	1.0 ± 0.2	0.7 ± 0.2	1.5 ± 0.4	1.2 ± 0.1
24.6	24:1	3.3 ± 1.7	3.7 ± 1.3	3.3 ± 1.5	3.8 ± 1.4
24.9	22:4	8.2 ± 0.6	8.5 ± 0.3	5.8 ± 0.4	6.4 ± 1.0
25.4	22:5	2.4 ± 0.4	2.6 ± 0.8	1.5 ± 0.5	2.0 ± 0.2
26.0	26:0	1.6 ± 0.6	0.8 ± 0.3	1.2 ± 0.3	1.3 ± 0.1
26.6	22:6	2.9 ± 0.8	2.1 ± 0.2	2.5 ± 0.2	2.8 ± 0.3

[1] Expressed as a percentage of the total acids and aldehydes detected.
[2] Number before the colon represents the carbon chain length, that after it represents the number of double bonds. Fatty aldehydes are designated by the suffix *al*.
[3] Mean of 3 analyses ± SD of mean.

TABLE 3

Fatty acid composition[1] of the choline-containing phospholipids

Carbon no.	Identity[2]	Group 1	Group 2	Group 3	Group 4
		Corn oil + vitamin E	Corn oil, no vitamin E	Lard + vitamin E	Lard, no vitamin E
14.0	14:0	0.3 ± 0.0[3]	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
15.1	16:0 al	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.1
16.0	16:0	42.9 ± 2.8	43.3 ± 2.2	43.3 ± 2.3	41.9 ± 3.0
16.7	16:1	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
17.1	18:0 al	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.6 ± 0.2
18.0	18:0	16.2 ± 2.1	16.6 ± 1.6	17.2 ± 2.6	17.6 ± 2.5
18.6	18:1	7.1 ± 1.1	7.5 ± 1.1	13.1 ± 1.8	13.8 ± 1.5
19.6	18:2	11.9 ± 1.5	11.4 ± 1.0	7.5 ± 1.7	7.5 ± 1.9
20.6	20:1	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.0	0.3 ± 0.0
20.9		0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
21.5	20:2	0.3 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
22.0	22:0	0.4 ± 0.0	0.5 ± 0.0	1.0 ± 0.2	1.0 ± 0.3
22.2	20:3	0.4 ± 0.2	0.4 ± 0.2	0.8 ± 0.3	0.2 ± 0.2
22.8	20:4	11.1 ± 0.8	10.6 ± 0.7	9.3 ± 1.0	10.2 ± 0.9
24.0	24:0	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.7 ± 0.1
24.6	24:1	3.1 ± 1.6	2.8 ± 1.8	2.5 ± 0.6	2.0 ± 0.7
24.9	22:4	0.7 ± 0.3	0.6 ± 0.4	0.5 ± 0.1	0.5 ± 0.1
25.4	22:5	1.3 ± 0.4	1.3 ± 0.4	0.5 ± 0.1	0.6 ± 0.1
26.0	26:0	—	—	—	—
26.6	22:6	1.5 ± 0.4	1.4 ± 0.5	1.0 ± 0.4	1.0 ± 0.2

[1] Expressed as a percentage of the total acids and aldehydes detected.
[2] Number before the colon represents the carbon chain length, that after it represents the number of double bonds. Fatty aldehydes are designated by the suffix *al*.
[3] Mean of 3 analyses ± SD of mean.

the isolation of the stroma and extraction of the lipids, some hemoglobin appeared to be associated with the lipids. The cholesterol content of the stroma lipids was 27.6, 26.8, 26.8, and 26.8% for the 4 groups, respectively. Less than 1% of the cholesterol was esterified. The phosphorus content of the lipids from the 4 groups was 2.30, 2.38, 2.54 and 2.35% by weight, respectively.

Column chromatography. The neutral lipid fraction consisted primarily of cholesterol. Cerebroside was present together with traces of triglyceride and cholesterol esters. Fraction B, the noncholine-containing lipids, contained phosphatidyl ethanolamine and phosphatidyl serine with traces of phosphatidyl choline. Cerebroside was again detected. Phosphatidyl choline, sphingomyelin and traces of lysophosphatidyl choline were found in fraction C, the latter component being eluted by the water-methanol solvent.

Fractions A, B, and C comprised 37, 11 and 52%, respectively, by weight of the total eluted material, and fractions B and C contained 15 and 85%, respectively, of the total eluted phosphorus. The phosphorus content of the lipids in fractions B and C was 2.8 and 3.7%, respectively. The latter is consistent with the theoretical value for naturally occurring phospholipids, but the former is low, reflecting the presence of nonphosphorus-containing lipids (cerebroside) in fraction B.

Gas chromatography. The fatty acid compositions of the dietary fats are presented in table 1. Corn oil was rich in linoleic acid, whereas oleic acid was the major component in lard. Sufficient linoleic acid was present in the lard to fulfill the essential fatty acid requirements of the animals.

The results obtained in gas chromatography of the methyl esters which were isolated from the phospholipid fractions are presented in tables 2 and 3. Samples of saturated methyl esters, methyl palmitoleate, oleate, nervonate, linoleate, arachidonate, docosapentaenoate and docosahexaenoate were available as standards. The methyl ester of the monoenoic 20-carbon acid was identified from the carbon number — log retention time curve constructed from the monoenoic ester standards. The component represented by carbon number 24.9 behaved as an unsaturated 22-carbon ester on hydrogenation. By analogy with the relative retention time of the methyl arachidonate - arachidate system, it was tentatively identified as methyl docosatetraenoate. No standard was available for the 20-carbon trienoic methyl ester. The component having a carbon number of 22.2 was unsaturated and has been observed in the methyl esters prepared from rat liver lipids. In the latter case, a diet low in essential fatty acids resulted in a considerable increase in the amount of this component.[6] Since the percentage of eicosatrienoic acid is increased in cases of essential fatty acid deficiency, the methyl ester with the above carbon number was tentatively identified as such.

The compounds having carbon numbers of 15.1, 17.1 and 17.8 were alkali-stable and were identified as dimethyl acetals of fatty aldehydes. Hexadecanal and octadecanal were prepared by reduction of the corresponding acyl chlorides and were converted to dimethyl acetal derivatives. These compounds had carbon numbers of 15.1 and 17.1, respectively, on the polyethylene glycol succinate column. On hydrogenation, the component with a carbon number of 17.8 behaved as an unsaturated 18-carbon compound and was therefore identified as octadecenal.

DISCUSSION

Monsen et al. (2) have reported that the amount of cholesterol in the red blood cell is not subject to dietary influence. The present study confirmed this report and, in addition, showed that the total extractable lipid content of the stroma and the phosphorus content of this lipid are also independent of the dietary variations used. Over 99% of the cellular cholesterol was present as the free alcohol. This is in marked contrast with the situation existing in the plasma where a major fraction of the cholesterol is esterified.

Thin-layer chromatography indicated a successful separation of the noncholine- and choline-containing phospholipids. However, the noncholine- containing fraction contained cerebroside which was ap-

[6] Unpublished data.

parent from the low phosphorus content of this fraction and from the thin-layer chromatograms. The cerebroside was eluted from the column in both the neutral lipid and initial phospholipid fractions. This tendency of cerebroside to elute with the ethanolamine and serine phosphatides fraction has been reported by De Gier and Van Deenen (14).

The distribution of the lipids between the 3 fractions was found to be independent of the dietary changes studied, as was the distribution of the lipid phosphorus between the choline- and noncholine-containing lipids.

In addition to fatty acids, long-chain aldehydes were detected in the phospholipids (tables 2 and 3). This is to be expected in view of the occurrence of plasmalogens in the red cell lipids (12, 15, 16). The aldehydes occurred principally in the noncholine-containing lipids, constituting 16 to 17% of the total long-chain residues. On this basis, the plasmalogens would comprise over 30% of this fraction. Farquar (12) has reported finding 67% of the ethanolamine phosphatides of human erythrocytes in this form. After saponification and re-chromatography of the methylation mixture, in which the aldehydes were present as dimethyl acetals, a number of minor components were detected in addition to the 3 aldehydes reported in tables 1 and 2. They were not investigated in the present study.

In the noncholine-containing phospholipids, arachidonic acid was the major component, constituting over 30% of the total long-chain residues. The compound tentatively identified as docosatetraenoic acid was present to the extent of 8% in this fraction when corn oil was the dietary fat. The unsaturated acids as a group accounted for about 65% of the total long-chain residues.

The choline-containing lipids contained relatively little aldehyde in comparison with the noncholine-containing fraction. Under the operating conditions used, the separation of octadecenal dimethyl acetal from methyl stearate was not achieved when the latter was in great excess. Consequently no figure is reported for this aldehyde in table 2. It was, in fact, present to the extent of about 0.4% in this fraction, as was evident from an analysis of the nonsaponifiable fraction of the methylation mixture. The fatty acids of this phospholipid fraction were predominantly saturated acids (over 60%) and palmitic acid was the major component. Linoleic acid was present in higher concentrations than in the noncholine fraction. The polyenoic 22-carbon compounds were found in much lower concentrations in this choline containing fraction. This was particularly evident in the case of the compound identified as the tetraene.

The distribution of acids presented here is consistent with data published for species other than the rat. Hanahan et al. (17), reporting on human erythrocyte phosphatides, found that the phosphatidyl ethanolamine contained predominantly unsaturated acids and that arachidonic acid was the major component of this fraction. In the more saturated choline phosphatides, linoleic acid was the predominant polyunsaturated acid. Farquar (12) and Kates and James (18) obtained similar results for the fatty acid distribution in human and fowl erythrocytes, respectively.

Monsen et al. (2) reported that the arachidonic acid content of the total rat erythrocyte phospholipids was independent of the dietary fat, whereas linoleic acid was higher when a fat such as safflower oil, which is high in this acid, was substituted for one low in linoleic acid content, such as hydrogenated coconut oil. With the more saturated dietary fat, oleic acid was higher. Stearic and palmitic acids were unchanged. Witting and his co-workers (1) obtained similar results when the total lipid fatty acids were examined. In the present study, the response of linoleic acid level to dietary fat was evident in both of the phospholipid fractions, but was more apparent in the choline containing lipids, which contained a higher percentage of linoleic acid than the noncholine-containing lipids. An increase in oleic acid content was also more noticeable in the choline fraction when lard was the dietary fat. The longer chain polyunsaturated acids were also responsive to diet. When corn oil was used in the diet, the docosatetraene and, to a lesser

extent, the pentaene were present in higher concentrations than when lard was the dietary fat. Palmitic, stearic and arachidonic acids showed only slight variations with diet as did the aldehyde components of the lipids. Apparently, arachidonic acid is of sufficient importance to the structure of the red cell that it is synthesized at the expense of linoleic acid when the dietary supply of the latter is restricted. Vitamin E deficiency did not appear to exert any influence on the composition of the stroma phospholipids (19).

The analyses were carried out as rapidly as possible to minimize any oxidation of the unsaturated lipids. In most cases, the fatty acid analyses were completed within 36 hours of the exsanguination of the animals. Preliminary studies indicated that storage of the lipids for any extended period of time resulted in the appearance of additional peaks on the gas chromatogram. The compounds represented by these peaks had relatively long retention times (carbon numbers in excess of 27.0); when they appeared, there was a concurrent decrease in the amount of unsaturated methyl esters present. The nature of these oxidation products and the conditions controlling their formation is under investigation.

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Effect of NaF on the Growth of Rats with Varying Vitamin and Calcium Intakes

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ABSTRACT Weanling rats fed an otherwise adequate diet for 28 days were given a vitamin supplement supplying levels of thiamine, riboflavin, pantothenic acid, pyridoxine and vitamin A that limited growth to two-thirds or one-half of the maximal rate. Weight gains and food efficiency values measured at 28 days were not affected by the inclusion of 0.5 or 1.0 mg of fluoride as NaF in the daily vitamin supplement. When the calcium levels of the diets were reduced to one-third or two-thirds that of the usual diet, the fluoride supplements also had no effect on growth of the rats. Fecal and total carcass fluoride revealed no significant effect of the level of dietary calcium on fluoride absorption and retention. The weight, ash and calcium content of the femur increased with the level of calcium in the diet. The total fluoride per femur was the same at all calcium levels, but the fluoride concentration in the femur was decreased as the level of calcium in the diet was increased. Incisor weights, and calcium content per incisor were not affected by the dietary calcium levels; both concentration and amount of fluoride per incisor were decreased with increasing dietary calcium.

The reluctance of many American communities to accept fluoridation of the water supply as a safe and useful way of reducing the incidence of dental caries has led to alternative methods for administering daily fluoride supplements to infants and children. Studies have shown that daily fluoride supplements are of benefit in decreasing caries development in children. Arnold et al. (1) have demonstrated that children receiving one milligram of fluoride daily as a tablet or in solution had a reduction in decay rate similar to that in children drinking fluoridated water. Ericsson (2) in a review of the work of a number of investigators of the effect of fluoride tablets in the prophylaxis of dental caries concluded that fluoride tablets are effective in reducing the incidence of dental caries. Rusoff (3) noted a significant reduction in caries in school children who drank each day a glass of milk (240 ml) containing one milligram of fluoride added as NaF.

Studies have also been directed to the interrelationships of fluoride with vitamins and other minerals. Hauck et al. (4) demonstrated that vitamin D reduced the toxicity of fluoride in rats fed a diet low in calcium. Phillips and Chang (5) reported that orange juice, presumably due to its ascorbic acid content, would prolong the survival period of young growing rats when fed NaF. Muhler (6) demonstrated that vitamin C increased the storage of fluoride in guinea pigs as compared with levels observed in control animals not receiving vitamin C.

The extent to which calcium may interfere with fluoride absorption has been studied in many laboratories. Machle and Largent (7) demonstrated in man that when fluoride was administered as a solution of NaF or CaF_2, 95% was absorbed, whereas when powdered CaF_2 was given, 60% of the fluoride was absorbed, and when supplied in bone meal, only 40% was absorbed. They concluded that fluoride absorption was influenced by the solubility of the fluoride salt, by its physical form when given and by the presence or absence of calcium in the gut. Muhler and Weddle (3) observed no difference in the retention of fluoride by rats when 5 or 10 ppm F (as NaF) were supplied in milk or water. However, with 2 ppm fluoride, milk significantly decreased fluoride retention and this was attributed to the calcium content of the milk. Wagner and Muhler (9)

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studied the retention of fluoride when 2 mg of calcium were given 15, 30, and 60 minutes after the administration of 2 mg fluoride as NaF. They found that this low level of calcium reduced fluoride retention to 85% of the amount retained by the NaF treated controls without added calcium. The controls retained 54.8% of the fluoride administered once a day over the 8-day test, whereas the calcium-treated groups retained 46.7%.

The present studies were designed to determine whether the utilization of vitamins was affected by inclusion of fluoride in the vitamin supplement and whether the level of calcium in the diet affected absorption and retention of the fluoride supplement given once a day. Groups of rats were given a single daily supplement containing 10 vitamins. The concentrations of 5 of these were provided at levels which allowed weight gains of either two-thirds or one-half of the maximal growth rate. These vitamin supplements were given with and without fluoride and with diets differing in calcium content in order to determine the effect of fluoride on vitamin utilization as measured by growth and the effect of calcium on fluoride uptake.

TABLE 1

Experimental diet

Ingredients	
	g/100 g
Casein[1]	18.0
Corn oil	10.0
Sucrose	63.7
Non-nutritive fiber	4.0
Salt mixture[2]	4.0
Choline bitartrate	0.2
Inositol	0.1
	mg/100 g
p-Aminobenzoic acid	10.0
Folic acid	0.2
α-Tocopheryl acetate	5.0

	Supplied for	
	Two-thirds growth rate	One-half growth rate
Daily vitamin supplements[3]		
Thiamine, μg	12.5	6.2
Riboflavin, μg	25.0	12.5
Pyridoxine·HCl, μg	10.0	5.0
Pantothenic acid, μg	100.0	50.0
Vitamin A, units	4.0	2.0

[1] Labco Vitamin-free Casein, The Borden Company, New York.

[2] Jones and Foster (15) salt mixture with NaF added to give 4 μg F/10 g diet. In the second experiment, Ca and P levels of the salt mixture were modified so that all diets had 413 mg P/100 g, whereas Ca levels were 207, 413 or 620 mg Ca/100 g diet.

[3] Each supplement also contained 500 μg niacinamide, 2 μg biotin, 1 μg vitamin B_{12}, 12 units vitamin D and 3 mg vitamin C.

EXPERIMENTAL

Groups of male weanling rats of the McCollum-Wisconsin strain were selected according to body weight and litter for both experiments. Each animal was housed in an individual screen-bottom cage in an air conditioned rat room, and records were kept of food and water intake and weight gain. The composition of the experimental diet is shown in table 1.

Stock solutions of vitamins were made in a glycerine, water and polyoxyethylene sorbitan monooleate (Tween 80) vehicle. Each milliliter of the vitamin solution provided thiamine, riboflavin, pyridoxine, pantothenic acid and vitamin A at levels which allowed about two-thirds or one-half of the maximal growth rate. These levels were derived from studies of the vitamin requirements for the growth of rats as reviewed by Brown and Sturtevant (10) and from studies in this laboratory. Other vitamins were supplied in excess either in the supplement or in the diet. Rats received one milliliter of the vitamin supplement each morning by stomach tube with or without NaF (0.5 and 1.0 mg F/rat/day).

Calcium determinations were performed by a titrimetric method with permanganate (11) and phosphorus by color development as phosphomolybdate (12). Fluoride was determined by a modification of the method of Icken and Blank (13). Calcium, phosphorous and fluoride determinations for each group were made on pooled samples.

Experiment 1. In the first experiment, 4 groups of 10 rats each were fed the experimental diet and were given each day for 28 days a vitamin supplement (with either zero or 1 mg F) permitting growth rates of two-thirds or one-half of the maximal rate. The data summarizing the weight gains, food efficiency values and fluoride levels in the femurs and incisors of these animals are given in table 2.

Virtually identical weight gains and food efficiency values were observed with and

TABLE 2

Four-week weight gains, food efficiency values and femur and incisor fluoride levels in male weanling rats (exp. 1)

Group no.	Vitamin supplement restriction	Fluoride supplement	Wt gain, 4 weeks	Caloric efficiency	Femur				Incisor			
					Wt	Fluoride			Wt	Fluoride		
		mg/rat/day	*g*	*g gain/1000 kcal*	*mg*	*mg/femur*	*mg/g femur*		*mg*	*mg/incisor*	*mg/g incisor*	
1	For one-half growth rate	0	63 ± 11[1]	78 ± 12[1]	310	0.02	0.06		76	0.01	0.09	
2	For one-half growth rate	1	64 ± 6	81 ± 10	290	0.55	1.90		72	0.12	1.64	
3	For two-thirds growth rate	0	93 ± 17	94 ± 10	338	< 0.01	< 0.03		87	< 0.01	< 0.03	
4	For two-thirds growth rate	1	90 ± 15	97 ± 6	325	0.50	1.54		90	0.12	1.34	

[1] SD.

without fluoride at each level of vitamin intake. Rats given the one-half growth-limiting vitamin supplements gained 63 and 64 g with and without fluoride and those receiving the two-thirds growth-limiting supplements gained 93 and 90 g with and without fluoride. (Weight gains observed with this diet fully supplemented with adequate vitamins usually average about 140 g in 4 weeks.) If sodium fluoride had any deleterious effect on vitamin utilization, it would be evident at the limiting vitamin levels used in the present study.

The total amounts of fluoride per femur and per incisor were similar in groups 2 and 4. Since the rats in group 4, receiving the higher vitamin intakes, gained more weight and had greater femur and incisor weights than did rats in group 2, receiving one-half of these vitamin levels, the concentration of fluoride in the femurs and incisors of group 4 was lower than in group 2. The concentration of fluoride noted in the femurs in this study are in fair agreement with those reported by Buttner and Muhler (14), using a semi-purified diet (cane sugar, casein, cottonseed oil, salts, and vitamin mixture and 2 levels of fat). These investigators also administered one milligram of fluoride (as NaF) per rat per day for 28 days and found femur fluoride levels of 1.82 ± 0.17 and 2.26 ± 0.05 mg F/femur.

Experiment 2. Since it has been suggested that diets high in calcium, as used in the first experiment (620 mg/100 g diet), may decrease fluoride absorption and mask any possible effect of fluoride on vitamin utilization, a second experiment was set up with varying levels of calcium in the diet using the vitamin levels which permitted two-thirds of the maximal growth rate. Calcium was added to each diet as calcium carbonate to supply 217, 413 and 620 mg/100 g of diet. Phosphorus was added as KH_2PO_4, so that the total diet including the contribution from casein would supply 413 mg. The Ca/P ratios in the 3 diets were 0.5, 1.0 and 1.5.

The 4-week weight gains and food efficiency values shown in table 3 were similar for all 9 groups. These data show that with only one-third or two-thirds of the level of dietary calcium used in the first

experiment, supplements of 0.5 and 1.0 mg F/rat/day had no deleterious effects on weight gain and food utilization in rats limited in growth by vitamin intake.

Feces were collected from all animals during the fourth week of this study and analyzed for fluoride, calcium, and phosphorus in order to calculate the net absorption of these minerals, as shown in table 4. At the 2 lower calcium levels (207 and 413 mg Ca/100 g diet), over 95% of the administered fluoride was absorbed, whereas at the highest dietary calcium level, 84 to 90% was absorbed. Data on carcass fluoride content and on the percentage of fluoride retained for the entire study confirm that at all 3 levels of calcium in the diet, calcium had little effect on the absorption of fluoride. Approximately 60% of the fluoride administered during the course of the experiment was retained in the carcass with a dose of 0.5 mg F/day and about 50% when 1.0 mg/day was given.

The percentage of calcium absorbed during the fourth week was greatest at the intermediate dietary level (413 mg Ca/100 g diet). The percentage of calcium ab-

TABLE 3

Four-week weight gains, food intakes and food efficiency values of male weanling rats given sodium fluoride-vitamin mixtures with different levels of dietary calcium (exp. 2)

Treatment	Weight gain	Food intake	Caloric efficiency
	g	*g*	*g gain/ 1000 kcal*
Calcium, 207 mg/100 g diet			
No fluoride	83 ± 11[1]	233 ± 29[1]	86 ± 8[1]
0.5 mg fluoride/day	89 ± 11	235 ± 22	91 ± 5
1.0 mg fluoride/day	87 ± 10	230 ± 16	91 ± 4
Calcium, 413 mg/100 g diet			
No fluoride	89 ± 10	240 ± 30	89 ± 6
0.5 mg fluoride/day	85 ± 18	238 ± 23	86 ± 12
1.0 mg fluoride/day	86 ± 10	235 ± 21	88 ± 6
Calcium, 620 mg/100 g diet			
No fluoride	88 ± 9	242 ± 18	86 ± 8
0.5 mg fluoride/day	81 ± 16	238 ± 24	81 ± 12
1.0 mg fluoride/day	88 ± 10	240 ± 23	88 ± 6

[1] SD.

TABLE 4

Fourth week fluoride, calcium and phosphate uptake and carcass fluoride levels (exp. 2)

Treatment	Net absorption — fourth week				Fluoride	
	F	Ca	P	Ca	Carcass	Retention[1]
	% of intake			*mg*	*mg*	*% of intake*
Calcium, 207 mg/100 g diet (Ca/P ratio: 0.5)						
No fluoride	—	75.3	87.6	116	0.05	—
0.5 mg fluoride/day	94.9	79.3	96.0	119	8.32	59.4
1.0 mg fluoride/day	97.0	74.2	96.0	112	13.18	47.1
Calcium, 413 mg/100 g diet (Ca/P ratio: 1.0)						
No fluoride	—	90.5	96.2	286	0.05	—
0.5 mg fluoride/day	95.1	91.1	96.2	288	8.85	63.2
1.0 mg fluoride/day	97.7	88.7	88.7	274	14.18	50.6
Calcium, 620 mg/100 g diet (Ca/P ratio: 1.5)						
No fluoride	—	65.1	83.4	312	0.06	—
0.5 mg fluoride/day	84.2	67.2	79.9	311	8.67	61.9
1.0 mg fluoride/day	90.1	62.5	85.0	293	13.44	48.0

[1] Total carcass fluoride/administered fluoride.

TABLE 5

Femur and incisor weights, ash and calcium levels and fluoride concentrations (exp. 2)

Treatment	Femur			Femur fluoride		Incisor		Incisor fluoride	
	Wt	Ash	Ca			Wt	Ca		
	mg	*mg/ femur*	*mg/ femur*	*mg/ femur*	*mg/g femur*	*mg*	*mg/ incisor*	*mg/ incisor*	*mg/g incisor*
Calcium, 207 mg/100 g diet									
No fluoride	218	77.0	24.0	0.01	0.04	43.3	10.1	0.01	0.24
0.5 mg fluoride/day	209	73.1	22.9	0.31	1.50	51.0	12.1	0.07	1.36
1.0 mg fluoride/day	223	70.0	21.4	0.49	2.20	56.6	11.0	0.16	2.41
Calcium, 413 mg/100 g diet									
No fluoride	252	85.6	26.4	0	0	52.0	12.1	0.01	0.24
0.5 mg fluoride/day	248	86.5	28.4	0.34	1.39	53.8	11.4	0.08	1.33
1.0 mg fluoride/day	247	83.9	26.7	0.54	2.19	47.1	10.7	0.10	2.04
Calcium, 620 mg/100 g diet									
No fluoride	312	106.9	34.7	0.02	0.06	46.7	11.7	0.01	0.19
0.5 mg fluoride/day	299	107.5	32.8	0.31	1.04	51.1	11.6	0.05	0.85
1.0 mg fluoride/day	302	103.0	33.8	0.51	1.70	51.8	10.8	0.09	1.41

sorbed averaged 90% at this level, 76% at the lower calcium level and 65% at the higher calcium level. On an absolute basis, the amount of calcium absorbed was almost as great from the diet intermediate in calcium as from the diet with the higher calcium level.

The percentage of dietary phosphorus absorbed was decreased at the highest dietary level of calcium (also highest Ca/P ratio). Phosphorus absorption averaged 93% at the low and intermediate levels of dietary calcium and 83% at the highest calcium level. Absorption of both calcium and phosphorus from all diets was apparently unaffected by fluoride supplementation.

The femur weights and their ash and calcium content were increased as the dietary calcium level was increased (table 5). The milligrams of fluoride per femur were similar at the 3 calcium levels and about 60% higher in rats receiving one milligram of fluoride that in rats receiving 0.5 mg. When 0.5 mg F was given/day, 0.31, 0.34, and 0.31 mg/femur were observed and with one milligram of F, 0.49, 0.54, and 0.51 mg F/femur were noted at the 3 calcium intake levels, respectively. When the femur fluoride was calculated as concentration in milligrams per gram of femur, the concentration of F decreased as the femur weights increased. With increasing levels of calcium in the diets, these values were 1.50, 1.39, and 1.04 mg F/g femur in rats receiving 0.5 mg F/day and 2.20, 2.19, and 1.70 mg F/g femur in rats receiving one milligram of F/day.

The weights of the incisors and their calcium content were similar at all 3 levels of calcium. With increasing levels of dietary calcium, there was some decrease in the fluoride content of the incisor either when calculated as amount of fluoride per incisor or concentration per gram of incisor.

DISCUSSION

The data indicate that the daily oral administration of one milligram F as NaF to the weanling rat did not alter the utilization of vitamins or of the diet as measured by growth rates. Buttner and Muhler (10) also observed that fluoride (up to 2 mg F/day) did not affect growth of rats receiving a complete diet. When 5 vitamins which readily affect growth in the rat were fed at levels permitting two-thirds or one-half of the normal growth rate, fluoride also had no effect.

Levels of calcium from 207 to 620 mg calcium/100 g of diet had little effect on the absorption and retention of fluoride, given as a single daily dose. At each level of dietary calcium the same amounts of fluoride were retained in the femurs and in the carcasses. However, the greater rate of bone growth permitted by the diets with greater calcium levels resulted in a decrease in the concentration of fluoride in the femurs of these animals when expressed as milligrams of fluoride per gram of femur.

These observations are in contrast with the data on incisors where the weights of the incisors were not affected by the calcium level of the diet. Both the level and concentration of fluoride per incisor decreased with increasing calcium level in the diet.

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Water Addition to Purified Diets

II. EFFECTS OF WATER AND CARBOHYDRATE ON PROTEIN EFFICIENCY RATIO IN RATS

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ABSTRACT Paired feeding studies with rats have shown that the increased protein efficiency ratio resulting from the addition of 20% water to a 9% protein purified diet was not due to increased protein consumption. It was shown further that when sucrose was the source of carbohydrate, increments of water addition of from 5 to 35% resulted in increases in the protein efficiency ratio of casein. Furthermore when cornstarch or dextrin was used as the carbohydrate source an increased PER was not obtained with casein when 20% water was added, but was observed when 50% water was added. The effect of adding 20% water to a dry purified diet containing sucrose as the carbohydrate was also observed when isolated soybean protein, cottonseed protein or enriched white flour was used as the source of protein.

Previous studies (1) have shown that the addition of 20% water to purified diets containing 6 to 12% protein resulted in an increase in growth rate and a higher protein efficiency ratio[1] in rats than that obtained when no water was added to the diet. To investigate these observations further, a series of experiments was undertaken to determine what other factors might influence this effect of water and if possible to determine the cause of this effect.

METHODS

Twenty one-day-old rats, descendants of the Sprague-Dawley strain[2] were used in all experiments. The animals were housed individually in screen-bottom cages kept in an air-conditioned room maintained at 23 to 25°C with a relative humidity of 45 to 55%. Food and water were supplied ad libitum except in the paired feeding experiments. Daily records were kept of the amount of food consumed by each rat. The animals were weighed individually at weekly intervals. The diets were prepared as previously reported (1). The diets were stored in airtight containers in the refrigerator to minimize possible moisture loss. Each protein efficiency ratio determination was measured over a 4-week period.

Paired feeding experiments. Studies were undertaken to determine whether the increased rate of gain and higher protein efficiency ratio (PER) observed when 20% water was added to a dry purified diet was the result of increased protein consumption. Ten rats were fed a 9% protein diet with no water added and 10 rats of similar weight were pair-fed the same diet to which 20% water was added. The composition of the dry diet is shown in table 1. The daily solids intake of each rat that received the wet diet was limited to the solids intake of its paired mate receiving the dry diet. The experiment was replicated twice. The results are shown in table 2. These data indicate that although the rats that had received the water-containing diet consumed no more protein than those receiving the dry diet, the PER was significantly higher $P < 0.01$ as measured by the t test (2).

Water increment studies. Since it was known that the effect observed could be obtained with 20% water, the effect of various increments of water addition on PER was investigated. Water was added to the basal dry diet at levels of 5, 10, 15, 20, 25, 30, and 35%. The experiment was replicated 4 times using 5 rats/group. The combined results were then subjected to

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[1] Grams gained per gram protein consumed.

[2] S. and K. Animal Company, Jamesburg, New Jersey.

TABLE 1

Composition of diet

	g/100 g
Casein[1]	10.16
Sucrose	71.64
Cottonseed oil	8.00
Vitamin mix	0.10
Choline chloride	0.10
d-α-Tocopheryl succinate	100 units
Cellulose	5.0
Mineral mix[2]	5.0
Vitamin A palmitate[3]	2000 IU
Vitamin D_2[3]	200 IU
Vitamin mix	
Thiamine·HCl	0.500
Riboflavin	0.800
Niacin	4.000
Pyridoxine·HCl	0.500
Ca-D-pantothenate	4.000
Biotin	0.040
Folic acid	0.200
Menadione	0.500
Cyanocobalamin	0.003
p-Aminobenzoic acid	10.000
i-Inositol	10.000
Cornstarch to make	100.000

[1] ANRC High Nitrogen Casein — 30 mesh, Sheffield Chemical Division, Norwich, New York.
[2] Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets high and low in phosphorus. J. Nutrition, 24: 394.
[3] Myvax, dry vitamin A palmitate with vitamin D_2, Distillation Products Industries, Rochester, New York.

appropriate statistical analysis by the *t* test (2). The results are shown in table 3. Except for 20 to 25% water addition each increment resulted in some increase in PER. All increments of water addition resulted in a highly significant increase in PER ($P < 0.01$) over that obtained when no water was added to the diet. There was no significant difference between 5 and 10% water addition, but all increments of 15% and above were highly significant over 5% ($P < 0.01$). The addition of 15% water was significantly higher than 10% ($P < 0.05$) and increments of 20 to 35% yielded PER's that were highly significant over 10% ($P < 0.01$). There was no significant difference between 15, 20, or 25% water addition. There was no significant difference between the PER's at increments of 20 to 35%.

Carbohydrate experiments. Another series of experiments was undertaken to determine whether the addition of water at 20 or 50% would yield an increase in PER when carbohydrate sources other than sucrose were used. Sucrose was included in the experiment as a standard. The diet previously described was used with either cornstarch or dextrin replacing sucrose. The experiment was replicated 4 times using 5 rats/group. The results were combined and analyzed statistically as previously described. The PER values are shown in table 4. These data indicate that, as before, the addition of 20% water yielded a highly significant ($P < 0.01$) increase in PER when sucrose was used as the carbohydrate. This level of water addition did not yield a significant increase in PER when cornstarch or dextrin was used as the source of carbohydrate. In the presence of the latter carbohydrates, however, the addition of 50% water did yield a highly significant increase in PER over that obtained when either zero or 20% ($P < 0.01$) water was added. It also was found that at 0% water addition there was no significant difference between the sucrose and starch or between starch and dextrin. However, dextrin yielded a highly significant increase in PER over that obtained with sucrose ($P < 0.01$). When 20% water was added a highly significant increase in PER was obtained with sucrose compared with either starch or dextrin ($P < 0.01$). There was no significant difference between the PER's obtained with either starch or dextrin at this level of water.

TABLE 2

Protein efficiency ratio (PER) values in paired feeding studies

Trial	Diet	Avg protein intake	Avg wt gain	Avg PER
1	Dry	18.38	39.6	2.15 ± 0.06[1]
1	Wet	18.23	48.5	2.65 ± 0.05[2]
2	Dry	21.62	53.8	2.48 ± 0.06
2	Wet	21.22	60.7	2.85 ± 0.07[2]

[1] Mean value ± SE.
[2] Highly significant ($P < 0.01$) over dry weight.

TABLE 3

Protein efficiency ratio (PER) values from water increment studies

Water added	Avg PER
%	
0	1.84 ± 0.06[1]
5	2.61 ± 0.05
10	2.66 ± 0.07
15	2.88 ± 0.06
20	2.94 ± 0.05
25	2.91 ± 0.06
30	3.02 ± 0.01
35	3.04 ± 0.04

[1] Mean value ± SE.

TABLE 4

Protein efficiency ratio (PER) values obtained with various carbohydrate sources

Water added	Average PER		
	Sucrose	Starch	Dextrin
%			
0	2.31 ± 0.06[1]	2.59 ± 0.12	2.71 ± 0.08
20	3.08 ± 0.06	2.82 ± 0.10	2.75 ± 0.09
50	2.07 ± 0.14	3.11 ± 0.06	3.24 ± 0.07

[1] Mean value ± SE.

When 50% water was added to the diet, both starch and dextrin yielded a significantly higher ($P < 0.01$) PER than sucrose and there was no significant difference in PER between starch and dextrin.

Protein source studies. Since all previous studies had been carried out using high nitrogen casein[3] as the source of protein, it was decided to determine whether the effect of water addition would be found when other protein sources were used. A diet similar to that already described was used. These diets contained 9% protein from either enriched white flour, isolated soybean protein, or cottonseed protein in place of casein. The nonprotein components of the diets were so adjusted that the proximate composition of all diets was the same. The experiment was replicated twice using 10 rats/group each time. The data were combined and analyzed statistically as previously described. The results are shown in table 5. With isolated soybean protein and cottonseed protein the addition of 20% water resulted in a highly significant ($P < 0.01$) increase in PER over that obtained when no water was added to the diet. When enriched white flour was used as the source of protein, the addition of 20% water resulted in a significant ($P < 0.02$) increase in PER over that obtained when no water was added to the diet.

TABLE 5

Protein efficiency ratio (PER) values obtained with various protein sources

Water added	Average PER		
	Soybean protein	Cottonseed protein	Enriched white flour
%			
0	1.42 ± 0.08[1]	1.44 ± 0.08	0.497 ± 0.0283
20	2.08 ± 0.04	2.11 ± 0.05	0.602 ± 0.0251

[1] Mean value ± SE.

DISCUSSION

The reasons for the increased rate of gain and higher PER obtained when water is added to a dry purified diet have not been explained. The paired feeding studies preclude the possibility that an increased protein consumption is the causative factor. This effect was not observed with either cornstarch or dextrin at the 20% level of water addition, but was observed at the 50% level. It should be pointed out that when either of these carbohydrates was used, the diets did not appear as moist with 20% added water as did the sucrose diet. This was due to the greater water binding capacity of cornstarch and dextrin. The "dry" diets did contain some moisture. In the case of the sucrose diets this amounted to about 1.5% and in the case of cornstarch and dextrin about 8%. However, since at the 0% level of water addition dextrin gave a significant increase in PER over that obtained with sucrose and starch did not, the difference in moisture content of the "dry" diets is probably not as important as the difference in water-binding capacity of the different carbohydrates. Womack et al. (3) and Marshall and Womack (4) have shown that adult rats fed diets containing low levels of amino acids and dextrin utilized nitrogen more efficiently than similar animals receiving comparable diets containing sucrose. This was confirmed by Spivey et al. (5) using a 9% casein diet with young growing rats. Our results with sucrose and dextrin in the absence of water addition confirmed these

[3] ANRC High Nitrogen Casein — 30 mesh, Sheffield Chemical Division, Norwich, New York.

results. However when 20% water was added a reversal of this observation was obtained. Cizek (6), as well as Harper and Spivey (7), has shown that the osmotic pressure of food in the gastrointestinal tract may have an effect on growth. This factor may be involved in the effect of added water on protein efficiency ratio.

The difference in the appearance of the sucrose vs. cornstarch or dextrin diets with various moisture levels and the resulting difference in PER values might indicate that the effect may be a physical one. That this effect has been observed with casein, isolated soybean protein, cottonseed protein and enriched white flour, indicates that it is not limited to one kind of protein.

The results obtained indicate clearly that the water content of the diet and the carbohydrate source of the diet and their interrelationship must be taken into consideration when protein quality is to be measured. The results observed in these experiments emphasize the need for further investigations of the factors involved in protein efficiency ratio determinations.

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Invitation for Nominations for 1964 American Institute of Nutrition Awards

Nominations are requested for the 1964 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1963*, to be considered for the 1964 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

1964 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
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1950 – H. C. Sherman
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1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black

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1964 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
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1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
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1957 – G. R. Cowgill
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1964 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award for Research in Nutrition has been re-established by the Mead Johnson Company. This award of $1000 and a certificate will be presented each year to an investigator who has not reached his 46th birthday during the calendar year in which the award is given and will be based on a single outstanding piece of research in nutrition published in the year preceding the annual meeting, or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

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Invitation for Nominations for 1964 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows will be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

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Icie Macy Hoobler (1960)
Paul E. Howe (1960)
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C. Glen King (1963)
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Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

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