White House Conference on Food, Nutrition and Health

The White House Conference on Food, Nutrition and Health will be held December 2, 3, and 4, 1969, in Washington, D. C. The purpose of the Conference is to advise the President and lay the foundation for a national nutrition policy. More than 2,500 educators, scientists, medical and health professionals, representatives of agriculture and the food industry, and spokesmen for consumer and social action groups, including the poor, will join with federal, state and local government officials at the meeting. Twenty-six pre-Conference panels will meet to consider a wide range of food and nutrition problems, and to draft provisional recommendations. The Conference participants will begin with these recommendations. In working sessions they will review the various proposals critically and discuss them with the panels that prepared them. From this interchange the Conference expects to produce its final conclusions — a carefully developed and broadly acceptable body of recommendations for the President, government, the private sector of the economy and the American people.

SECTION ONE

SURVEILLANCE AND EVALUATION OF THE STATE OF NUTRITION OF THE AMERICAN PEOPLE

1. *A Continuing Monitoring System of Dietary and Nutritional Evaluation.*

   Chairman: Dr. James P. Carter, Assistant Professor of Nutrition and Instructor in Pediatrics, Vanderbilt University School of Medicine, Nashville, Tenn.

   Vice Chairman: Dr. Andrew W. Greeley, Professor of Sociology and Program Director, National Opinion Research Center, University of Chicago, Chicago, Ill.

2. *Standards of Dietary and Nutritional Evaluation.*

   Chairman: Dr. D. Mark Hegsted, Professor of Nutrition, School of Public Health, Harvard University, Boston, Mass.

   Vice Chairman: Dr. Robert E. Shank, Danforth Professor of Preventive Medicine, Washington University School of Medicine, St. Louis, Mo.

3. *Federal and State Administrative Structure of Monitoring Organizations.*

   Chairman: William D. Carey, Senior Staff Consultant, Arthur D. Little, Inc., Washington, D.C. Former Assistant Director, Bureau of the Budget.

   Vice Chairman: Dr. John H. Browe, Director, Bureau of Nutrition, New York State Department of Health, Albany, N. Y.

SECTION TWO

ESTABLISHING GUIDELINES FOR THE NUTRITION OF VULNERABLE GROUPS (WITH SPECIAL REFERENCE TO THE POOR)

1. *Pregnant and Nursing Women and Infants.* Subpanel: Nutrition and Mental Development.

   Chairman: Dr. Charles U. Lowe, Scientific Director, National Institute of Child Health and Human Development, Department of Health, Education and Welfare, Bethesda, Md.

   Vice Chairman: Dr. Howard N. Jacobson, Department of Obstetrics, School of Medicine, Harvard University, Boston, Mass.

2. *Children and Adolescents.* Subpanels: 1) Day Care and Preschool Nutrition. 2) Fitness.

   Chairman: Dr. Samuel J. Fomon, Professor of Pediatrics, School of Medicine, University of Iowa, Iowa City, Iowa.

   Vice Chairman: Dr. Robert J. Haggerty, Professor and Chairman, Department of Pediatrics, University of Rochester, and Pediatrician-in-Chief, Strong Memorial Hospital, Rochester, N. Y.

3. *Adults in Affluence:* The Middle Aged and Degenerative Diseases. Subpanels: 1) Obesity. 2) Nutrition and Heart Disease. 3) Nutrition and Dental Problems. 4) Fitness.

   Chairman: Dr. Ancel Keys, Director of Physiology and Hygiene Laboratory, School of Public Health, University of Minnesota, Minneapolis, Minn.

   Vice Chairman: Dr. Irvine H. Page, physician, and Senior Consultant, Research Division, Cleveland Clinic Foundation, Cleveland, Ohio.

4. *The Aging.*

   Chairman: Dr. Edward L. Bortz, Senior Consultant in Medicine, Lankenau Hospital, Philadelphia, Pa. Former President, American Medical Association.

   Vice Chairman: Dr. Donald M. Watkin, Staff Physician, Veterans Administration Hospital, West Roxbury, Mass. Formerly Program Chief, Research in Nutrition and Clinical Research in Gerontology, Veterans Administration.

5. *The Sick:* Nutrition and Public Health. Nutrition and Hospital Care. The Role of Outpatient Services. Outreach into the Community. Medical Care.

   Chairman: Dr. W. H. Sebrell, Jr., R. R. Williams Professor of Nutrition and Director, Institute of Nutrition Sciences, Columbia University, New York, N. Y. Former Director, National Institutes of Health.

   Vice Chairman: Dr. George J. Christakis, Associate Dean and Professor of Community Medicine (Nutrition), Mt. Sinai School of Medicine, New York, N. Y.

6. *Groups for Whom the Federal Government Has Special Responsibilities:* District of Columbia. Puerto Rico. Virgin Islands. Guam. Samoa. Indians. Eskimos. Migrant workers.

   Chairman: Dr. William J. Darby, Professor and Chairman, Department of Biochemistry, and Professor of Medicine in Nutrition, Vanderbilt University School of Medicine, Nashville, Tenn.

   Vice Chairman: Dr. Nevin S. Scrimshaw, Professor of Nutrition and Head, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass.

SECTION THREE

THE PROVISION OF FOOD AS IT AFFECTS THE CONSUMER: GUIDELINES FOR FEDERAL ACTION

1. *Traditional Foods:* Guidelines for Trends Affecting Nutrition and Nutritionally Improved Foods. Trends in Modification and Merchandising of Traditional Foods.

   Chairman: William B. Murphy, President, Campbell Soup Company, Camden, N. J.

   Vice Chairman: Dr. Alfred E. Harper, Head, Department of Nutritional Sciences, University of Wisconsin, Madison, Wis.

2. *New Foods:* Standards of Food Identities that Simulate Traditional Foods. Impact of New Technologies on Nutritional Value. Nutritional Supplements.

   Chairman: Dr. Richard S. Gordon, Vice President and General Manager, New Enterprise Division, Monsanto Company, St. Louis, Mo.

   Vice Chairman: Dr. Gladys A. Emerson, Department of Nutrition, School of Public Health, University of California at Los Angeles, Los Angeles, Calif.

3. *Food Safety:* Food Additives. Administrative Structure to Assure Safety.

Chairman: Donald M. Kendall, President and Chief Executive Officer, Pepsico, Inc., New York, N. Y.

Vice Chairman: Dr. Julius M. Coon, Professor and Head, Department of Pharmacology, Jefferson Medical College, Philadelphia, Pa.

4. *Food Quality:* Guidelines and Suggested Administrative Structure.

Chairman: Dr. Emil M. Mrak, Chancellor Emeritus and Professor Emeritus of Food Technology, University of California at Davis, Davis, Calif.

Vice Chairman: Dr. Richard L. Hall, Director of Research and Development, McCormick and Company, Inc., Baltimore, Md.

SECTION FOUR

NUTRITION AND NUTRITION EDUCATION

1. *Nutrition Teaching in Elementary and High Schools.*

Chairman: Dr. George M. Briggs, Professor of Nutrition and Chairman, Department of Nutritional Sciences, University of California at Berkeley, Berkeley, Calif.

Vice Chairman: Dr. Ercel Eppright, Head, Department of Home Economics, Iowa State University, Ames, Iowa.

2. *Advanced Academic Teaching of Nutrition:* Medical and Dental Schools. Nursing Schools. Dietitians, Public Health Nutritionists and Food Technologists.

Chairman: Dr. Grace A. Goldsmith, Dean, School of Public Health, Tulane University, New Orleans, La.

Vice Chairman: Dr. Theodore B. Van Itallie, Director of Medicine, St. Luke's Hospital Center, New York, N. Y.

3. *Community Nutrition Teaching.*

Chairman: Dr. Cecile H. Edwards (Mrs. Gerald A. Edwards), Chairman, Department of Home Economics, and Professor of Nutrition and Research, North Carolina Agricultural and Technical University, Greensboro, N. C.

Vice Chairman: Helen E. Walsh, Chief, Nutrition Service, Bureau of Nutrition, California Department of Public Health, Berkeley, Calif.

4. *Popular Education and How to Reach Disadvantaged Groups.* Subpanels: 1) The Role of Radio and Television. 2) The Press and Other Media. 3) Legal and Other Aspects of Quackery and Misinformation.

Chairman: Dr. Philip L. White, Secretary, Council on Foods and Nutrition, American Medical Association, Chicago, Ill.

Vice Chairman: Dr. Cortez F. Enloe, Jr., editor and publisher in medicine and nutrition, Washington, D. C.

SECTION FIVE

FOOD DELIVERY AND DISTRIBUTION AS A SYSTEM

1. *Problems of Budgeting, Marketing and Food Pricing.* Subpanel: Food Distribution in Poverty Areas.

Chairman: Dr. David L. Call, H. E. Babcock Professor of Food Economics, Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Vice Chairman: Robert D. Stuart, Jr., President and Chief Executive Officer, Quaker Oats Company, Chicago, Ill.

2. *The Family as a Delivery System:* The Role of Nutrition in Reinforcing the Family Structure. Special Problems of Poor Parents.

   Chairman: Dr. Kenneth B. Clark, Professor of Psychology, College of the City of New York, New York, N. Y. President, American Psychological Association.

   Vice Chairman: Katherine Beal (Mrs. Thaddeus R. Beal), Washington, D. C.

3. *Delivery Systems of Food and of Money for Food:* Federal, State and Local Income Maintenance, Food Stamps and Commodity Distribution. The School as a Food Delivery System. Other Food Delivery Systems. The Monitoring of Delivery Systems for Administrative and Nutritional Effectiveness.

   Chairman: Dr. Stanley Gershoff, Associate Professor of Nutrition, School of Public Health, Harvard University, Boston, Mass.

   Vice Chairman: Jean Fairfax, Director, Division of Legal Information and Community Service, NAACP Legal Defense and Educational Fund, New York, N. Y.

4. *Large-Scale Meal Delivery Systems:* Department of Defense. Veterans Administration. Penal Institutions. School Lunch Program. Subpanel: Restaurant Chains, Catering and Industrial Feeding.

   Chairman: Harvey T. Stephens, Executive Vice President, Automatic Retailers of America, Inc., Philadelphia, Pa.

   Vice Chairman: Dr. Hartley W. Howard, Vice President for Research and Development, Borden, Inc., New York, N. Y.

SECTION SIX

VOLUNTARY ACTION TO HELP THE POOR

What Can Be Done by Farmers and the Food Industry?

1. *Agricultural Production.*

   Chairman: Robert A. Brogoitti, Lazy B Ranch, La Grande, Ore.

   Vice Chairman: Edward H. Covell, Jr., President, Bayshore Food, Inc., subsidiary of Kane Miller Corporation, Easton, Md.

2. *Food Manufacturing and Processing.*

   Chairman: C. W. Cook, Chairman and Chief Executive Officer, General Foods Corporation, White Plains, N. Y.

   Vice Chairman: Gordon Edwards, Chairman, Kraftco Corporation, New York, N. Y.

3. *Food Distribution and Retailing.*

   Chairman: Donald S. Perkins, President, Jewel Companies, Inc., Melrose Park, Ill.

   Vice Chairman: Clarence Adamy, President, National Association of Food Chains, Washington, D. C.

4. *Food Packaging and Labeling.*

   Chairman: Augustine R. Marusi, Chairman and President, Borden, Inc., New York, N. Y.

   Vice Chairman: H. C. Harder, Chairman and Chief Executive Officer, CPC International, Inc., Englewood Cliffs, N. J.

5. *Promotion and Advertising.*

   Chairman: James P. McFarland, President, General Mills, Inc., Minneapolis, Minn.

   Vice Chairman: Lee S. Bickmore, Chairman and Chief Executive Officer, National Biscuit Company, New York, N. Y.

The Influence of Chronic Vitamin C Deficiency on Fatty Acid Composition of Blood Serum, Liver Triglycerides and Cholesterol Esters in Guinea Pigs

E. GINTER, R. ONDREIČKA, P. BOBEK AND V. ŠIMKO
Institute of Human Nutrition Research, Bratislava, Czechoslovakia

ABSTRACT Chronic hypovitaminosis C (2–week scorbutogenic regime followed by administration of maintenance dose of 0.5 mg ascorbic acid for 24 hours) in guinea pigs produced hypercholesterolemia and an increased accumulation of cholesterol in liver. Deficiency of ascorbic acid increased significantly the content of saturated fatty acids (up to chain length C_{16}) and decreased content of mono- and polyunsaturated fatty acids in cholesterol esters of liver. Similar changes were observed after administration of the same diet supplemented with 0.25% of cholesterol to guinea pigs saturated with vitamin C. In blood serum of vitamin C-deficient guinea pigs, the cholesterol esters contain an increased amount of linoleic acid and a decreased amount of palmitic and oleic acids. The spectrum of fatty acids in triglycerides of blood serum and liver was not significantly affected by hypovitaminosis C. It is surmised that the primary cause of the observed changes is the disturbance of cholesterol metabolism, produced by chronic vitamin C deficiency.

Considerable amounts of data have been accumulated in the literature on the effect of ascorbic acid on the metabolism of fatty acids. Various authors (1–3) have described the stimulatory effect of ascorbic acid on oxidation of polyunsaturated fatty acids and a decreased oxidation of linolenic acid in the tissues of scorbutic guinea pigs. Catalytic effect of ascorbic acid on oxidation of fatty acids in nonbiological systems (4), on peroxidation of lipids in brain microsomes (5) and on decarboxylation of 2-keto fatty acids in brain (6, 7) were reported. Synthesis of fatty acids from acetate-1-^{14}C is decreased in ascorbic acid deficiency (8, 9).

In view of these data, the question of how the lack of vitamin C will be reflected in the composition of fatty acids in tissue lipids is of great interest. In a preliminary study with acutely scorbutic guinea pigs we have found (10) that in liver and epididymal fat the fraction of polyunsaturated fatty acids increased. The acutely scorbutic guinea pigs consume a considerably smaller amount of food, and it can be assumed that starvation played a certain role in the observed changes because starvation has been found to increase the fraction of polyunsaturated fatty acids in the tissues of experimental animals (11, 12).

Owing to this fact we have used in the present study a model of chronic deficiency of vitamin C, during which the consumption of food and growth of animals is not significantly influenced (13). Apart from the fatty acid composition of triglycerides we have studied also the fatty acid composition of cholesterol esters in view of the evident effect of vitamin C deficiency on cholesterol metabolism (14).

MATERIALS AND METHODS

Male guinea pigs were used, with average starting weight of 445 g. They were fed ad libitum the modified Lunde's scorbutogenic diet (15); see table 1. The control group was orally administered 10 mg of ascorbic acid per animal per 24 hours. In the experimental group the chronic lack of vitamin C was produced by a fortnightly desaturation of tissues by a scorbutogenic regime followed by administration of the maintenance dose of 0.5 mg of ascorbic acid for 24 hours (13). Between days 112 and 143 of the above-mentioned dietary

Received for publication April 14, 1969.

TABLE 1

Composition of Lunde's modified scorbutogenic diet

Oat flakes	10,000 g
Wheat bran	4,000 g
Dried milk	3,000 g
Butter	2,000 g
NaCl	200 g
Salt mixture (Hegsted) [1]	100 g
Cod-liver oil	25 ml
B vitamin complex (Spofa) [2]	100 pills

[1] Salt mixture composition: (in g) $CaCO_3$, 600; K_2HPO_4, 645; NaCl, 335; $MgSO_4 \cdot 7H_2O$, 204; $CaHPO_4 \cdot 2H_2O$, 150; Fe citrate $\cdot 6H_2O$, 55; KI, 1.6; $MnSO_4 \cdot 4H_2O$, 10; $CuSO_4 \cdot 5H_2O$, 0.6; $ZnCl_2$, 0.5.

[2] B vitamin complex (Spofa) composition: (in mg per pill) thiamine hydrochloride, 2; riboflavin, 2; pyridoxine hydrochloride, 1; Ca pantothenate, 3; nicotinamide, 20.

regime, animals from the control and hypovitaminotic groups were killed in parallel by decapitation after 17-hour starvation. Concentration of total cholesterol in blood serum and liver was determined by Liebermann-Buchard's reaction (16), and the concentration of vitamin C in liver and spleen was assayed (17).

Lipids were extracted from blood serum by ethanol–ether (3:1), and from liver after homogenization according to Folch et al. (18). Separation of lipids into classes was performed by thin-layer chromatography (Silica Gel CH Spolana, solvent mixture petrolether–diethylether–acetic acid 90:10:1) (19). The fraction of triglycerides and cholesterol esters was separated after transmethylation (20) by gas-liquid chromatography in a double-column gas chromatograph.[1] The column length is 200 cm, interior diameter 4 mm, content 14% ethylene glycol succinate on Chromosorb P (HMDS, 60–80 mesh). A flame-ionization detector was used. During the analyses, the temperature was programmed from 150 to 200°, increasing at a rate of 1° per minute. The temperature increase started 5 minutes after sample application. The flow of carrier gas through the analytical column was 30.2 ml/minute, and through the reference column 33.1 ml/minute. Nitrogen was used as a carrier gas. Percentage composition of the fatty acids spectrum was determined by computing the areas of peaks on the graphs. All fractions were quantitatively evaluated, but the tables list only the data on the most important and regularly occurring fatty acids. In the same way the fatty acid composition of the scorbutogenic diet was determined.

The results were evaluated by the Student's *t* test on the computer.[2]

RESULTS AND DISCUSSION

There was no substantial difference between the weight curves of animals from the experimental and control groups (average increase of control group, + 298; of hypovitaminotic group, + 261 g; the difference is not significant). Relative liver weight (in respect to body weight) was significantly increased in the hypovitaminotic group (control group, $3.3 \pm 0.1\%$; hypovitaminotic group, $4.1 \pm 0.2\%$; $P < 0.002$). The weight of adrenals showed no significant change.

Concentration of vitamin C in liver and spleen in hypovitaminotic animals decreased with high significance (see table 2). The observed data are similar to those reported in our previous paper (13). With chronic deficiency of vitamin C, accumulation of cholesterol in liver and cholesterolemia were significantly increased (see table 3), in agreement with our previous results (14).

The spectrum of fatty acids in triglycerides of blood serum and liver revealed little individual variability. Composition of triglycerides of serum and liver was very

[1] Becker Research Gas Chromatograph, type 3810.
[2] Olivetti Programma 101.

TABLE 2

Vitamin C concentration (mg per 100 g of wet tissue) in control and vitamin C-deficient guinea pigs

Sample	Control	Hypovitaminosis C	Statistical significance
No. of animals	12	12	
Liver	7.6 ± 1.4 [1]	3.3 ± 0.5	$P < 0.01$
Spleen	19.0 ± 1.3	5.5 ± 1.2	$P < 0.001$

[1] Mean ± SEM.

TABLE 3

Total cholesterol concentration in blood serum and liver, and cholesterol content in whole liver of control and vitamin C-deficient guinea pigs

Sample	Control	Hypo-vitaminosis C	Statistical significance
No. of animals	12	12	
Blood serum, mg per 100 ml	118 ± 14 [1]	171 ± 18	$P < 0.05$
Liver, mg per 100 g	456 ± 56	627 ± 65	border $P = 0.05$
Liver, mg per organ	109 ± 12	181 ± 24	$P < 0.02$

[1] Mean ± SEM.

TABLE 4

Fatty acids patterns (percent of total fatty acids) in triglycerides of blood serum and liver in control and vitamin C-deficient guinea pigs

Fatty acids	Blood serum		Liver	
	Control	Hypovitaminosis C	Control	Hypovitaminosis C
No. of animals	9	11	12	12
C_{12}	4.3 ± 0.3 [1]	5.4 ± 0.4	6.2 ± 0.4	5.2 ± 0.03
C_{14}	1.3 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	1.3 ± 0.1
C_{16}	26.8 ± 1.1	27.4 ± 0.7	28.8 ± 0.5	27.8 ± 0.4
C_{18}	6.3 ± 0.2	6.5 ± 0.3	3.6 ± 0.2	4.3 ± 0.2
C_{20}	1.6 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
$C_{14:1}$	1.1 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
$C_{16:1}$	3.3 ± 0.2	3.1 ± 0.2	4.3 ± 0.2	4.0 ± 0.2
$C_{18:1}$	28.5 ± 0.8	27.0 ± 0.3	26.0 ± 0.6	28.5 ± 0.5
$C_{20:1}$	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
$C_{18:2}$	18.4 ± 0.5	18.3 ± 0.7	19.9 ± 0.4	20.0 ± 0.5
$C_{18:3}$	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
Saturated	40.3	41.7	41.5	39.9
Monounsaturated	34.5	33.0	33.5	35.5
Polyunsaturated	19.9	19.8	21.2	21.2

[1] Mean ± SEM.

similar; more than 70% of all fatty acids were represented by palmitic, oleic and linoleic acids. Comparable results were obtained by Ostwald and Shannon (21), except that they found a considerably higher fraction of linoleic acid than we did; this is probably a result of the diet they used, the fatty component of which contained about 60% linoleic acid. The linoleic acid content in the diet used in our experiments was substantially lower, the main source of fatty acids being milk lipids (table 6).

Table 4 shows that chronic deficiency of vitamin C has practically no effect upon the composition of triglycerides in blood serum and liver. The effects of ascorbic acid on oxidation or biosynthesis of fatty acids, discussed in the introduction, are in no no way reflected on composition of triglycerides of serum and liver in guinea pigs with chronic vitamin C deficiency. These data, found in animals whose weight was not substantially influenced by the lack of vitamin C, indicate that changes in the structure of liver lipids, described in acutely scorbutic guinea pigs refusing food (10), are of nonspecific character and are produced by starvation.

Composition of cholesterol esters of blood serum of control guinea pigs differed from cholesterol esters of liver by a considerable content of linoleic acid, which is in agreement with Ostwald and Shannon (21). They described higher content of linoleic acid in cholesterol esters of liver and blood serum, which again, as in the case of triglycerides, can be explained by a different type of fat in the diet used. Chronic hypovitaminosis C affected significantly the composition of cholesterol esters of liver and blood serum (see table 5). With a low supply of ascorbic acid, the fraction of saturated fatty acids (C_{12}, C_{14}

and C_{16}) increased in cholesterol esters of the liver, whereas the content of mono- and polyenic fatty acids was decreased.

Changes in composition of cholesterol esters of blood serum show to a certain degree a reverse trend: in hypovitaminotic guinea pigs the fraction of linoleic acid is increasing, whereas the content of palmitic and oleic acids is significantly lower.

The difference in composition of cholesterol esters of serum and liver is not surprising, because it has been shown that the place and mechanism of formation of cholesterol esters may vary for blood serum and liver (22). Deficiency of ascorbic acid caused an increase of cholesterolemia and an increase of the fraction of linoleic acid in cholesterol esters of blood serum; similar changes were reported for rabbits and rats fed cholesterol diet (23, 24), but not for guinea pigs (21). It is possible that the increase of linoleic acid in cholesterol esters of blood serum in animals with experimentally induced hypercholesterolemia is connected with the transport function of polyunsaturated fatty acids.

Chronic lack of vitamin C caused an increased accumulation of cholesterol in liver, conditioned probably by a decrease in catabolism of cholesterol (14). Even more pronounced accumulation of cholesterol in guinea pig liver can be attained by administration of Lunde's diet with addition of cholesterol (25). When comparing the results obtained in guinea pigs fed Lunde's diet with addition of 0.25% cholesterol during 150 days (26) we can see that the trend of changes in the composition of liver cholesterol esters is similar in guinea pigs with lack of ascorbic acid and in vitamin C-saturated guinea pigs fed the same diet with addition of cholesterol. Table 6 indicates that in both cases there occurs an increase in the fraction of saturated fatty acids (C_{14} and C_{16}), a slight decrease of oleic acid and a decrease of the linoleic acid, apparent especially in the group with an increased supply of exogenous cholesterol. Ostwald and Shannon (21) observed in guinea pigs fed a diet with high content of linoleic acid an opposite effect of dietary cholesterol on liver cholesterol ester fatty acids (concentration of saturated fatty acid decreased, linoleic acid increased). Also in our experiment the composition of fatty acids in liver cholesterol esters in guinea pigs, fed cholesterol diet, approaches the composition of fatty acids in this diet.

The increased accumulation of cholesterol in liver (conditioned in one case by vitamin C deficiency, in the second case by cholesterol feeding) is apparently connected with an increased esterification of

TABLE 5

Fatty acids patterns (percent of total fatty acids) in cholesterol esters of blood serum and liver in control and vitamin C-deficient guinea pigs

Fatty acids	Blood serum		Liver	
	Control	Hypovitaminosis C	Control	Hypovitaminosis C
No. of animals	9	10	9	11
C_{12}	5.3 ± 0.2 [1]	4.6 ± 0.4	5.9 ± 0.6	10.0 ± 0.5 [b]
C_{14}	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	2.7 ± 0.1 [b]
C_{16}	20.2 ± 0.4	17.8 ± 0.5 [b]	18.7 ± 1.4	23.9 ± 0.6 [b]
C_{18}	4.4 ± 0.1	3.7 ± 0.4	5.4 ± 0.2	4.9 ± 0.2
C_{20}	1.1 ± 0.2	1.4 ± 0.2	1.9 ± 0.2	1.0 ± 0.2 [b]
$C_{14:1}$	1.5 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	2.6 ± 0.1 [b]
$C_{16:1}$	3.6 ± 0.2	3.8 ± 0.2	4.5 ± 0.3	4.5 ± 0.4
$C_{18:1}$	21.0 ± 0.5	17.2 ± 0.3 [b]	24.2 ± 0.8	20.5 ± 0.7 [b]
$C_{20:1}$	1.2 ± 0.2	1.5 ± 0.1	3.0 ± 0.2	1.2 ± 0.2 [b]
$C_{18:2}$	32.4 ± 0.7	38.7 ± 0.6 [b]	20.9 ± 1.4	17.5 ± 0.5 [a]
$C_{18:3}$	1.6 ± 0.2	2.0 ± 0.2	2.4 ± 0.3	1.4 ± 0.2 [b]
$C_{20:4}$	0.9 ± 0.3	0.5 ± 0.1	1.5 ± 0.3	0.7 ± 0.1 [b]
Saturated	32.1	28.5	33.1	42.5
Monounsaturated	27.3	23.8	33.6	28.8
Polyunsaturated	34.9	41.2	24.8	19.6

[1] Mean ± SEM.
[a] Difference significant at $P < 0.05$ level.
[b] Difference significant at $P < 0.01$ or 0.001 level.

TABLE 6

Fatty acid composition of Lunde's scorbutogenic diet and composition of liver cholesterol esters (percent of total fatty acids) in normal, vitamin C-deficient and cholesterol-fed guinea pigs

Fatty acids	Scorbutogenic diet	Control	Hypovitaminosis C	Cholesterol-fed group
No. of animals		9	11	10 [1]
C_{14}	11.5	1.2	2.7	10.9
C_{16}	31.6	18.7	23.9	22.7
C_{18}	6.9	5.4	4.9	1.4
C_{20}	0.2	1.9	1.0	1.4
$C_{14:1}$	1.3	1.9	2.6	3.5
$C_{16:1}$	1.8	4.5	4.5	4.6
$C_{18:1}$	25.2	24.2	20.5	22.2
$C_{18:2}$	12.7	20.9	17.5	8.5

[1] Analyses were performed on pooled samples (26).

cholesterol by those fatty acids which are received by the organism in food. This conclusion is further strengthened by results reported by other authors, describing changes in composition of cholesterol esters of liver during administration of cholesterol diet to guinea pigs (21) and rabbits (27). Due to the fact that composition of triglycerides in guinea pig liver is not affected by the lack of ascorbic acid, whereas the composition of cholesterol esters is evidently changing, we think it probable that changes in composition of cholesterol esters of liver of hypovitaminotic guinea pigs are conditioned by disturbed primary metabolism of cholesterol. This conclusion is further strengthened by our unpublished observation that in female guinea pigs, 85 days of vitamin C hyposaturation does not increase the cholesterol levels in blood and tissues, and does not cause any changes in the composition of cholesterol esters of blood serum and liver. It remains an open question whether sex difference or a shorter period of vitamin C deficiency was operating in this experiment.

LITERATURE CITED

1. Abramson, H. 1949 The oxidation of unsaturated fatty acids in normal and scorbutic guinea pigs. J. Biol. Chem., *178:* 179.
2. Kuck, S. K. D. 1950 Further studies on the factors which affect the lipid oxidation in the rat liver as estimated by the thiobarbituric acid test. Arch. Biochem., 26: 351.
3. Radsma, W., and H. E. M. van Groningen 1959 Catalytic action of ascorbic acid on the oxidation of fatty components in liver tissue. Acta Physiol. Pharmacol. Neer., *8:* 15.
4. Geyer, R. P., S. Kydd and M. Ryan 1957 Fatty acid oxidation by means of ascorbic acid and oxygen. Arch. Biochem. Biophys., *70:* 129.
5. Robinson, J. D. 1965 Structural changes in microsomal suspensions. III. Formation of lipid peroxides. Arch. Biochem. Biophys., *112:* 170.
6. Lewis, G. M. 1965 The possible role of ascorbic acid in the a-hydroxyacid decarboxylase of brain microsomes. Biochim. Biophys. Acta, *99:* 194.
7. Davies, W. E., A. K. Hajra, S. S. Parmar, N. S. Radin and J. F. Mead 1966 Decarboxylation of 2-keto fatty acids by brain. J. Lipid Res., *7:* 270.
8. Guchhait, R., B. C. Guha and N. C. Ganguli 1964 Metabolic studies of scorbutic guinea pigs. IV. Fatty acid metabolism in vitro. Z. Ernährungswiss., *5:* 21.
9. Ginter, E., P. Bobek, M. Gerbelová, R. Ondreička and L. Bilisics 1965 Lipid metabolism under conditions of acute and chronic *l*-ascorbic acid deficiency in guinea pigs. Physiol. Bohemoslov., *14:* 282.
10. Ginter, E., P. Majer, R. Nemec, Š. Hajzer and M. Gerbelová 1966 Einfluss einer experimentellen C-Avitaminosis auf die Zusammensetzung von Gewebelipoiden bei Meerschweinchen. Acta F. R. N. Univ. Comen, Chimia, *11:* 79.
11. Williams, N. A., K. T. Tamai and D. J. McIntosh 1967 Effect of fasting on liver lipids in rats fed a purified diet. Biochim. Biophys. Acta, *137:* 187.
12. Angiolini, V., R. Aresu and G. Ligneri 1967 Variazioni della concentrazione in acidi grassi polinsaturi nei lipidi, estratti da alcuni organi di ratto albino, indotte da un breve periode di digiuno. Boll. Soc. Ital. Biol. Sper., *43:* 1151.
13. Ginter, E., P. Bobek and M. Ovečka 1968 Model of chronic hypovitaminosis C in guinea pigs. Int. J. Vitamin Res., *38:* 104.
14. Ginter, E., P. Bobek, Z. Kopec, M. Ovečka and K. Čerey 1967 Metabolic disorders in guinea pigs with chronic vitamin C hyposaturation. Z. Vers., *9:* 228.
15. Hanke, H. 1943 Vitamine und Chirurgie. G. Thieme, Leipzig, p. 143.

16. Richterich, R. 1965 Klinische Chemie. Theorie und Praxis. S. Karger, Basel (Schweiz), New York.
17. Roe, J. H., and C. A. Kuether 1943 Determination of ascorbic acid in whole blood and urine through the 2.4-dinitrophenyl-hydrazine derivate of dehydroascorbic acid. J. Biol. Chem., *147:* 339.
18. Folch, J., M. Lees and G. H. S. Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497.
19. Malins, D. C., and H. K. Mangold 1960 Analysis of complex lipid mixtures by thin layer chromatography and complementary methods. J. Amer. Oil Chem. Soc., 37: 576.
20. Feldman, G. L., and G. Rouser 1965 Ultramicro-fatty acid analysis of polar lipids. GLC after column and thin layer chromatography. J. Amer. Oil Chem. Soc., 42: 290.
21. Ostwald R., and A. Shannon 1964 Composition of tissue lipids and anaemia of guinea pigs in response to dietary cholesterol. Biochem. J., *91:* 146.
22. Goodman, De W. S. 1965 Cholesterol ester metabolism. Physiol. Rev., 45: 747.
23. Aftergood, L., and R. B. Alfin-Slater 1967 Sex differences in plasma cholesterol-esterifying activity in rats. J. Lipid Res., *8:* 126.
24. Warenbourg, H., M. Bertrand, G. Biserte and G. Sezille 1966 Les acides gras plasmatiques et aortiques au cours de l'athérome nutritionnel du lapin. 1. Les acides gras des fractions lipidiques du plasma. Rev. Fr. Étud. Clin. Biol., *11:* 719.
25. Ginter, E., J. Babala, P. Bobek and Z. Dumbalová 1968 Model of experimental hypercholesterolaemia and atherosclerosis in the guinea pigs. Cor Vasa, *10:* 126.
26. Šimko, V., R. Ondreička, P. Bobek and J. Babala 1969 Ultraviolet light irradiation and fat metabolism in guinea-pigs subjected to exogenous cholesterol feeding. J. Atheroscler. Res., *8:* 935.
27. Swell, L., M. D. Law and C. R. Treadwell 1962 Tissue cholesterol ester and triglyceride fatty acid composition of rabbits fed cholesterol diets high and low in linoleic acid. J. Nutr., 76: 429.

Lysine Metabolism in Rats Fed Lysine-free Diet

KANAE YAMASHITA AND KIYOSHI ASHIDA
Laboratory of Nutritional Biochemistry, Department of Agricultural Chemistry, Nagoya University, Chikusa, Nagoya, Japan

ABSTRACT The specificity in the metabolic fate of lysine and threonine was studied in rats fed either a lysine-free or a threonine-free diet. It was found that rats fed the lysine-free diet ad libitum lost less body weight than those fed the threonine-free diet. The intake of the lysine-free diet was considerably greater than of the threonine-free diet. By determining the content of free lysine and threonine in plasma, liver and muscle, the level of threonine in all of these tissues in rats fed the threonine-free diet was found to decline markedly. The reduction of free-lysine content in rats fed the lysine-free diet, however, was not so great. By examining the metabolic transformation of lysine-U-^{14}C in rats fed the lysine-deficient diet, and of threonine-U-^{14}C in rats fed the threonine-deficient diet, a notable difference was observed between the two rat groups. The percentage recovery of ^{14}C from lysine-U-^{14}C in a) respiratory CO_2, b) blood sugar, c) liver glycogen, d) liver lipids and e) carcass lipids in rats fed the lysine-free diet decreased considerably, whereas that of threonine-U-^{14}C in these fractions of rats fed the threonine-free group did not alter much compared with the controls. The ratio of the percentage recovery of ^{14}C in the protein fraction to that in the respiratory CO_2 was higher in the lysine-free diet-fed groups than in the threonine-free diet groups. Overall results indicate that in rats fed the lysine-free diet there may operate a certain homeostatic mechanism to maintain the level of lysine in the body compared with that of threonine in the animal body on the threonine-free diet.

Studies by several investigators have established that feeding animals ad libitum with diets completely deficient in a single essential amino acid causes a) decreased food intake, b) loss of body weight, and c) shortening of the life span (1–4). Markedly different experimental results were observed, however, using diets deficient in each different essential amino acid. It was further found that the weight loss due to lysine deficiency was the least among the amino acids tested. As a possible interpretation for this unique effect of lysine deficiency, it can be speculated that there may operate a certain homeostatic mechanism in animals fed the lysine-deficient diet, so that the retention of lysine in the animal body is much greater compared with other amino acids. In order to test this hypothetical view, the metabolism of lysine was examined by injecting ^{14}C-lysine to rats on the lysine-deficient diet. Since it is known that feeding a threonine-deficient diet causes a severe amino acid-deficient syndrome (1–4), we examined in parallel the effect of threonine deficiency using ^{14}C-threonine. Another objective of selective lysine and threonine as the test compound in the present experiment lies in the fact that several previous workers (5–7) have examined the nature of nutritional improvement of white polished rice protein by addition of these two amino acids. Experimental results on the metabolic patterns of lysine and threonine are compared and discussed in the light of their nutritional effect.

EXPERIMENTAL

Animals and diets. Male rats of the Donryu strain were used throughout the experiment. Compositions of the diets are shown in table 1. In each of the lysine-free and threonine-free diets, either lysine or threonine in the basal diet was replaced by sucrose. Three experiments were performed.

Experiment 1. Rats weighing 60 to 70 g were fed the basal diet ad libitum for 7 days before starting experiments. They were then divided into three groups (five rats per group). Group 1 was continued on the basal diet, while groups 2 and 3 were fed lysine-free and threonine-free diets, respectively, ad libitum for 6 days. Body weight and food intake were recorded

Received for publication May 12, 1969.

during this period. Afterwards, rats were fasted for 24 hours and the diets described above were fed for another 3 hours. The animals were anesthetized with Nembutal, and blood was withdrawn by a cardiac puncture. Both liver and part of the muscle were removed immediately for analyzing the individual constituents. After the centrifugation of blood, an aliquot of plasma was removed and mixed with 5 volumes of 1% picric acid to precipitate the protein. The resulting precipitate was removed by centrifugation, and the protein-free picric acid extract was used for the analysis of either lysine or threonine following the method of Stein and Moore (8), using an amino acid analyzer.[1] The contents of these free amino acids in liver and muscle were also determined by the method reported previously (9).

Experiment 2. After 7-day feeding on the basal diet, rats weighing 60 to 70 g were divided into four groups (five rats per group). Rats of groups 1 and 3 were continued on the basal diet. Groups 2 and 4 were fed the lysine-free and threonine-free diets, respectively, for 5 days. The animals were fasted for 24 hours and then continued on their respective diets for another 3 hours. Afterwards, rats of groups 1 and 2 were given intraperitoneally a single injection of L-lysine-U-^{14}C, while those of groups 3 and 4 were given L-threonine-U-^{14}C (5 μCi per 100 g body weight). One hour after the amino acid injection, rats were killed by decapitation and both liver and muscle were removed immediately. A portion of these tissues was used for the determination of nitrogen by the Kjeldahl method. The protein content of the tissues was determined by subtracting the nitrogen content of the TCA-soluble fraction from the total nitrogen content. A portion of the tissues was used separately for the determination of radioactivity in the protein fraction. Although the recovery of total muscle weight in rat body was not complete, it was determined by weighing muscle torn from the body. It was in a range of 25 to 30%. The radioactivity measurements in the protein, as well as the respiratory CO_2 collected during the hour after the injection of amino acid, were based on the method described previously (9). All the measurements were in duplicate and conducted using a liquid scintillation spectrometer.[2]

Experiment 3. In this experiment, rats fed a commercial stock diet and weighing

[1] Beckman Model 120B, Beckman Instruments, Inc., Spinco Division, Palo Alto, California.
[2] Packard Instrument Co., Inc., Downers Grove, Illinois.

TABLE 1

Composition of basal, lysine-free, and threonine-free diets

	Basal diet	Lysine-free diet	Threonine-free diet
		g/kg diet	
Amino acid mixture lacking lysine and threonine [1]	44.04	44.04	44.04
Lysine·HCl	4.15	—	4.15
Threonine	2.64	2.64	—
Vitamin mixture [2]	8.50	8.50	8.50
Choline chloride	1.50	1.50	1.50
Salt mixture [3]	40.00	40.00	40.00
Corn oil	50.00	50.00	50.00
$NaHCO_3$	1.91	—	1.91
Sucrose	847.27	853.33	849.91
Vitamin E	0.10	0.10	0.10
		IU/kg diet	
Vitamin A	6000	6000	6000
Vitamin D	600	600	600

[1] The amino acid mixture contained: (in g/kg diet) L-arginine, 1.58; L-histidine, 1.58; L-isoleucine, 2.75; L-leucine, 4.00; L-methionine, 1.95; L-cystine, 1.15; L-phenylalanine, 3.48; L-tyrosine, 1.15; L-tryptophan, 0.94; L-valine, 3.48; L-alanine, 2.18; L-aspartic acid, 4.20; L-glutamic acid, 9.62; glycine, 1.59; L-proline, 2.18; and L-serine, 2.18.
[2] The vitamin mixture contained: (percentage in the mixture) thiamine, 0.059; riboflavin, 0.059; niacin, 0.294; Ca pantothenate, 0.235; pyridoxine·HCl, 0.029; menadione, 0.006; biotin, 0.001; folic acid, 0.002; vitamin B_{12}, 0.0002; inositol, 1.176; ascorbic acid, 0.588; and lactose, 97.551.
[3] Harper, A. E. 1959 J. Nutr., 68: 405.

180 to 200 g were divided into four groups (five rats per group). After 24 hours fasting they were given the above described experimental diets for 3 hours. Then, L-lysine-U-^{14}C and L-threonine-U-^{14}C (5 μCi per rat) were given intraperitoneally to rats of groups 1 and 2, and groups 3 and 4, respectively. One hour after the injection, blood was collected by cardiac puncture under Nembutal anesthesia and liver was taken out to determine the glycogen and lipid content.

Blood was treated by the method of Nadkarini et al. (10) and Somogyi (11), and an aliquot was used to determine the blood sugar by the phenol-sulfuric acid method (12). The residue was passed through a column of Amberlite IR 120 and Amberlite 4B to remove amino acids and organic acids, and the radioactivity was determined by plating an aliquot on planchets. The volume of blood was calculated on the basis of 8.3% of the body weight according to the method of Wiseman and Irving (13).

Liver glycogen was isolated and purified by the method of Stetten and Boxer (14) with a minor modification. For the radioactivity measurements, glycogen was extracted by adding an equal volume of 10% TCA and precipitated again by adding 1.2 volumes of ethanol. The resulting precipitate was further purified by repeating the H_2O-dissolution and ethanol precipitation.

The isolation procedures of total lipids in liver were essentially based on the method of Folch et al. (15). To determine the radioactivity in the lipid fraction, an aliquot of the lipid sample dissolved in a small volume of chloroform–methanol (2:1, v/v) was plated on planchets.

The frozen carcass was ground several times by passing through a meat grinder. The method for determining the carcass lipid fraction was basically the same as that used for the liver lipid analysis. The radioactivity measurements in experiment 3 were conducted using a windowless gas-flow counter and corrected for the self absorption.

RESULTS

Growth rate, food intake and control of free amino acids (experiment 1). The growth curves of rats fed basal, lysine-free and threonine-free diets are shown in figure 1. Although both lysine- and threonine-free diets caused a growth retardation, the weight loss due to the threonine deficiency was found to be more pronounced than that due to the lysine deficiency.

Results presented in table 2 show that the food intake of rats fed the threonine-free diet decreased markedly throughout the 6-day experimental period. The content of free lysine and threonine in plasma, liver and muscle was determined and results are shown in table 3. The content of threonine was found to decline greatly in these tissues of rats on the threonine-free diet, whereas the lysine content in the tissues of rats fed the lysine-free diet did not decline as much.

Metabolic fate of lysine and threonine (experiments 2 and 3). Results presented in table 4 deal with the radioactivity incorporation of either ^{14}C-lysine or ^{14}C-threonine into a) respiratory CO_2, b) liver protein, and c) muscle protein of rats fed either lysine-free or threonine-free diets for 5 days. The values in the table denote the percentage recovery in each fraction from the initially given dose. The magnitude of lysine-^{14}C breakdown to respiratory $^{14}CO_2$

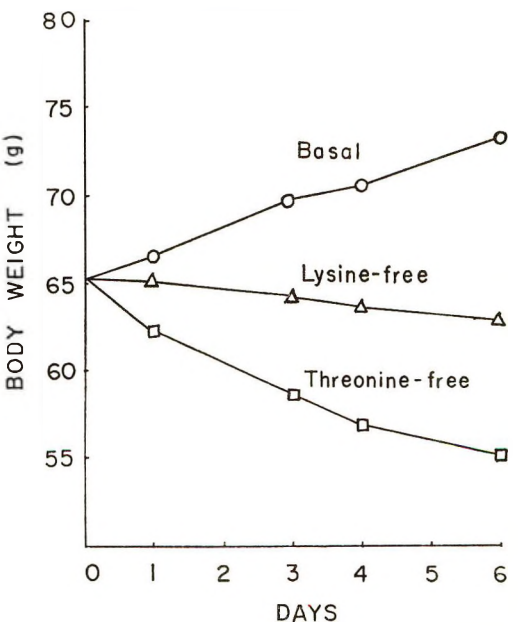

Fig. 1 Growth curves of rats fed basal, lysine-free and threonine-free diets.

TABLE 2

Food intake of rats fed lysine- and threonine-free diets (experiment 1)

Group no.	Diet type	Food intake [1] Growth period (day)			Sum
		1–2	3–4	5–6	
		g/day	*g/day*	*g/day*	*g/6 days*
1	Basal	8.18 ± 0.21 [2]	8.50 ± 0.12	8.35 ± 0.07	50.5
2	Lysine-free	5.55 ± 0.24	4.97 ± 0.14	5.67 ± 0.22	32.4
3	Threonine-free	3.48 ± 0.28	4.17 ± 0.18	4.52 ± 0.17	24.4

[1] Food intake was measured every 2 days.
[2] Mean ± SEM.

TABLE 3

Content of lysine and threonine in plasma, liver and muscle of rats fed lysine- and threonine-free diets (experiment 1)

Group no.	Diet type	Content of amino acids [1]					
		Plasma		Liver		Muscle	
		μmoles/ml plasma, %		*μmoles/g liver, %*		*μmoles/g muscle, %*	
				Lysine			
1	Basal	0.192	100	0.200	100	0.476	100
2	Lysine-free	0.072	38	0.168	84	0.364	76
				Threonine			
1	Basal	0.196	100	0.500	100	0.550	100
3	Threonine-free	0.011	6	0.054	10	0.084	15

[1] Samples were pooled from 5 rats/group.

TABLE 4

Percentage recovery of radioactivity in respiratory CO_2, liver and muscle protein after injection of lysine-U-^{14}C and threonine-U-^{14}C (experiment 2)

Group no.	Diet type	^{14}C-Amino acid injected	Radioactivity recovery			Ratio [(B) + (C)]/(A)
			Respiratory CO_2 (A)	Liver protein (B)	Muscle protein (C)	
			%	%	%	
1	Basal	Lysine-U-^{14}C	2.70 ± 0.37 [1]	8.11 ± 0.69	4.54 ± 0.52	4.72 ± 0.60
2	Lysine-free	Lysine-U-^{14}C	1.33 ± 0.14	14.81 ± 1.03	3.04 ± 0.38	14.20 ± 0.89
3	Basal	Threonine-U-^{14}C	2.33 ± 0.34	7.64 ± 0.19	3.31 ± 0.34	4.95 ± 0.45
4	Threonine-free	Threonine-U-^{14}C	2.14 ± 0.17	11.34 ± 0.44	3.04 ± 0.32	6.95 ± 0.40

[1] Mean ± SEM.

within 1 hour after its injection to rats fed the lysine-free diet decreased to about one-half that of the controls. In the case of threonine-^{14}C, however, there was no significant difference in the recovery of respiratory $^{14}CO_2$ between rats on the threonine-free diet and those on the control diet. The radioactivity recovery in the liver protein fraction was found to increase in both the lysine-free and threonine-free diet groups, especially in rats fed the former diet. The recovery in the muscle protein declined in both groups.

The greater percentage recovery of radioactivity in the protein fraction does not necessarily reflect an enhanced synthesis of protein molecules, because the pool size of amino acid may readily influence the incorporation of radioactive amino acids into protein. Therefore, we have calculated

the ratio of percentage recovery of ^{14}C in protein over that in CO_2, to determine whether amino acids in the pool might have been retained in the body or excreted. Results readily show that in rats on both types of deficient diet the ratio has increased, particularly in rats on the lysine-free diet. Overall results thus strongly indicate that the retention of lysine in rats fed the lysine-free diet appears to be much greater than that of threonine in the threonine-deficient group.

Results of the transformation of ^{14}C-lysine and ^{14}C-threonine into blood sugar, liver glycogen, liver lipids, and carcass lipids of rats fed lysine-free and threonine-free diets are shown in table 5. It can be seen that the transformation of these two amino acids into carbohydrates and lipids was not great. In rats on the basal diet, however, the utilization of ^{14}C-threonine to the carbohydrate synthesis appears to be greater than that of ^{14}C-lysine. It was also noted that the transformation of ^{14}C-lysine to lipids is greater than that of ^{14}C-threonine. Another observation of interest was a significant decrease of the radioactivity recoveries in all constituents in rats fed the lysine-free diet compared with those fed the basal diet, whereas the values were not much affected by the threonine-free diet. A rather dramatic change of this type in metabolic activity, over a period of only 3 hours of feeding the lysine-free diet, together with the results presented in table 4, strongly indicates depression of lysine catabolism in rats on the lysine-free diet.

DISCUSSION

The present experiment was undertaken to investigate the biochemical nature of the less marked weight loss of rats fed ad libitum the lysine-free diet in comparison with that of rats on the threonine-free diet. As shown in table 2, the consumption of the lysine-free diet was greater than that of the threonine-free diet, although these two diets were consumed less than the basal diet. Similar results have been reported by Frazier et al. (1). Accepting the view of Harper et al. (16), who have postulated that the depressed food intake is related to the lower concentration of the plasma amino acids, the level of lysine in plasma may be higher than that of threonine when rats are fed the respective amino acid-free diets. Our results shown in table 3 indeed support this presumption. However, Sidransky and co-workers (2, 3, 17) and Sugimura et al. (18) have reported that the weight loss of rats forcibly fed a lysine-free diet was still less than that of rats fed a threonine-free diet. One can, therefore, surmise that one or more factors other than the difference in food intake might control the rate of weight loss. Consequently we undertook an isotopic experiment to seek the metabolic fate of lysine and threonine in rats fed diets deficient in these two amino acids. Results presented in tables 4 and 5 clearly demonstrate a marked difference between the two groups in the radioactivity recoveries in a) respiratory CO_2, b) blood sugar, c) liver glycogen, d) liver lipids, and e) carcass lipids. Radioactivity recovery from lysine-^{14}C in these fractions was found to decline considerably in rats fed the lysine-free diet, whereas that from threonine-^{14}C in the threonine-free rats did not alter much in comparison with the controls. All these findings strongly indicate that the lysine

TABLE 5

Percentage recovery of radioactivity in blood sugar, liver glycogen, liver lipids and carcass lipids after injection of lysine-U-^{14}C and threonine-U-^{14}C (experiment 3)

Group no.	Diet type	^{14}C-Amino acid injected	Radioactivity recovery			
			Blood sugar	Liver glycogen	Liver lipids	Carcass lipids
			%	%	%	%
1	Basal	Lysine-U-^{14}C	0.06 ± 0.003 [1]	0.08 ± 0.014	0.24 ± 0.030	0.75 ± 0.020
2	Lysine-free	Lysine-U-^{14}C	0.01 ± 0.003	0.04 ± 0.001	0.12 ± 0.017	0.40 ± 0.045
3	Basal	Threonine-U-^{14}C	0.08 ± 0.008	0.11 ± 0.017	0.11 ± 0.014	0.63 ± 0.104
4	Threonine-free	Threonine-U-^{14}C	0.08 ± 0.001	0.17 ± 0.063	0.11 ± 0.020	0.53 ± 0.054

[1] Mean ± SEM.

catabolism of rats fed the lysine-free diet is depressed, whereas the threonine catabolism in rats fed the threonine-free diet might not be greatly affected. In addition, we can conclude that the large value of the ratio of radioactivity in protein to that in respiratory CO_2 might reflect the conservation of the lysine molecule in rats fed the lysine-free diet. A question will be raised, however, as to the extrapolation of the present data to amino acids other than threonine. Because we examined only the effect of lysine- and threonine-deficient purified diets, we cannot conclude with certainty that among the essential amino acids threonine is unique in being poorly conserved. The nature of other amino acids should be tested under analogous conditions.

It is also an intriguing subject of future investigation to elucidate the uniqueness of amino acid catabolism presently observed at the enzymic basis. Since it is known that the nutritional effect of amino acid imbalance can be elicited using a purified diet containing 5% amino acid mixture (7, 9, 16), we chose to add this diet throughout the experiment. In this context, experimental results of Harper (19) pertaining to the rise of threonine dehydratase activity in rats responding to diets of various protein contents merit description. He has demonstrated that the enzyme activity begins to increase only when the diet contains 15 to 20% protein. Thus, a purified diet containing 5% amino acid mixture exerts only a greatly restricted growth rate on rats (see figure 1), and under the experimental conditions employed the activity of threonine dehydratase is not considered to be greatly affected. It should be stressed, however, that such a predicted constancy of the enzyme activity might well explain our present data concerning the content of amino acids in tissues (see table 3) as well as the breakdown of threonine-^{14}C (see tables 4 and 5).

Sidransky and Farber (17), Waterlow (20) and Nimni and Bavetta (21) have determined the radioactivity distributions by giving the ^{14}C-labeled amino acids to rats fed either a low-protein diet or a diet lacking one of the essential amino acids. In these investigations, it was found that the magnitude of the incorporation of ^{14}C-amino acids into organs such as liver was much greater than that into the less essential organs such as muscle. Consequently, Sidransky and Farber (17) have proposed that in animals fed diets deficient in the essential amino acids, amino acids derived from the breakdown of muscle protein will be utilized for the synthesis of proteins such as those in liver. In regard to the reutilization of amino acids, Sugimura et al. (18) have also reported that in the tumor-bearing rats fed a lysine-free diet, lysine might be reutilized for the tumor growth more efficiently than other amino acids. Lavers et al. (22) have also reported the ready regeneration of liver tissues in a partially hepatectomized rat fed a lysine-free diet as well as a complete diet. Thus our present study showing the high radioactivity recovery in liver protein and the low recovery in muscle protein in rats fed the lysine-free diet is considered to be basically in agreement with results of these previous workers (17, 18, 20–22).

LITERATURE CITED

1. Frazier, L. E., R. W. Wissler, C. H. Steffee, R. L. Woolridge and P. R. Cannon 1947 Studies in amino acid utilization. 1. The dietary utilization of mixtures of purified amino acids in protein-depleted adult albino rats. J. Nutr., *33:* 65.
2. Sidransky, H., and E. Farber 1958 Chemical pathology of acute amino acid deficiencies. 1. Morphologic changes in immature rats fed threonine-, methionine-, or histidine-devoid diets. Arch. Pathol., *66:* 119.
3. Sidransky, H., and T. Baba 1960 Chemical pathology of acute amino acid deficiencies. III. Morphologic and biochemical changes in young rats fed valine- or lysine-devoid diets. J. Nutr., *70:* 463.
4. Ousterhout, L. E. 1960 Survival time and biochemical changes in chicks fed diets lacking different essential amino acids. J. Nutr., *70:* 226.
5. Pecora, L. J., and J. M. Hundley 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. J. Nutr., *44:* 101.
6. Rosenberg, H. R., R. Culik and P. E. Eckert 1959 Lysine and threonine supplementation of rice. J. Nutr., *69:* 217.
7. Yoshida, A., and K. Ashida 1968 Supplementation of amino acids to a diet containing an amino acid mixture simulating rice protein. J. Jap. Soc. Food Nutr., *20:* 432.
8. Stein, W. H., and S. Moore 1954 The free amino acids of human blood plasma. J. Biol. Chem., *211:* 915.

9. Yamashita, K., and K. Ashida 1969 The fate of threonine and leucine in rats fed threonine-deficient diets. J. Nutr., *97:* 367.
10. Nadkarni, G. B., B. Friedmann and S. Weinhouse 1960 Gluconeogenesis from glycine and serine in the rat. J. Biol. Chem., 235: 420.
11. Somogyi, M. 1945 Determination of blood sugar. J. Biol. Chem., *160:* 69.
12. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith 1956 Colorimetric method for determination of sugars and related substances. Anal. Chem., *28:* 350.
13. Wiseman, R., Jr., and C. C. Irving 1963 A rapid micro blood volume method in laboratory animals. Proc. Soc. Exp. Biol. Med., *114:* 728.
14. Stetten, D., Jr., and G. E. Boxer 1944 Studies in carbohydrate metabolism. 1. The rate of turnover of liver and carcass glycogen, studied with the aid of deuterium. J. Biol. Chem., *155:* 231.
15. Folch, J., M. Lees and G. H. S. Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497.
16. Harper, A. E., P. Leung, A. Yoshida and Q. R. Rogers 1964 Some new thoughts on amino acid imbalance. Federation Proc., *23:* 1087.
17. Sidransky, H., and E. Farber 1958 Chemical pathology of acute amino acid deficiencies. II. Biochemical changes in rats fed threonine- or methionine-devoid diets. Arch. Pathol., 66: 135.
18. Sugimura, T., S. M. Birnbaum, M. Winitz and J. P. Greenstein 1959 Quantitative nutritional studies with water-soluble, chemically defined diets. VIII. The forced feeding of diets each lacking in one essential amino acid. Arch. Biochem. Biophys., *81:* 448.
19. Harper, A. E. 1968 Diet and plasma amino acids. Amer. J. Clin. Nutr., *21:* 358.
20. Waterlow, J. C. 1959 Effect of protein depletion on the distribution of protein synthesis. Nature, *184:* 1875.
21. Nimni, M. E., and L. A. Bavetta 1961 Dietary composition and tissue protein synthesis. I. Effect of tryptophan deficiency. Proc. Soc. Exp. Biol. Med., *108:* 38.
22. Lavers, M. K., L. E. Hallanger and M. O. Schultze 1958 Regeneration of liver in lysine-deficient, partially hepatectomized rats. Proc. Soc. Exp. Biol. Med., *97:* 621.

Effect of Maternal Protein Deficiency on Cellular Development in the Fetal Rat [1,2]

FRANCES J. ZEMAN AND ELLEN C. STANBROUGH
Department of Nutrition, University of California, Davis, California

ABSTRACT The effects of maternal protein deficiency in rats on cell number and size in the offspring were investigated. Pregnant rats were fed a diet containing 30% or 6% casein. DNA, RNA, and total protein were determined in whole carcasses of 16-day-old fetuses and in liver, kidney, heart, thymus, brain, and carcass of 18- and 20-day-old fetuses and newborn young. Young of protein-deficient females had smaller organ and carcass weights at all ages studied. Total DNA was significantly decreased in livers of 18-day fetuses and in all tissues studied of 20-day fetuses and newborn young. Total protein was significantly reduced in liver and carcass at each age and in the other organs, beginning at 20 days. Total RNA was reduced in all tissues except brain beginning at 20 days. Weight/DNA ratio in the livers of newborn protein-deficient animals was increased. There were no differences in weight/DNA ratios in other organs, or in protein/DNA or RNA/DNA ratios. The data indicated that the effect of maternal protein deficiency on body and organ size was primarily a result of a decrease in cell number in the last 4 days of gestation.

Studies of normal growth have shown that it may consist of an increase in the number of cells, an increase in the size of the cells, an increase in the amount of extracellular material, or various combinations of these [3] (1, 2). The effect of postnatal food restriction on cell number and cell size in various organs in the rat has been investigated (3), showing that cell size or cell number or both may be affected at different times in the various organs.

The young of rats fed a protein-deficient diet during pregnancy have been shown to be smaller than normal at birth (4–16). In addition, certain organs are disproportionately small (15). The specific effects of maternal protein malnutrition, however, on growth of these young in terms of increases in number and size of cells have not been elucidated. The present study was undertaken to determine the effects of maternal protein restriction on cell division and cell growth in the fetal rat.

MATERIALS AND METHODS

Fifty-one virgin female rats of the Sprague-Dawley strain, weighing 180 to 200 grams, were mated with normal males. Pregnancy was assumed to have begun when vaginal plugs or sperm were found. The day following the night of mating was considered day zero of pregnancy.

The pregnant females were divided into 2 groups and fed a semipurified diet ad libitum starting on day zero. The control diet was fed to 26 animals and consisted of dextrose,[4] 56%; casein,[5] 30%; corn oil, 8%; and salt mix,[6] 6%. The restricted protein diet, fed to 25 animals, contained 80% dextrose and 6% casein, and was otherwise identical to the control diet. Each animal in both groups received a vitamin supplement mixture [7,8] three times weekly. Both

Received for publication June 9, 1969.

[1] Supported by Grant no. 375 from the Nutrition Foundation, Inc.

[2] Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., 1969.

[3] Enesco, M. 1957 Increase in cell number and size and in extra-cellular space during postnatal growth of several organs of the albino rat. Ph.D. Thesis, McGill University, Montreal, Canada.

[4] Cerelose, Corn Products Company, New York.

[5] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[6] Salt content of diet: (g/kg) $CaCO_3$, 18.0; K_2HPO_4, 19.5; $CaHPO_4$, 3.6; NaCl, 10.08; $FeSO_4 \cdot 7H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 7.5; KI, 0.015; $ZnCO_3$, 0.048; $CuSO_4 \cdot 5H_2O$, 0.018; and $MnSO_4 \cdot H_2O$, 0.138.

[7] Calculated on a per day basis, each pregnant animal received: (in milligrams) choline chloride, 20.0; inositol, 10.0; ascorbic acid, 2.0; calcium pantothenate, 1.0; (in micrograms) *p*-aminobenzoic acid, 200.0; pyridoxine, 600.0; nicotinic acid, 600.0; thiamin, 600.0; menadione, 500.0; riboflavin, 200.0; folic acid, 12.0; biotin, 5.0; vitamin B_{12}, 0.6; and (in IU) vitamin A palmitate, 300; vitamin D, 30; and *dl*-*α*-tocopherol, 2.2.

[8] We wish to express our appreciation to Commercial Solvents Corp., Terre Haute, Ind., for supplying choline chloride; Merck and Co., Rahway, N. J., for ascorbic acid, Ca pantothenate, thiamine·HCl, riboflavin, pyridoxine, and vitamin B_{12}; and Hoffmann-LaRoche, Inc., Nutley, N. J., for vitamins A, D, and E, and biotin.

diets were continued until the fetuses were taken or until the animal littered.

On day 16 of pregnancy, uteri were removed from 6 decapitated females in the control group and 7 in the experimental group and placed on a bed of crushed ice. Each fetus was then removed, weighed, and frozen on a piece of aluminum foil on a bed of dry ice. These were then wrapped in the foil, put into a snap-cap vial, sealed in a moisture-proof plastic bag, and stored at −16°.

On days 18 and 20 of pregnancy, fetuses were obtained in similar fashion from 6 females in each group. Each fetus was then decapitated and the liver, kidneys, heart, thymus, brain, and remaining carcass containing the other viscera were weighed, frozen, and stored as described above.

Newborn young of the remaining 8 females in the control group and 6 females in the experimental group were dissected as above within 4 hours of birth.

The various organs and carcasses, chosen at random from each litter, were assayed for DNA (17), RNA (18), and protein (19). Tissues of 4 animals from the same litter were pooled for assays of 18-day fetuses. In 20-day fetuses, tissues from 2 animals were pooled. Ten samples from each group at each age were assayed, no more than 2 samples being obtained from any one dam. Data for total carcass were obtained by addition of data for each organ plus the value for the carcass remaining after dissection.

Data were analyzed by Student's t test (20).

RESULTS

Carcass and organ weights at each age studied are shown in figure 1. In the carcass, brain, kidneys, heart, and thymus, there was a progressive increase in weight over the total time period studied, but there was a decline in the rate of increase between 20 and 22 days when compared to the rate in the 18 to 20-day period. This occurred in both groups. In the liver, this growth leveled off at 20 days: there were no significant differences in total liver weights of 20-day fetuses and of newborns within each diet group.

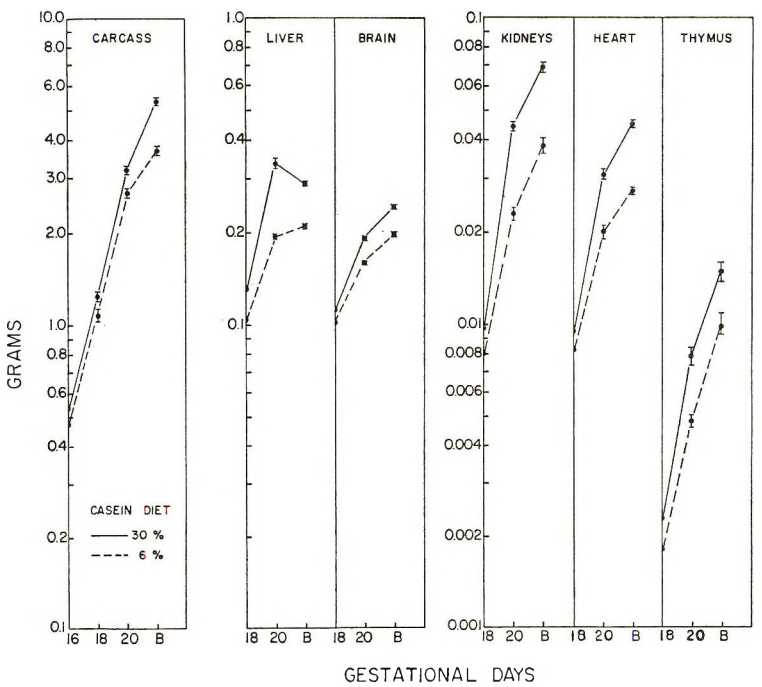

Fig. 1 Organ and carcass weights of the young. Vertical bars represent ± SE.

Total carcass weights were significantly less at each age in the young of protein-deficient dams when compared to control young. Weights of all organs studied were also significantly less at each age, with the exception of the heart and thymus at 18 days gestation.

Total DNA content of these tissues is shown in figure 2. There was a progressive increase in total DNA in kidney, heart, thymus, and carcass, with a slower increase between 20 and 22 days. In the brain, the DNA content leveled off at 20 days while, in the liver, there was an actual decline between 20 and 22 days. This decline was statistically significant in the deficient group only.

In deficient young, the DNA levels of all tissues from 20-day fetuses and newborns, and from livers only of 18-day fetuses were significantly decreased. There were no significant differences between the two groups in DNA content in 16- or 18-day tissues and organs, other than the liver.

Total protein (fig. 3) increased progressively in both groups in all tissues studied except liver between 20 and 22 days. In the liver, there was, as was the case with DNA, no significant increase in this period. In the other tissues, the rates of increase slowed to varying degrees between 20 days and birth, when compared to the rate in the previous 2-day period.

Total protein content of carcass and liver from deficient young was significantly less than from controls at all ages studied. The kidneys, heart, thymus, and brain protein were significantly decreased at 20 days and in newborns, but not at 18 days.

Only liver and thymus showed changes in protein/DNA or weight/DNA ratios (table 1). In the liver, the protein/DNA ratio increased progressively with age in both groups throughout the study period. In the thymus, the ratio decreased significantly between 18 and 20 days gestation and increased in the following 2 days in both groups. In all other organs studied, there were no significant changes with age. There were no differences between the control and protein-deficient groups in protein/DNA ratios in any organs studied.

In the liver of newborn deficient young, the weight/DNA ratio was significantly

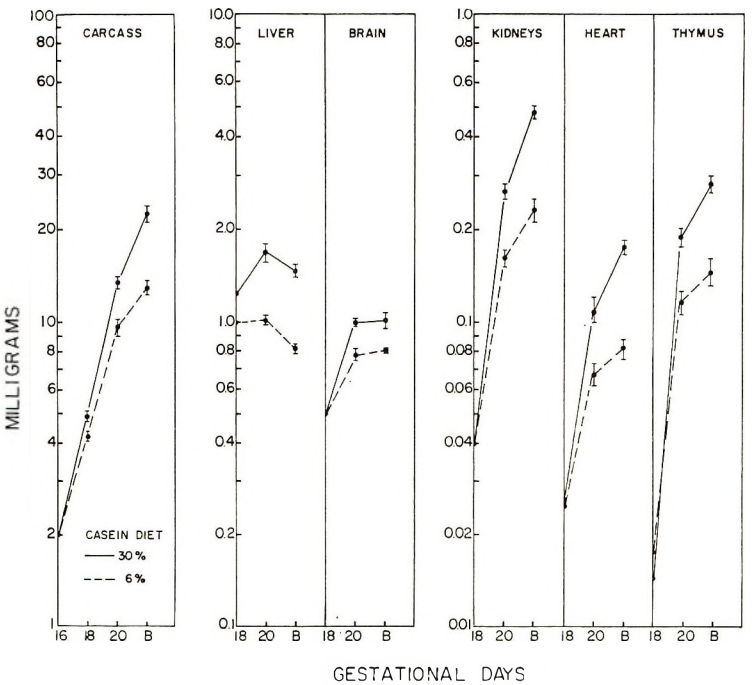

Fig. 2 DNA content of organs and carcasses in the young. Vertical bars represent ± SE.

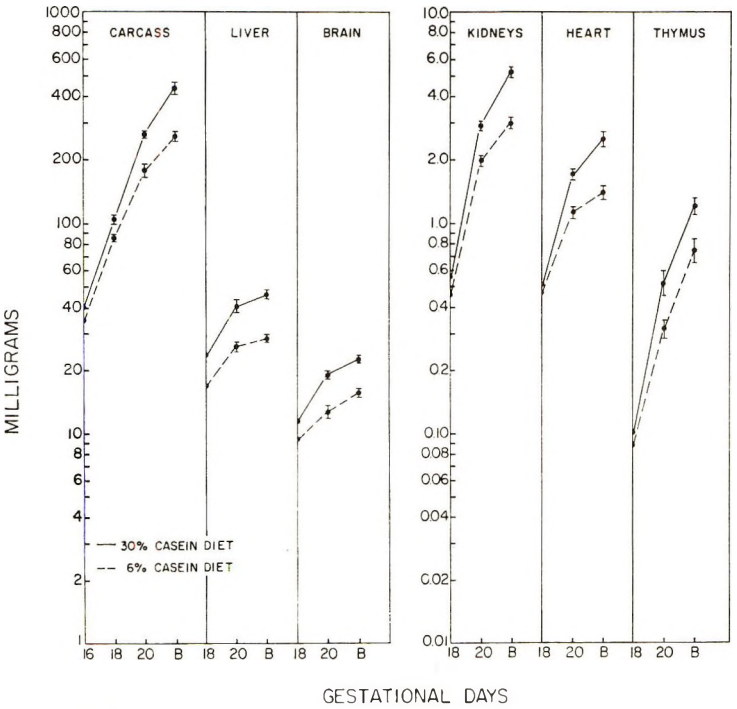

Fig. 3 Total protein content of organs and carcasses in the young. Vertical bars represent ± SE.

greater than that found in the controls. Other organs were unaffected by diet, as was the liver at 18 and 20 days gestation. There were no changes with gestational age in the carcass, heart, kidneys, or brain. In the liver, weight/DNA ratio increased between 18 and 20 days in both groups and between 20 days and birth in the deficient group. Conversely, in the thymus, there was a decline in weight/DNA ratio between 18 and 20 days gestation (table 1).

Total RNA levels (fig. 4) increased progressively in both groups, with a slight decline in rate between 20 and 22 days in carcass, and a sharp decrease in rate in brain, kidney, heart, and thymus. The RNA content leveled off at 20 days in the control livers and decreased between 20 and 22 days in deficients.

In protein-deficient young, total RNA was reduced in liver, kidney, heart, thymus, and carcass of 20-day fetuses and newborns. There were no significant differences between the two groups at 16 or 18 day in any of the organs, or in brain at any age.

There were no differences between the two groups in RNA/DNA ratios in any organs. RNA/DNA ratios changed with age in the liver and thymus (table 1), but not in the other tissues assayed. The RNA content per liver cell increased significantly in the 18 to 20-day period and then leveled off in both groups. In the thymus, there was a decrease in RNA content per cell in the 18 to 20-day period in both groups and no significant change thereafter.

DISCUSSION

The DNA content of the diploid nucleus in the cells of the rat has been shown to be constant[9] (21–27). Therefore, a change in total DNA content of a tissue or organ can be considered to indicate a change in cell number. The weight/DNA ratios and protein/DNA ratios are then indicative of the total amount of cellular material per cell and the amount of protein

[9] See footnote 3.

TABLE 1

Effect of diet and gestational age on weight, protein, and RNA per cell in liver and thymus

Gestational age, days	Liver						Thymus					
	Protein/DNA [1]		Weight/DNA [2]		RNA/DNA [3]		Protein/DNA [1]		Weight/DNA [2]		RNA/DNA [3]	
	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient
18	18.7826 ± 0.7071 [4]	17.7676 ± 1.3157	0.1002 ± 0.0030	0.1055 ± 0.0050	1.1731 ± 0.1061	1.4826 ± 0.1027	8.423 ± 1.890	5.221 ± 1.050	0.1777 ± 0.0176	0.1115 ± 0.0090	1.1825 ± 0.2516	0.7261 ± 0.0594
20	25.4205 ± 1.1384 [5]	26.3316 ± 1.8631 [6]	0.1967 ± 0.0161 [5]	0.1991 ± 0.0130 [5]	1.964 ± 0.1694 [5]	2.292 ± 0.1386 [6]	2.771 ± 0.255 [6]	2.917 ± 0.335 [6]	0.0492 ± 0.0024 [6]	0.0470 ± 0.0042 [5]	0.5115 ± 0.0500 [7]	0.4955 ± 0.0628 [7]
22	32.9097 ± 2.2180 [6]	35.8478 ± 1.7145 [5]	0.2113 ± 0.0190	0.2645 ± 0.0134 [6,8]	2.1045 ± 0.2836	2.3090 ± 0.3502	4.351 ± 0.391 [7]	5.221 ± 0.755 [7]	0.0558 ± 0.0055	0.0673 ± 0.0118	0.4153 ± 0.0352	0.5582 ± 0.0906

[1] Milligrams protein per mg DNA.
[2] Grams organ weight per mg DNA.
[3] Milligrams RNA per mg DNA.
[4] Mean ± SE.
[5] Significance of difference from previous age, $P < 0.001$.
[6] Significance of difference from previous age, $P < 0.01$.
[7] Significance of difference from previous age, $P < 0.05$.
[8] Significance of difference with diet, $P < 0.05$.

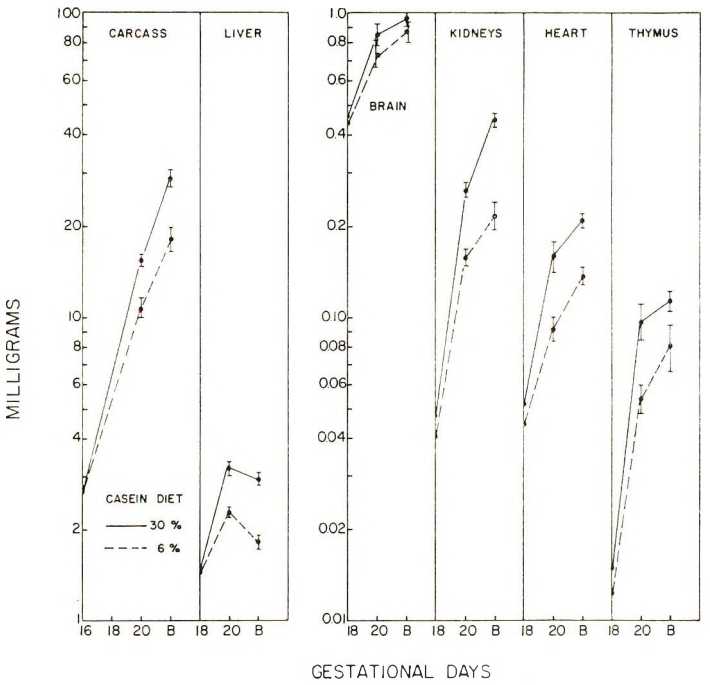

Fig. 4 Total RNA content of organs and carcasses in the young. Vertical bars represent sent ± SE.

per cell, respectively. These have been used to estimate cell size.

Maternal protein deficiency in this study resulted in smaller young with smaller organs. However, some organs were affected earlier in gestation than others. Figure 1 also indicates, as would be expected, that the growth retardation became more severe as pregnancy progressed. In general the effect was quite late in the gestation period, appearing in the last 4 days.

The curves for the DNA levels in kidney, heart, brain, and whole animal are very similar in shape to curves plotted from data taken from a table of numbers of nuclei per organ given by Winick and Noble (2). He did not give values in the table for liver at any age or for thymus in the prenatal period.

The similarity of the curves for weight and for DNA in this report indicate that the decreased growth rate in the last 2 days is the result of a decrease in the rate of cell division in both groups of animals. The slowing of the rate of cell division at the same time in both groups of animals suggests that the rate of cell multiplication in these young is, to a great extent, time-dependent.

In the deficient animals, the lower DNA content indicates that the decreased organ size is a consequence of a decreased cell number in 18-day fetal livers, and in all tissues studied from 20-day fetuses and newborn young. For the carcass, kidney and brain at 18 days, the picture is less clear. In these tissues, there is a decrease in DNA content, or cell number, and also a decreased cell size, neither of which is statistically significant. The combined effects of these presumably account for the statistically significant decreases in total weight. In the 16-day carcass, there is no evidence of decrease in the number of cells. Cell size, as indicated by weight/DNA and protein/DNA ratios is somewhat reduced, although the difference is not statistically significant.

The decrease in DNA content in the liver from 20 days to term requires some explanation. It had been suggested that the procedure of putting uteri with 20-day fetuses on ice during the dissection process,

whereas newborn young were dissected without cooling, might account for the difference because cooler animals presumably would not bleed as freely when decapitated. Consequently, one group of newborn rats was dissected using the procedure previously described, and the second group was first cooled on a bed of crushed ice. The results indicated no significant difference in the mean weights of the livers from the two groups. Paraffin sections of livers cut at 6 μ and stained with hematoxylin and eosin did not show a difference in the blood-cell concentrations in the livers.

Comparison of H and E-stained sections of 20-day fetal livers and newborn livers indicated a more marked decrease in the proportion of blood cells in livers of deficient young which may account for the decrease in the number of cells.[10]

The decreased DNA content in the brain of the newborn young of protein-deficient females is in agreement with the data reported by Zamenhof et al. (28) who fed a protein-deficient diet starting 1 month before mating and continuing throughout gestation. In the present project, interference with cell reproduction occurred in a much shorter time. It seems likely that the larger average size of the females and the less severe protein restriction used by Zamenhof et al. increased the time interval before the deficient diet affected the young.

Cell size in the animals in this study, as indicated by weight/DNA ratio or protein/DNA ratio, was not affected by the protein deficit, with the exception of the liver. In the liver of deficient newborns, lipid accumulation apparently accounts for the increased weight per cell.[11]

Only two organs showed changes in cell size with age. In the liver, the cells became progressively larger between 18 and 20 days, as indicated by both weight per cell and protein per cell. The increase in cell size leveled off at 20 days in controls but continued in deficients as the result of fat accumulation. In the thymus, there is a drop in cell size between 18 and 20 days gestation. Between 20 days and birth, the protein per cell increases, but not the weight per cell. It seems probable that the former is the result of an increase in the proportion of smaller lymphocytes typical of the developing organ (29). The increase in protein per cell in the last 2 days of gestation without the concomitant weight increase suggests a decrease in water content in these cells.

In the liver, protein per cell increases linearly in both groups. The weight per cell does not increase as rapidly between 20 and 22 days in either group, especially if the contribution of fat in protein-deficient newborns is considered. This suggests, then, that the water content in the liver also decreases during this period. In all other tissues studied, the water content apparently remains relatively constant since neither weight per cell nor protein per cell changes during the period studied.

The increase in RNA content of the tissues paralleled the increase in DNA content. The resulting DNA/RNA ratio did not vary with time, indicating that the constant RNA/DNA ratio for each organ previously described in postnatal animals (2) is also found prenatally as early as 18 days gestation. The higher ratio in liver compared to the thymus has also been reported to exist postnatally (2).

The presence of large amounts of RNA in fetal tissues has been considered to be associated with rapid protein synthesis (30). The tendency for RNA per cell and protein per cell to change in the same general direction in the tissues in this study lends support to this view.

A major question, of course, concerns the adequacy of function of the affected organs. The function of the kidney has been shown to be impaired at birth and early postnatal life in the protein-deficient young (31), but the degree of recovery possible has not as yet been established.

In view of accumulated evidence concerning the effect of early postnatal malnutrition on brain function in both man (31–35) and animals (36–42), it is interesting to speculate on effects of prenatal protein deficiency on brain function. Brain cells at birth in the rat are mostly neurons (43) suggesting that the decrease in the total number of cells reflects a decrease in neurons. Postnatally, there is evidence of a further increase in numbers of short-

[10] Schrader, R. E., and F. J. Zeman 1969 Unpublished observation.
[11] See footnote 10.

axoned neurons only (44, 45). Therefore, it seems possible that a deficit of long-axoned neuronal cells at birth is a permanent impairment.

It has been stated (46) that early postnatal malnutrition produced by restriction in total food intake interferes with cell division, and that this can be overcome if the animal is adequately fed before the normal period of cell reproduction in each organ ends. This suggests, then, that the growth retardation in organs where cells are multiplying can be overcome if proper nutrition is instituted early enough in the postnatal period. This may be the case in the kidney. However, 94 to 97% of the number of nerve cells of the adult brain are found in the brain of the newborn rat (47). Presumably then, postnatal cell division in neuronal cells is quite limited, suggesting again that the decrease in the numbers of these cells at birth may be a permanent effect. It has been suggested that young rats malnourished prenatally may be more sensitive to postnatal malnutrition with a more severe effect on total cell count than occurs with either prenatal or postnatal malnutrition alone (48). A further detailed study of the effects of prenatal protein deficiency on the brain is in progress.

ACKNOWLEDGMENT

The authors acknowledge the assistance of Laurence Burdick in preparation of diets and in the breeding, care, and feeding of animals.

LITERATURE CITED

1. Enesco, M., and C. P. Leblond 1962 Increase in cell number as a factor in the growth of the organs and tissue of the young male rat. J. Embryol. Exp. Morph., *10:* 530.
2. Winick, M., and A. Noble 1955 Quantitative changes in DNA, RNA, and protein during prenatal and postnatal growth in the rat. Develop. Biol., *12:* 451.
3. Winick, M., and A. Noble 1966 Cellular response in rats during malnutrition at various ages. J. Nutr., *89:* 300.
4. Macomber, D. 1933 Studies of reproduction in the rat. I. The effect of changes in the protein upon fertility, pregnancy, and lactation. New Engl. J. Med., *209:* 1105.
5. Seegers, W. H. 1937 The effect of protein deficiency on the course of pregnancy. Amer. J. Physiol., *119:* 474.
6. McCoy, R. H. 1940 Effects of different levels of protein in the diet of the rat. J. Biol. Chem., *133:* lxiv.
7. Curtiss, C. 1953 Effects of a low protein intake on the pregnant rat. Metabolism, *2:* 344.
8. Nelson, M. M., and H. Evans 1953 Relation of dietary protein levels to reproduction in the rat. J. Nutr., *51:* 71.
9. Kosterlitz, H. W., and R. M. Campbell 1957 Nutrition and gestation. In: International Congress of Nutrition, 4th. Centre National de la Recherche Scientifique, Paris, France, p. 85.
10. Leathem, J. M. 1959 Extragonadal factors in reproduction. In: Recent Progress in the Endocrinology of Reproduction, ed., C. W. Lloyd. Academic Press, New York, p. 179.
11. Ambegaokar, S. D., and K. Chadran 1959 Physiological effects of low protein diet. I. Studies of nitrogen balance, growth, reproduction, and lactation, and basal metabolism in the rat. Indian J. Med. Res., *47:* 539.
12. Shaw, J. W., and D. Griffiths 1963 Dental abnormalities in rats attributable to protein deficiency during reproduction. J. Nutr., *80:* 123.
13. Platt, B. S., C. R. C. Heard and R. J. C. Stewart 1964 Experimental protein-calorie deficiency. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 445.
14. Berg, B. N. 1967 Maintenance of pregnancy in protein-deprived rats by transitory supplements during early gestation. J. Nutr., *92:* 66.
15. Zeman, F. J. 1967 Effect on the young rat of maternal protein restriction. J. Nutr., *93:* 167.
16. Caldwell, D. R., and J. A. Churchill 1967 Learning ability in the progeny of rats administered a protein deficient diet during the second half of gestation. Neurology, *17:* 95.
17. Webb, J. M., and H. B. Levy 1955 A sensitive method for the determination of deoxyribonucleic acid in tissues and micro-organisms. J. Biol. Chem., *213:* 107.
18. Webb, J. M. 1955 A sensitive method for the determination of ribonucleic acid in tissues and micro-organisms. J. Biol. Chem., *221:* 635.
19. Lowry, O. H., N. J. Rosebrough, H. L. Farr and R. J. Randall 1951 Protein measurement with Folin phenol reagent. J. Biol. Chem., *193:* 265.
20. Croxton, F. E. 1953 Elementary Statistics with Applications in Medicine and the Biological Sciences. Dover Publications, Inc., New York, p. 228.
21. Davidson, J. N. 1945 Cytoplasmic ribonucleoproteins. Biochem. J., *39:* lix.
22. Boivin, A., R. Vendrely and C. Vendrely 1948 L'acide desoxyriboncleique du noyau cellulaire, depositaire des caractères hereditaires; arguments d'ordre analytique. C. R. Acad. Sci., *226:* 1061.
23. Campbell, R. M., and H. W. Kosterlitz 1948 The effect of dietary protein on the turnover of phospholipides, ribonucleic acid, and desoxyribonucleic acid in the liver. J. Biol. Chem., *175:* 989.

24. Vendrely, R., and C. Vendrely 1948 La teneur du noyau cellulaire en acide desoxyribonucleique à travers les organes, les individs, et les espèces animales. Experientia, *4:* 434.
25. Mirsky, A. E., and H. Ris 1949 Variable and constant components of chromosomes. Nature, *163:* 666.
26. Campbell, R. M., and H. W. Kosterlitz 1952 The absence of dietary effects on the DNA content of liver nuclei of the adult rat. Science, *115:* 84.
27. Thomson, R. Y., F. C. Heagry, W. C. Hutchinson and J. N. Davidson 1953 The deoxyribonucleic acid content of the rat cell nucleus, and its use in expressing the results of tissue analysis, with particular reference to composition of liver tissue. Biochem. J., *53:* 460.
28. Zamenhof, S., E. van Marthens and F. L. Margolis 1968 DNA (cell number) and protein in neonatal brain: Alteration by maternal dietary protein restriction. Science, *160:* 322.
29. Harland, M. 1940 Early histogenesis of the thymus in the white rat. Anat. Rec., *77:* 247.
30. Caspersson, T. O. 1950 Cell Growth and Cell Function. W. W. Norton & Co., Inc., New York, p. 140.
31. Hall, S. M., and F. J. Zeman 1968 Kidney function of the progeny of rats fed a low protein diet. J. Nutr., *95:* 49 .
32. Viteri, F., M. Béhar and G. Arroyave 1964 Clincal aspects of protein malnutrition. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 523.
33. Cravioto, J. 1966 Malnutrition and behavioral development in the preschool child. In: Pre-School Child Malnutrition: Primary Deterrent to Human Progress. Pub. 1282, NAS-NRC, Washington, D.C., p. 74.
34. Jelliffe, D. B. 1966 Effect of malnutrition on behavioral and social development. In: Proceedings of Western Hemisphere Nutrition Congress. American Medical Association, Chicago, p. 24.
35. Mönckeberg, F. 1968 Effect of early marasmic malnutrition on subsequent physical and psychological development. In: Malnutrition, Learning, and Behavior, eds., N. S. Scrimshaw and J. E. Gordon. M.I.T. Press, Cambridge, Mass., p. 269.
36. Stoch, M., and P. M. Smythe 1968 Undernutrition during infancy and subsequent brain growth and intellectual development. In: Malnutrition, Learning, and Behavior, eds., N. S. Scrimshaw and J. E. Gordon. M.I.T. Press, Cambridge, Mass., p. 278.
37. Platt, B. S., C. R. C. Heard and R. J. C. Stewart 1964 Experimental protein-calorie deficiency. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 498.
38. Stewart, R. J. C., and B. S. Platt 1968 Nervous system damage in experimental protein-calorie malnutrition. In: Malnutrition, Learning, and Behavior, eds., N. S. Scrimshaw and J. E. Gordon. M.I.T. Press, Cambridge, Mass., p. 168.
39. Barnes, R. H., A. V. Moore, I. M. Reid and W. G. Pond 1968 Effect of food deprivation on behavioral patterns. In: Malnutrition, Learning, and Behavior, eds., N. S. Scrimshaw and J. E. Gordon. M.I.T. Press, Cambridge, Mass., p. 203.
40. Franková, S., and R. H. Barnes 1968 Influence of malnutrition in early life on exploratory behavior of rats. J. Nutr., *94:* 477.
41. Franková, S., and R. H. Barnes 1968 Effect of malnutrition in early life on avoidance conditioning and behavior of adult rats. J. Nutr., *96:* 485.
42. Howard, E., and D. M. Granoff 1968 Effect of neonatal food restriction in mice on brain growth, DNA, and cholesterol, and on adult delayed response learning. J. Nutr., *95:* 111.
43. Brizee, K. R., J. Vogt and X. Kharetchko 1963 Postnatal changes in glia/neuron index with a comparison of methods of cell enumeration in the white rat. Progr. Brain Res., *4:* 136.
44. Altman, J., and G. D. Das 1966 Autoradiographic and histological studies of postnatal neurogenesis. J. Comp. Neur., *126:* 337.
45. Kuckuk, B. 1967 Über die Entwicklung und Chemodifferenzierung des Kleinhirns der Ratte. Histochemie, *9:* 217.
46. Winick, M., I. Fish and P. Rosso 1968 Cellular recovery in rat tissues after a brief period of neonatal malnutrition. J. Nutr., *95:* 623.
47. McIlwain, H. 1966 Biochemistry and the Central Nervous System, ed. 3. Little, Brown and Company, Boston, Mass., p. 274.
48. Winick, M. 1969 Malnutrition and brain development. J. Pediat., *74:* 667.

Catabolism in vitro of 2-^{14}C-Thiazole-labeled Thiamine by Rat Tissues [1]

P. N. DUA AND W. N. PEARSON [2]
Division of Nutrition, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee

ABSTRACT The catabolism in vitro of 2-^{14}C-thiazole-labeled thiamine was studied by incubating it with rat liver homogenates. The criterion of thiamine breakdown was the production of $^{14}CO_2$. The results show that the rat tissues contain an enzyme system capable of causing the release of carbon dioxide from the 2 position of the thiazole moiety of thiamine. The amount of thiamine degraded in this manner is very small, however. Of the total amount of thiamine broken down, about 85% was degraded during the first 4 hours of incubation. Heating the homogenates for varying periods of time caused a considerable decrease in the activity of the enzyme. The amount of thiamine broken down by the liver of stock-fed rats was significantly greater ($P < 0.01$) than that from the thiamine-deficient or stock-fed animals fasted for 46 hours. The kidney was found to be 90% as active as liver; other tissues had much less activity. Several compounds including ethanol, methanol, thiazole (4-methyl-5-β-hydroxyethyl thiazole), mercaptoethanol and folic acid were found to inhibit the enzyme activity. On an equivalent protein basis, mitochondrial and microsomal fractions had a greater enzyme activity than the nuclear and the supernatant fractions.

The thiamine molecule is extensively degraded in the animal body, and several urinary metabolites have been shown to exist (1–5). To date, however, no enzyme system capable of degrading thiamine has been reported in mammals. The present studies were therefore initiated to determine if there is an enzyme system in mammals capable of metabolizing thiamine. In these studies the criterion of thiamine breakdown was the production of $^{14}CO_2$ from 2-^{14}C-thiazole thiamine incubated with rat tissues. Previous studies (6, 7) have shown that the thiazole ring of labeled thiamine is broken down in vivo with the liberation of radioactive carbon dioxide.

EXPERIMENTAL

The purity of thiazole-2-^{14}C-labeled thiamine [3] was checked by thin-layer chromatography. Adult female rats of the Sprague-Dawley strain or male rats of the Holtzman strain, kept on a stock diet were used in these experiments. In the experiment involving the use of livers from thiamine-deficient rats, the animals were fed a thiamine-deficient diet for a period of 4 weeks before their livers were removed. The composition of this diet has been described previously (8).

The animals were killed by decapitation and the desired tissues were removed immediately and kept on ice. Ten percent homogenates of the various tissues were prepared in cold 0.05 M phosphate buffer (pH 7.4), using a Potter-Elvehjem homogenizer. In all cases, the final volume of the incubation medium, which consisted of 0.05 M phosphate buffer (pH 7.4), was 10 ml. A small amount of penicillin was added to all incubations to check any possible bacterial growth which might lead to the release of carbon dioxide from thiamine. The concentration of thiamine used varied from 5.7×10^{-5} M to 9.0×10^{-2} M and the specific activity of thiamine ranged between 0.1 and 710 μCi/mmole. The thiamine solution was made up in phosphate buffer and its pH was adjusted to 7.4 before adding to the incubation flasks.

The incubations were carried out in a shaking water bath at 37°, in stoppered 125-ml Erlenmeyer flasks. Special center wells capable of containing a liquid scintillation vial were constructed in the flasks.

Received for publication June 11, 1969.
[1] Supported in part by Public Health Service Research Grant no. AM-07709 and AM-05441 from the National Institute of Arthritis and Metabolic Diseases.
[2] Deceased.
[3] Purchased from Nuclear Chicago Corporation, Chicago, Ill.

The flasks were stoppered with rubber stoppers having a hole in the middle where small serum stoppers were fixed. At the end of the incubation period, which was usually 4 hours in most experiments, 1 ml of 1 M Hyamine [4] was injected by a hypodermic needle into the scintillation vial. This was followed by the addition of 1 ml of 1 M H_2SO_4 to the medium, whereupon the flasks were shaken for an additional half-hour to complete the liberation of $^{14}CO_2$ from the buffer. The scintillation vials were then removed, 10 ml of scintillation fluid was added, and the radioactivity determined in a liquid scintillation counter.[5] The scintillation fluid used consisted of a mixture of 4 g of PPO (2,5-diphenyloxazole), 100 mg of POPOP (1,4-bis-2-[5-phenyloxazole]-benzene), and sufficient toluene to make 1 liter. The number of moles of thiamine broken down was calculated from the amount of $^{14}CO_2$ collected after the incubation.

The method of Hogeboom (9) was used for the fractionation of liver into nuclear, mitochondrial, microsomal, and soluble fractions. Protein was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard. When the data were examined statistically, the Student's *t* test was used to test the significance of the differences.

RESULTS

Several preliminary experiments were conducted to determine the optimum conditions for the incubation. The pH optimum for the release of $^{14}CO_2$ from thiamine was found to be in the range of 7 to 7.8 and a pH of 7.4 was therefore used in all subsequent experiments. The production of $^{14}CO_2$ was found to increase linearly when the amount of tissue per flask varied from 40 mg to 1.5 g, but beyond this concentration (150 mg/ml), a marked decrease in production occurred.

Figure 1 shows the breakdown of thiamine as a function of time of incubation. The values were obtained by averaging the results from two separate experiments run in duplicate and represent the cumulative breakdown of thiamine. Of the total amount of thiamine broken down, 85% was degraded within 4 hours after the start

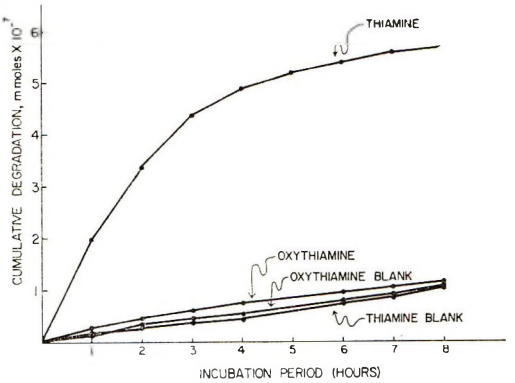

Fig. 1 The time course of the breakdown of thiamine and oxythiamine by a rat liver homogenate. The values are the averages of 2 separate experiments run in duplicate. Each flask contained 1 g of liver tissue. The concentration of thiamine or oxythiamine was 5.7×10^{-5} M. The blanks were prepared by heating the liver homogenates at 100° for 1 minute before adding thiamine or oxythiamine.

of the incubation. In addition to ^{14}C-thiamine, the metabolism of 2-^{14}C-thiazole-labeled oxythiamine [6] with time was also examined. Oxythiamine was added to each flask at the same concentration and had the same specific activity as thiamine. The amount of $^{14}CO_2$ released from ^{14}C-oxythiamine was not appreciably different than that from the blank. The blanks contained the liver homogenate which was heated to 100° for 1 minute prior to the addition of thiamine or oxythiamine. The incubations were also carried out in 0.05 M phosphate buffer (pH 7.4) in which the liver tissue was absent. In such cases, only a negligible breakdown of thiamine occurred.

To determine the stability of the enzyme system to heat, the liver homogenates were heated at 50° or 70° for 0, 2, 5, 10 and 20 minutes before incubation. Heating the homogenates at 70° for 2 minutes or longer caused a marked decrease in the activity of the enzyme (fig. 2). A gradual decrease in the thiamine catabolizing activity was obtained when the homogenates were heated at 50° for increasing periods of time.

[4] Hyamine hydroxide in methanol obtained from Nuclear Chicago.

[5] See footnote 3.

[6] Synthesized from 2-^{14}C-thiazole-labeled thiamine according to the method of Rydon, H. N. 1951 Biochem. J., *48*: 383.

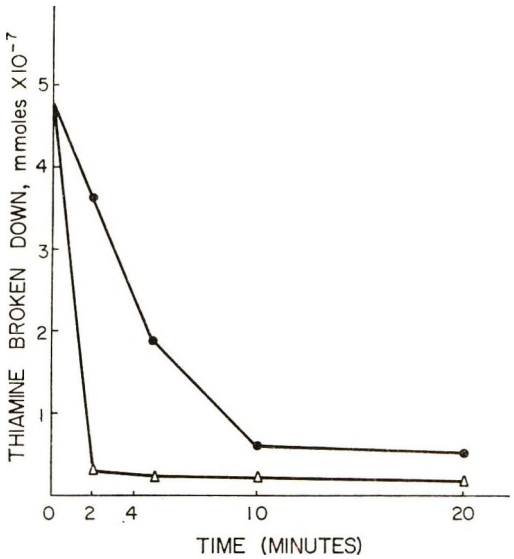

Fig. 2 Stability of the activity of the enzyme as a function of time of heating. Liver homogenates were heated to 50° (●) or 70° (△) before the addition of thiamine and the start of incubation. The incubation was continued for 4 hours. Values are the averages of duplicate groups. Each flask contained 1 g of liver tissue. The total volume of the incubation medium was 10 ml and the concentration of thiamine was 5.7×10^{-5} M.

The effect of the thiamine nutritional status of the animal on the ability of liver tissue to catabolize thiamine was determined. Liver samples were taken from stock-fed, thiamine-deficient, and stock-fed rats fasted for 46 hours. The concentration of thiamine in the incubation flasks in this experiment and in the subsequent experiments was 9.0×10^{-2} M. The amount of thiamine broken down by the liver of stock-fed rats was significantly greater ($P < 0.01$) than that from thiamine-deficient or fasted animals (table 1). The activity of liver tissue from thiamine-deficient and fasted animals was almost the same.

Tissues other than liver were also examined for their ability to degrade ^{14}C-thiamine to $^{14}CO_2$. The results are presented in table 2. The values were calculated on a milligram protein basis and are expressed relative to the liver. Kidney was 90% as active as liver; all other tissues examined had much less activity compared with liver.

TABLE 1

Catabolism of ^{14}C-thiamine using liver homogenates from stock-fed and thiamine-deficient rats

Diet and feeding pattern	Thiamine degraded [1]
	mmoles $\times 10^{-3}$
Stock-fed [2]	2.06 ± 0.04 [3]
Thiamine-deficient	1.54 ± 0.02
Stock-fed fasted [4]	1.57 ± 0.01

[1] The values were calculated per 100 mg protein and represent the average of 3 animals per group.
[2] Differs significantly ($P < 0.01$) from the other two groups.
[3] SEM.
[4] Fasted for 46 hours.

TABLE 2

The activity of different tissues of rat in breaking down thiamine

Tissue	Relative activity [1]
	%
Liver	100
Kidney	90
Heart	29
Brain	20
Gastrocnemius muscle	10

[1] Tissue homogenates; values were calculated on protein basis and are expressed relative to liver. Each value is a mean of 4 separate experiments.

The effect of different compounds on the catabolism of thiamine by liver homogenates was also determined. The results are shown in table 3. Of all the compounds tested, only ethanol, methanol, thiazole (4-methyl-5-β-hydroxyethyl thiazole), mercaptoethanol and folic acid were found to inhibit the enzyme activity to any great extent.

TABLE 3

Effect of certain compounds on the breakdown of thiazole-labeled thiamine [1] by liver homogenate

Compound	Final concentration	Inhibition
	m	%
Control	—	—
Methanol	1×10^{-3}	32
Ethanol	1×10^{-3}	19
Malonic acid	1×10^{-3}	4
L-Cysteine	1×10^{-3}	9
Glutathione	1×10^{-3}	3
Mercaptoethanol	1×10^{-3}	39
p-Chloromercurybenzoate	1×10^{-4}	14
4-Methyl-5-β-hydroxyethyl thiazole	9×10^{-2}	51
Folic acid	1×10^{-3}	32

[1] The compounds were dissolved in water before adding to the incubation medium.

The subcellular localization of the enzyme system in liver tissue was examined (table 4). The greatest activity was found in the mitochondrial and microsomal fractions, although there was also considerable activity in the nuclear and supernatant fractions.

DISCUSSION

These data indicate that there is present in rat tissues an enzyme system capable of causing the release of carbon dioxide from the 2 position of the thiazole moiety of thiamine. The activity of the enzyme is, however, very low.

There was very little production of $^{14}CO_2$ from oxythiamine, a structural analogue of thiamine by liver homogenates. This observation coincides with the in vivo studies, in which there was considerably less $^{14}CO_2$ released from 2-^{14}C-thiazole oxythiamine than from 2-^{14}C-thiazole thiamine (11).

The enzyme does not seem to be localized in any particular fraction, although the mitochondrial and microsomal fractions have a greater activity than the nuclear and supernatant fractions. On the hypothesis that a mixed-function oxidase enzyme system might be involved in the breakdown of thiamine, the influence of a NADPH-generating system was studied. There was, however, no appreciable increase in the breakdown of thiamine in the presence of a NADPH-generating system. Also, there was no induction of the enzyme activity following treatment of rats for 4 days with phenobarbital at the level of 40 mg/kg, a treatment which is reported to increase the activity of some mixed-function oxidase enzyme systems three- to fivefold (12, 13).

TABLE 4

The breakdown of thiazole-labeled thiamine by different subcellular fractions of rat liver

Fraction	Thiamine degraded [1]
	mmoles $\times 10^{-3}$
Nuclei	0.9
Mitochondria	1.9
Microsomes	1.6
Supernatant	1.0

[1] The values were calculated on 100 mg protein and are means of 4 separate experiments.

The decreased activity in liver tissue from thiamine-deficient and fasted animals suggests that this enzyme system might be inducible by thiamine. Feeding excessive amounts of thiamine (1 g/kg of diet), however, did not increase the enzyme activity in liver tissue over that observed in the livers of rats fed a stock diet (about 11 mg of thiamine per kg of diet).

Addition of thiazole at an equimolar concentration inhibited the metabolism of thiamine by a liver homogenate. In a separate experiment, it was found that 2-^{14}C-thiazole [7] is also metabolized under similar conditions with the production of $^{14}CO_2$. It is possible that the inhibition of thiamine metabolism by thiazole is a result of the thiazole competing with thiamine for the active site on the enzyme.

In one experiment, the generation of $^{14}CO_2$ by liver and kidney homogenates from rats which had been injected with labeled thiamine, but to which no ^{14}C-thiamine was added, was also examined. These animals had been injected with 60 μg of ^{14}C-thiazole-labeled or ^{14}C-pyrimidine-labeled thiamine daily for a period of 6 weeks. A release of $^{14}CO_2$ was detected in homogenates of the organs of the rats injected with ^{14}C-thiazole thiamine but not in those of the rats injected with ^{14}C-pyrimidine thiamine. No release of $^{14}CO_2$ was detected in vivo in rats injected with ^{14}C-pyrimidine thiamine (1).

It is suggested that thiamine is broken down in vivo either by the attack of some oxygen species on the 2 position followed by opening of the thiazole ring and the liberation of carbon dioxide, or by the cleavage of thiamine into pyrimidine and thiazole moieties followed by the liberation of CO_2 from the thiazole moiety by a similar mechanism. It is possible that one or both of these pathways may also be involved in the in vitro system reported here.

ACKNOWLEDGMENTS

The authors would like to express their appreciation to Dr. Robert A. Neal for his advice in carrying out some of these experiments and also for his help in the

[7] Prepared by the cleavage of 2-^{14}C-thiamine according to the method of Williams et al. 1935 J. Amer. Chem. Soc., 57: 536.

preparation of this manuscript. We also wish to acknowledge the expert technical assistance of Mr. Thomas R. Fuller, Jr.

LITERATURE CITED

1. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. I. Metabolic products found in urine. J. Nutr., *83:* 343.
2. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. II. Isolation and identification of 2-methyl-4-amino-5-pyrimidinecarboxylic acid as a metabolite of thiamine in rat urine. J. Nutr., *83:* 351.
3. Balaghi, M., and W. N. Pearson 1967 Comparative studies of the metabolism of ^{14}C-pyrimidine-labeled thiamine, ^{14}C-thiazole-labeled thiamine and ^{35}S-labeled thiamine in the rat. J. Nutr., *91:* 9.
4. Suzuoki, Z., F. Tominaga, T. Matsuo, Y. Sumi and M. Miyakawa 1968 Metabolism of thiamine and thiamine tetrahydro-furfuryl disulfide to 4-methylthiazole-5-acetic acid in conventional and germfree rats under various dosing conditions. J. Nutr., *96:* 433.
5. Ariaey-Nejad, M. R., and W. N. Pearson 1968 4-Methylthiazole-5-acetic acid — a urinary metabolite of thiamine. J. Nutr., *96:* 445.
6. Balaghi, M., and W. N. Pearson 1966 Metabolism of physiological doses of thiazole-2-^{14}C-labeled thiamine by the rat. J. Nutr., *89:* 265.
7. Pearson, W. N., and W. J. Darby, Jr. 1967 Catabolism of ^{14}C-labeled thiamine by the rat as influenced by dietary intake and body thiamine stores. J. Nutr., *93:* 491.
8. Pearson, W. N., E. Hung, W. J. Darby, Jr., M. Balaghi and R. A. Neal 1966 Excretion of metabolites of ^{14}C-pyrimidine-labeled thiamine by the rat at different levels of thiamine intake. J. Nutr., *89:* 133.
9. Hogeboom, G. H. 1955 Fractionation of cell components of animal tissues. In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
11. Ariaey-Nejad, M. R., and W. N. Pearson 1968 Catabolism of ^{14}C-thiazole-labeled oxythiamine and its effects on thiamine catabolism in the rat. J. Nutr., *96:* 206.
12. Ernster, L., and S. Orrenius 1965 Substrate-induced synthesis of the hydroxylating enzyme system of liver microsomes. Federation Proc., *24:* 1190.
13. Orrenius, S. 1965 Further studies on the induction of the drug-hydroxylating enzyme system of liver microsomes. J. Cell Biol., *26:* 725.

The Role of Dietary Fat in Protecting the Rat against Oxythiamine-produced Thiamine Deficiency [1,2]

CARL D. BENNETT,[3] JAMES H. JONES AND JUNE NELSON
Laboratories of Biochemistry, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

ABSTRACT Dietary fat decreases the thiamine requirements of the rat and provides extended protection against the thiamine antagonist oxythiamine. We have measured the changes in the activities of three major thiamine-requiring enzymes of the brain and liver in rats fed low fat and high fat oxythiamine diets. Brain activities were unchanged with both diets. Liver and blood transketolase activities were decreased to the same extent with both diets. With the high fat oxythiamine diet, the oxidation of pyruvate by the liver was decreased to 51% of the control, whereas with the low-fat oxythiamine diet, it was decreased to 26% of the control (significantly lower than for the high-fat oxythiamine diet). These data provide additional evidence to support the idea that dietary fat spares thiamine by supplying energy through a route which requires a minimum of thiamine. On the basis of the results from our study, it appears that the cause of death in the thiamine deficiency produced by oxythiamine is a decreased ability of the rat to convert dietary carbohydrate into utilizable energy. The importance of the hexose monophosphate shunt in the metabolism of fat is discussed.

Dietary fat decreases the thiamine requirement of the rat (1, 2). Oxythiamine is an analogue of thiamine which produces death in rats without convulsions; while pyrithiamine, another analogue of thiamine, produces convulsions before death (3). A high level of dietary fat provides extended protection against oxythiamine, but no protection against pyrithiamine (4). Balaghi and Pearson (5) have shown that dietary fat decreases the breakdown of thiamine in the body and increases the excretion of free thiamine in the urine. They concluded that dietary fat reduces thiamine breakdown by the body. Gruber (6) observed that on a ration free of thiamine the depletion time was increased by a high level of fat intake.

These observations raise the question of the effect of a high fat diet on the activities of the enzymes requiring thiamine diphosphate. Do they decrease in the presence of oxythiamine as they do in animals on the low fat oxythiamine diet or do the enzymes remain at normal levels? If it is the former, how does fat substitute for the enzyme?

In the present paper we report the activities of pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and transketolase in brain and liver, and of transketolase in blood of rats fed diets high and low in fat, with and without oxythiamine. Our data show that the high fat diet offers some, but limited, protection against the decrease in the activity of the first two enzymes but not of the third.

EXPERIMENTAL

Diets. The low and high fat diets are the same as those used previously (4). The low fat diet contained 73.8% by weight of sucrose and 1.25 μg of thiamine chloride·HCl per gram of diet. The high fat diet contained 35.0% by weight of hydrogenated vegetable oil [4] and 27.2% sucrose. The other constituents of the high fat diet were increased so that, on a caloric basis, this ration contained the same amount of protein, salts, vitamins and other ingredi-

Received for publication June 16, 1969.

[1] A preliminary note presenting some of the data given here has been published (Nature, 220: 1236, 1968).

[2] Supported in part by Public Health Service Grant no. NB-03626 from the National Institute of Neurological Diseases and Blindness.

[3] Fellow of the Institute of Neurological Sciences, University of Pennsylvania, United States Public Health Service Trainee-5T1-GM 281, and National Institute of Neurological Diseases and Blindness Postdoctoral Fellow F2-NB-32,947. Present address: Dept. of Biochemistry, School of Medicine, Yale University, New Haven, Connecticut 06510.

[4] Light Spry, Lever Brothers, New York.

ents as the low fat diet. Thus, 1.80 μg of thiamine chloride·HCl were present per gram of high fat diet. The oxythiamine diets contained oxythiamine·HCl [5] at a molar ratio to thiamine of 80 to 1 or 89.6 μg oxythiamine·HCl/g of low fat diet, and 129 μg/g of high fat diet.

Experimental animals. Young female rats [6] were fed the inhibitor-free low fat diet for 4 days before being placed on the experimental diets. Group 1 (10 rats) was fed the low fat oxythiamine diet ad libitum. Group 2 (10 rats) was fed the low fat diet without oxythiamine, individually, paired with the animals in group 1. Group 3 (6 rats) was fed the high fat oxythiamine diet ad libitum and group 4 (6 rats) was fed the high fat diet without oxythiamine. Each rat in group 4 was individually paired with a rat in group 3. Because there was no significant difference between groups 2 and 4, the data relative to these 16 rats were combined with other data obtained from normal rats in earlier studies of the activities of the enzymes assayed. These data are presented as the control group in table 1 and the data of the experimental groups 1 (low fat oxythiamine diet) and 3 (high fat oxythiamine diet) are expressed as a percentage of the values of the control groups.

Enzyme analysis. When rats in group 1 appeared to be in such physical condition that they could not survive 24 hours, they were killed, along with their pair-fed mates. Blood, liver, and brain were analyzed for transketolase activity, and the liver and brain for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activity. This time was selected as it was felt that if there were a difference between experimental and control animals, this difference would probably be maximum at this point. As soon thereafter as possible, a rat on the high fat oxythiamine diet and its pair were also killed and their tissues analyzed.

The activities of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase in sucrose homogenates of brain and of liver mitochondria were measured as previously described (7). The amount of oxygen consumed by a tissue homogenate in the presence of a 10 mM concentration of the appropriate substrate was measured by an oxygen electrode apparatus. The pyruvate dehydrogenase assay also contained 3.33 mM malate as a source of oxaloacetate.

The activity of transketolase in the tissues was measured by our modification of the method of Dreyfus (8) and Dreyfus and Moniz (9), in which a glucose-depleted tissue extract is incubated with ribose-5-

[5] Obtained from Calbiochem, Los Angeles, Calif.
[6] Carworth Farms, New City, New York.

TABLE 1

Tissue enzyme activities

Substrate	Control,[1] no oxythiamine	Group 1, low fat plus oxythiamine	Group 3, high fat plus oxythiamine
		% of control	
Brain			
Pyruvate	14.8 ± 0.92 [2] (40) [3,4]	97.7±4.0(7)	98.2±5.4(6)
2-Oxoglutarate	9.67± 0.57 (29) [5]	98.3±4.3(7)	93.3±7.2(6)
Ribose-5′-phosphate	73.6 ± 4.5 (40) [6]	100.5±6.8(6)	96.7±4.0(6)
Liver			
Pyruvate	15.3 ± 0.85 (38) [4]	25.8±3.8(8) [7]	50.8±2.8(6) [8]
2-Oxoglutarate	24.4 ± 1.52 (32) [5]	72.2±6.4(8) [9]	86.5±6.3(6)
Ribose-5′-phosphate	43.2 ± 3.2 (36) [6]	16.4±4.2(8) [9]	15.5±3.4(6) [9]
Blood			
Ribose-5′-phosphate	468 ±31 (37)	34.9±6.0(8) [9]	15.8±3.0(6) [9]

[1] The data from groups 2 and 4 combined with results obtained from previous studies on normal controls.
[2] SEM.
[3] Number of rats in parentheses.
[4] Oxygen electrode assay, mμmoles O_2 uptake/mg protein per min with 10 mM pyruvate plus 3.33 mM malate.
[5] Oxygen electrode assay, mμmoles O_2 uptake/mg protein per min with 10 mM 2-oxoglutarate.
[6] Transketolase assay, μg sedoheptulose formed/mg protein per hour.
[7] Significantly different from control and from group 3.
[8] Significantly different from control and from group 1.
[9] Significantly different from control.

phosphate, and the formation of sedoheptulose-7-phosphate is measured.[7]

Statistical procedure. Differences between means were considered significant at the 1% level, based on Student's *t* test (10).

RESULTS

In confirmation of previous results it was found that the rats on the low fat oxythiamine diet grew poorly, developed anorexia and profuse diarrhea on day 2 on experiment; if allowed to continue they died on about day 11, having lost an average of 4 g from their starting weight. The rats fed the high fat oxythiamine diet, however, grew well and showed no abnormal signs at the time of sacrifice. Two of the low fat rats, and their pair-fed mates, died before analysis could be performed. The tissues from the high fat oxythiamine-fed rats were analyzed as soon as possible. During the time that they were fed the diet (average 21 days), they gained an average of 76 g.

The activities of the three enzymes of the brain (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and transketolase) did not differ significantly among the rats in all three groups (table 1).

Besides the enzymes of the brain, 2-oxoglutarate dehydrogenase of liver from group 3 (high fat oxythiamine) was the only one studied which was not significantly reduced below the controls by oxythiamine. Group 3 showed a significant decrease from the control in the activities of liver pyruvate dehydrogenase and transketolase and of blood transketolase. The activities of liver and blood transketolase from rats in group 1 (low-fat oxythiamine-fed) were significantly lower than the controls, but did not differ from those of group 3. However, the activity of pyruvate dehydrogenase from the livers of rats in group 1 was significantly lower than that from either the control rats or from the rats in group 3 (table 1). There was no apparent correlation between the decrease in enzyme levels on the high fat oxythiamine diet and the length of time that the rats were fed the diet (maximum, 27 days).

DISCUSSION

The results of our studies of rats fed the low fat oxythiamine diet (table 1) agree with previous reports on changes in the activities of thiamine-requiring enzymes in tissues of rats treated with various amounts of oxythiamine (11–14). At the time of death from oxythiamine, the activity of three major thiamine-requiring enzymes of the brain is unchanged, as is also the activity of liver 2-oxoglutarate dehydrogenase from group 3 (high fat oxythiamine diet).

The high fat diet does not protect the liver or the blood against loss of pyruvate dehydrogenase or transketolase activity, although it does prevent weight loss due to oxythiamine and it does extend the life of rats on both a mildly and a severely thiamine-deficient diet (4). In fact, outwardly it appeared as if the fat had completely substituted for the loss of thiamine pyrophosphate.

Since the enzyme activities of the tissues of the rats on the high fat oxythiamine diet did not show a progressive decline over the 14-day period during which they were analyzed, it is possible that they could have continued on the diet indefinitely. Jones (4) terminated the oxythiamine diet on day 98, with 4 out of the 5 rats in excellent health. (The fifth rat grew poorly from the start and died on day 56.) Oxythiamine does not penetrate the blood-brain barrier (15), and if sufficient thiamine is available in the diet the brain will not show the signs of thiamine deficiency from oxythiamine administration (table 1). Oxythiamine is phosphorylated slowly by the rest of the body (15), but when it is phosphorylated, it is an effective antagonist to thiamine diphosphate (16).

Balaghi and Pearson (5) have suggested that ". . . the conversion of the 2-^{14}C thiazole moiety to CO_2 may represent a 'wear-and-tear' loss of thiamine as a direct consequence of its participation in metabolic reactions in the body . . ." In the present study, in vitro pyruvate oxidation by liver mitochondria is decreased to a greater extent on the low fat oxythiamine diet (to 26% of control) than on the high fat oxythiamine diet (to 51%) of the control (table 1). This may be due to the fact that less thiamine is catabolized on the high fat diet, because the oxidation of fat bypasses

[7] Bennett, C. D., J. H. Jones and J. Nelson. Unpublished data.

the thiamine-requiring pyruvate dehydrogenase, thus decreasing the destruction of thiamine. Rindi et al. (15) have shown that even small amounts of thiamine markedly decrease the rate of oxythiamine phosphorylation.

In addition to this, fat may substitute for, or bypass the reaction or reactions inhibited by oxythiamine, thus prolonging life. The only thiamine-dependent enzyme necessary for the conversion of fat to acetyl CoA and then to CO_2 is 2-oxoglutarate dehydrogenase. The activity of this enzyme in the liver was significantly depressed in the rats fed on the low fat, oxythiamine-containing diet, but not on the high fat, oxythiamine diet (table 1). In neither case, however, did the decrease in activity approach that of the corresponding pyruvate enzyme. The activity of transketolase, on the other hand, was in no way protected by the high fat diet in either liver or blood.

It is possible that the action of oxythiamine, when present in a diet high in carbohydrates and containing some thiamine, follows its conversion, however slowly, to oxythiamine pyrophosphate. By competitive inhibition with thiamine pyrophosphate, oxythiamine pyrophosphate then decreases the activity of pyruvate dehydrogenase and transketolase in the body, except brain, to such a level that energy can no longer be derived from glucose in sufficient quantity to maintain life.

Datta and Racker (17) have shown that the affinity of oxythiamine pyrophosphate for the apoenzyme of transketolase is much greater than that of the natural coenzyme, whereas with the apoenzyme of pyruvate dehydrogenase the opposite is true. Our data substantiate these observations in that the activity of transketolase was depressed considerably more than that of pyruvate dehydrogenase.

Not only is thiamine pyrophosphate needed in the oxidation of fat, it also is needed in the synthesis of fatty acids. In this synthesis hydrogen in the form of NADPH is required. The chief source of reduced NADP appears to be the hexose monophosphate shunt, which in turn requires thiamine pyrophosphate for the complete cycle. Schoenheimer and Rittenberg (18, 19) have shown that there is a continuous conversion of carbohydrates to fatty acids and that this conversion takes place even when the animals (mice) are losing weight. Stetten and Boxer (20) have calculated that of the daily intake of glucose by normal rats fed a relatively high starch diet (60% by weight), approximately 30% was metabolized by way of fat. What is the purpose of this conversion to fatty acids? Is it a necessary step in metabolism?

Table 1 shows the very low level to which the transketolase activity has been reduced in the liver and the blood by the oxythiamine. In spite of an extremely low level of active enzyme, the animals on the high fat diet appeared to be perfectly normal, thus confirming that fat, either dietary or metabolic, has a specific function to perform in the animal organism. These data indicate that under such conditions a primary function of the hexose monophosphate shunt is the role it plays in the synthesis of fatty acids; for with a liberal supply of fat the rat remains *apparently* normal. Furthermore, it is clear that an enzyme, or enzymes, can be replaced in the body economy by the product(s) formed by the reaction catalyzed by the enzyme(s) and that in these experiments a vitamin deficiency was prevented by provision of the product normally synthesized by the enzyme which contains the coenzyme which in turn contains the vitamin.

LITERATURE CITED

1. Evans, H. M., and S. Lepkovsky 1929 Sparing action of fat on the antineuritic vitamin B. J. Biol. Chem., *83:* 269.
2. Salmon, W. D., and J. G. Goodman 1937 Alleviation of vitamin B deficiency in the rat by certain natural fats and synthetic esters. J. Nutr., *13:* 477.
3. Cerecedo, L. R., M. Soodak and A. J. Eusebi 1951 Studies on thiamine analogues. I. Experiments in vivo. J. Biol. Chem., *189:* 293.
4. Jones, J. H. 1962 A striking difference in the thiamine-sparing action of fat on deficiencies produced by oxythiamine and pyrithiamine. J. Nutr., *78:* 353.
5. Balaghi, M., and W. N. Pearson 1966 Alterations in the metabolism of 2-^{14}C-thiazole labeled thiamine by the rat induced by a high fat diet or thyroxine. J. Nutr., *90:* 161.
6. Gruber, M. 1953 The influence of carbohydrate on the origin of symptoms and the onset of death in thiamine-deficient pigeons. Biochim. Biophys. Acta, *10:* 136.

7. Bennett, C. D., J. H. Jones and J. Nelson 1966 The effects of thiamine deficiencies on the metabolism of the brain. I. Oxidation of various substrates in vitro by the liver and brain of normal and pyrithiamine fed rats. J. Neurochem., *13:* 449.
8. Dreyfus, P. M. 1962 Clinical application of blood transketolase determinations. N. Engl. J. Med., 267: 596.
9. Dreyfus, P. M., and R. Moniz 1962 The quantitative histochemical estimation of transketolase in the nervous system of the rat. Biochim. Biophys. Acta, 65: 181.
10. Croxton, F. E., and D. J. Cowden 1955 Applied General Statistics, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.
11. Gubler, C. J. 1961 Studies on the physiological functions of thiamine. I. The effects of thiamine deficiency and thiamine antagonists on the oxidation of 2-keto acids by rat tissues. J. Biol. Chem., 236: 3112.
12. Wolfe, S. J. 1957 The effects of administration of thiamine analogues (oxythiamine and pyrithiamine) on rat erythrocyte metabolism. J. Biol. Chem., 229: 801.
13. Brin, M. 1962 Effects of thiamine deficiency and of oxythiamine on rat tissue transketolase. J. Nutr., 78: 179.
14. Jones, J. H., and E. deAngeli 1960 Thiamine deficiency and the in vivo oxidation of lactate and pyruvate labeled with carbon-14. J. Nutr., 70: 537.
15. Rindi, G., L. de Giuseppe and U. Ventura 1963 Distribution and phosphorylation of oxythiamine in rat tissues. J. Nutr., *81:* 147.
16. Koedam, J. C., and E. P. Steyn-Parve 1960 The action of thiamine antagonists upon some enzymes involved in thiamine metabolism. Koninkl. Ned. Akad. Wetenschap. Proc. Ser. C, 63: 318.
17. Datta, A. V., and E. Racker 1961 Mechanism of the action of transketolase. I. Properties of the crystalline yeast enzyme. J. Biol. Chem., 236: 617.
18. Schoenheimer, R., and D. Rittenberg 1944 Deuterium as an indicator in the study of intermediary metabolism. VI. Synthesis and destruction of fatty acids in the organism. J. Biol. Chem., *114:* 381.
19. Schoenheimer, R., and D. Rittenberg 1935 Deuterium as an indicator in the study of intermediary metabolism. III. The role of the fat tissues. J. Biol. Chem., *111:* 175.
20. Stetten, DeW., Jr., and H. E. Boxer 1944 Studies in carbohydrate metabolism. III. Metabolic defects in alloxan diabetes. J. Biol. Chem., *156:* 271.

Triglyceride Configuration and Fat Absorption by the Human Infant [1]

L. J. FILER, JR., F. H. MATTSON AND S. J. FOMON
Department of Pediatrics, University of Iowa College of Medicine and Miami Valley Laboratories, The Procter and Gamble Company, Cincinnati, Ohio

ABSTRACT A metabolic balance study was performed with 11 newborn infants fed formulas identical except for the type of fat. Five infants received a formula containing natural lard (palmitic acid primarily in the 2-position of the triglyceride molecule) while six infants received a formula containing randomized lard (palmitic acid equally distributed among the 1-, 2-, and 3-positions of the triglyceride molecule). Excretion of fat by infants fed the formula containing natural lard averaged 0.30 g/kg per day (range 0.15 to 0.50) while that by infants fed randomized lard averaged 1.79 g/kg per day (range 1.09 to 3.11). All of the fatty acids of the natural lard were absorbed better, but this was most marked in the case of palmitic and stearic acids. The greater absorption of palmitic acid from lard is believed to result from the greater content of 2-monopalmitin and the lower content of free palmitic acid present in the intestine after hydrolysis. Although there is no obvious mechanism to explain the greater absorption of stearic acid from lard, it may be due to a more rapid rate or greater extent of micellization in the presence of greater amounts of 2-monopalmitin.

The effect of the nature of dietary fat on its absorbability was first studied extensively by Langworthy (1). He concluded that the absorbability of a fat was determined by its melting point. Subsequently, Hoagland and Snider (2) proposed that the determining factor was the content of saturated fatty acids having 18 or more carbon atoms. The experiments of Mattson (3), in which tristearin and safflower seed oil were randomly transesterified or merely blended, demonstrated that neither the melting point of the fat nor its content of stearic acid was the primary determinant of absorbability. Rather, in this instance it depended on the amount of triglycerides that contained only saturated, long chain fatty acids.

Studies of the mechanism of the digestion and absorption of triglycerides suggested that absorbability might be controlled by the distribution of certain fatty acids on the triglyceride molecule. It was found that pancreatic lipase specifically hydrolyzes the fatty acids esterified in the 1- and 3-positions of a triglyceride, with no hydrolysis of the majority of fatty acids that are esterified in the 2-position (4). Consequently, the lipid species absorbed into the intestinal wall are free fatty acids and 2-monoglycerides (5–7). Also, it was observed that the extent of absorption of palmitic acid depended on the form in which it was fed (5). Thus, the relative absorbability of palmitic acid when it was fed as the free fatty acid, as an ester at the 1-position of a triglyceride, or as an ester at the 2-position of a triglyceride was 55, 84 and 94%, respectively.

Although butterfat is poorly absorbed by the newborn infant, human milk fat is well absorbed. A possible reason for this difference is that palmitic acid is approximately equally distributed among all three positions of the triglyceride in butterfat but is predominately esterified at the 2-position in human milk fat (8). Thus, the free palmitic acid resulting from the lipolysis of butterfat would be poorly absorbed. On the other hand, the palmitic acid of human milk fat, being esterified at the 2-position of the triglyceride, would not be hydrolyzed and the digestion product, in this instance 2-monopalmitin, would be better absorbed. The selective esterification of palmitic acid is even more marked in lard than in human milk. It is possible to rearrange the fatty acid distribution of the triglycerides by chemical treatment. This

Received for publication June 23, 1969.
[1] Supported in part by Public Health Service Grant HD 00383.

process has the effect of redistributing the palmitic acid molecules randomly so that all three positions of the triglyceride molecule contain an equal portion of this acid. Thus lard, which contains 85% of its palmitic acid in the 2-position (9) and randomized lard, which contains 33% of its palmitic acid in the 2-position, afford an excellent pair of fats for studying the effect of triglyceride structure on the absorbability of fat.

The normal full-term human infant appeared to be a particularly suitable test subject. Although the commonly used dietary fats are well absorbed by weanling rats, adult rats, and adult human subjects (10), the human infant during at least the first few months of life has a sharply reduced ability to absorb certain fats. Butterfat, for example, is readily absorbed by human adults (10b) and children beyond infancy (11), is moderately well absorbed by older infants (11–14a), but is poorly absorbed by premature (15–22) and full-term (11, 14a,b, 17, 23, 24) infants during the early months of life.

SUBJECTS, MATERIALS AND METHODS

The subjects were normal, full-term infants born to unwed mothers and scheduled for adoptive placement by various social agencies. One 3-day metabolic balance study was carried out with each infant. Sex, body weight, and age on the first day of the metabolic balance study are given in table 1.

Prime steam lard was divided into two portions. One of these was used without alteration. The fatty acids of the other were randomly rearranged at steam bath temperature using 0.3% of sodium methoxide as a catalyst. At the end of 2 hours, the catalyst was removed by water washing and the randomized lard was steam deodorized at 200°.

The fatty acid composition of the lard and of the randomized lard was determined by gas-liquid chromatography. The distribution of the fatty acids of the triglycerides of these two fats was determined by hydrolysis with pancreatic lipase (25). As may be seen from table 2, 85% of the palmitic acid was esterified at the 2-position in the lard; after randomization, one-third of this fatty acid was esterified at the 2-position.

The experimental formulas, one containing lard and the other randomized lard, were the sole source of nutrients. These were provided [2] in ready-to-feed, disposable units and were fed ad libitum at 4-hourly intervals from 3 days before the onset of the balance study until its completion. As may be seen from table 3, the formulas

[2] Dr. Sidney Saperstein of the Borden Company kindly provided the formulas.

TABLE 1

Information concerning the infant subjects and their metabolic balances

Subject number and sex	Age	Body weight	Volume of formula intake	Metabolic balance data					
				Fat		Calcium		Magnesium	
				Intake	Excretion	Intake	Retention	Intake	Retention
	days	*g*	*ml/kg/day*	*g/kg/day*		*mg/kg/day*			
				Lard					
1 M	5	3000	178	6.47	0.30	113	56	10	3
2 F	5	3290	173	6.24	0.50	109	−18	9	−2
3 M	7	3060	179	6.50	0.29	113	39	10	4
4 F	5	3325	184	6.68	0.28	116	20	10	2
5 F	9	2650	164	5.96	0.15	103	40	9	3
		Average	176	6.37	0.30	111	27	10	2
				Randomized lard					
6 M	6	4305	131	4.86	1.11	80	20	7	2
7 M	8	3275	167	6.22	3.11	103	17	9	1
8 F	6	3415	186	6.92	1.80	113	30	10	3
9 F	6	3155	197	7.33	1.09	121	60	10	3
10 F	8	3270	158	5.89	1.25	97	32	8	0
11 M	6	2815	182	6.78	2.37	112	29	9	1
		Average	170	6.33	1.79	104	31	9	2

TABLE 2

Fatty acid composition and distribution of the fatty acids of the triglycerides of the experimental fats (weight percent)

Fatty acid	Lard			Randomized lard		
	Tri-glyceride	2-position	% in 2-position	Tri-glyceride	2-position	% in 2-position
$C_{16:0}$	24.8	63.6	85.3	23.8	24.2	33.9
$C_{16:1}$	3.1	6.4	68.8	2.9	3.3	37.6
$C_{18:0}$	12.6	3.0	7.9	12.2	12.0	32.4
$C_{18:1}$	45.0	16.5	12.2	47.2	47.4	33.5
$C_{18:2}$	9.8	5.1	17.4	9.5	9.8	34.4
Miscellaneous and unknown	4.7	5.4	—	4.4	3.3	—

TABLE 3

Composition of formulas

	Lard	Randomized lard
Concentration, kcal/100 ml	67	68
Major constituents, g/100 ml		
cow milk protein	1.53	1.60
fat	3.63	3.72
lactose	7.0	7.0
Content of minerals per liter		
calcium, mg	690	720
phosphorus, mg	360	373
sodium, mEq	11	11
potassium, mEq	15	16
chloride, mEq	13	14
magnesium, mg	56	58
iron, mg	8.8	8.8
Content of vitamins per liter		
vitamin A, IU	4550	4360
thiamine, μg	750	840
riboflavin, μg	1100	1150
niacin, mg	6.6	6.9
pyridoxine, μg	440	460
ascorbic acid, mg	96	96
vitamin D, IU	423	423

were nearly identical except for the fat. Procedures followed in the 3-day metabolic balance studies have been described in detail elsewhere (14c).

Fecal excretion of fat was determined by the method of Van de Kamer et al. (26). Fatty acid composition of fecal fat was determined by gas-liquid chromatography of the methyl esters prepared from the lipids obtained by the procedure of Van de Kamer et al.

RESULTS

One 3-day metabolic balance study was performed with each of the 11 infants (table 1). It may be seen that fecal excretion of fat averaged 0.30 g/kg per day (range 0.15 to 0.50 g/kg per day) when lard was fed and 1.79 g/kg per day (range 1.09 to 3.11 g/kg per day) when randomized lard was fed. The metabolic balances of calcium and magnesium were similar in the two feeding groups.

The percentage absorption of the four major dietary fatty acids is given in table 4. These accounted for more than 84% of the total fatty acids in the feces. The average weight of fatty acids excreted is also indicated. The absorbability of palmitic and stearic acids was markedly decreased as the result of randomization of the lard. The value for oleic acid is also lower but the difference is not great. The difference in percentage of linoleic acid

TABLE 4

Percent absorption of fatty acids

Subject	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	Total
			Lard		
1	95	88	97	97	95
2	91	79	94	97	92
3	94	89	97	98	96
4	95	90	97	98	96
5	97	96	99	100 [1]	98
Average	94	88	97	98	95
	(0.08) [2]	(0.09)	(0.09)	(0.01)	
			Randomized lard		
6	60	43	90	94	77
7	40	12	93	75	50
8	49	28	93	100 [1]	74
9	70	56	98	100 [1]	85
10	63	55	89	93	79
11	48	49	75	82	65
Average	58	40	90	91	72
	(0.68) [2]	(0.47)	(0.29)	(0.05)	

[1] No linoleic acid was detected in the feces.
[2] Values in parentheses indicate mean fecal excretion in g/kg body weight/day.

excreted by the two groups of infants is large but may be spurious because of the low absolute content of this acid both in the dietary fat and in the feces.

DISCUSSION

As may be seen from table 1, normal infants, 5 to 9 days of age on the first day of the metabolic balance study, were able to absorb about 95% of the lard but only about 70% of the randomized lard. These results bear out the hypothesis on which this work was based: that palmitic acid is better absorbed from the 2-position than from the 1- and 3-positions of the triglyceride. Our results with newborn human infants are similar to those of Renner and Hill (27), who observed with chicks that lard was better absorbed than was randomized lard. Although Tomarelli et al. (28) reported relatively good absorption of all of the fats tested in young rats, their data suggested that absorbability of dietary fat is favorably influenced by a high content of 2-palmitoyltriglyceride.

The change in absorbability of the individual fatty acids (table 4) does not point to a single mechanism. Randomization of the fatty acids on the triglycerides caused an apparent decrease in the absorbability of all of the fatty acids with palmitic and stearic acids being most markedly affected. This decrease cannot be due to a slower rate of hydrolysis of any of the fatty acids because all of them are hydrolyzed at the same rate (29).

A possible explanation for the poorer absorbability of the palmitic acid is that more of it would be released from the randomized fat as free fatty acid. Free palmitic acid reacts with dietary calcium and magnesium to form insoluble soaps. Several investigators (30, 31) have demonstrated that in the case of fats with high melting point, increases in dietary intake of calcium exert an adverse effect on absorption of fat. As may be seen from table 1, however, analysis of the feces of the subjects showed that fecal excretions of calcium and magnesium per kilogram of body weight were similar in the two feeding groups. If the formation of calcium and magnesium soaps was the mechanism, one would expect a significant increase in the amount of these cations in the feces of the subjects fed the randomized lard. This was not the case.

The greater absorbability of the stearic acid from lard than from randomized lard indicates that in this case the extent of hydrolysis to free fatty acid is not the responsible mechanism. Because of the distribution of fatty acids on the triglycerides of lard, more than 90% of its stearic acid will be present as free fatty acid when it

has been hydrolyzed by pancreatic lipase in the lumen of the intestinal tract. In the case of randomized lard, only two-thirds of the esterified stearic acid will be released. Greater absorption of stearic acid was observed, however, from lard than from randomized lard.

Possibly, the large proportion (65%) of 2-monopalmitin present as the result of digestion of lard is responsible for the greater absorption of all of the fatty acids. One of the steps in the process of absorption is the formation in the lumen of the intestinal tract of a micelle consisting of monoglycerides, free fatty acids, and bile salts (32). The formation of this micelle is influenced by its components (33). Unfortunately, adequate information is not available as to the relative rates and extent of micellization that would be expected with the different digestion products obtained from the two fats used in our study. In view of this lack of information, it is tempting to propose that differences in the rate or extent of micellization may be responsible for the differences in absorbability that we have observed.

LITERATURE CITED

1. Langworthy, C. F. 1923 The digestibility of fats. Ind. Eng. Chem., *15:* 276.
2. Hoagland, R., and G. G. Snider 1943 Digestibility of some animal and vegetable fats. J. Nutr., 25: 295.
3. Mattson, F. H. 1959 The absorbability of stearic acid when fed as a simple or mixed triglyceride. J. Nutr., 69: 338.
4. Mattson, F. H., J. H. Benedict, J. B. Martin and L. W. Beck 1952 Intermediates formed during the digestion of triglycerides. J. Nutr., *48:* 335.
5. Mattson, F. H., and R. A. Volpenhein 1962 Rearrangement of glyceride fatty acids during digestion and absorption. J. Biol. Chem., *237:* 53.
6. Mattson, F. H., and R. A. Volpenhein 1964 The digestion and absorption of triglycerides. J. Biol. Chem., 239: 2772.
7. Kayden, H. J., J. R. Senior and F. H. Mattson 1967 The monoglyceride pathway of fat absorption in man. J. Clin. Invest., *46:* 1695.
8. Freeman, C. P., E. L. Jack and L. M. Smith 1965 Intramolecular fatty acid distribution in the milk fat triglycerides of several species. J. Dairy Sci., *48:* 853.
9. Mattson, F. H., R. A. Volpenhein and E. S. Lutton 1964 The distribution of fatty acids in the triglycerides of the Artiodactyla (even-toed animals). J. Lipid Res., *5:* 363.
10. Deuel, H. J. 1955 The Lipids, vol. 2. Interscience Publishers, New York. (a) p. 221; (b) p. 216, 217.
11. Van de Kamer, J. H., and H. A. Weijers 1961 Malabsorption syndrome. Federation Proc., 20; 335.
12. Holt, L. E., A. M. Courtney and H. L. Fales 1919 Fat metabolism of infants and young children. II. Fat in the stools of infants fed on modifications of cow's milk. Amer. J. Dis. Child., *17:* 423.
13. Holt, L. E., Jr., H. C. Tidwell, C. M. Kirk, D. M. Cross and S. Neale 1935 Studies in fat metabolism. I. Fat absorption in normal infants. J. Pediat., *6:* 427.
14. Fomon, S. J. 1967 Infant Nutrition. W. B. Saunders, Philadelphia. (a) p. 92; (b) p. 91; (c) p. 282–289.
15. Tidwell, H. C., L. E. Holt, Jr., H. L. Farrow and S. Neale 1935 Studies in fat metabolism. II. Fat absorption in premature infants and twins. J. Pediat., *6:* 481.
16. Gordon, H. H., S. Z. Levine, M. A. Wheatley and E. Marples 1937 Respiratory metabolism in infancy and in childhood. XX. The nitrogen metabolism in premature infants — comparative studies of human milk and cow's milk. Amer. J. Dis. Child., *54:* 1030.
17. Gordon, H. H., and H. McNamara 1941 Fat excretion of premature infants. I. Effect on fecal fat of decreasing fat intake. Amer. J. Dis. Child., *62:* 328.
18. Morales, S., A. W. Chung, J. M. Lewis, A. Messina and L. E. Holt, Jr. 1950 Absorption of fat and vitamin A in premature infants. I. Effect of different levels of fat intake on the retention of fat and vitamin A. Pediatrics, *6:* 86.
19. Söderhjelm, L. 1952 Fat absorption studies in children. I. Influence of heat treatment of milk on fat retention by premature infants. Acta Paediat., *41:* 207.
20. Snyderman, S. E., S. Morales, A. W. Chung, J. M. Lewis, A. Messina and L. E. Holt, Jr. 1953 Absorption of fat and vitamin A in premature infants. III. Effect of surface active agents on the absorption of these substances. Pediatrics, *12:* 158.
21. Davidson, M., and C. H. Bauer 1960 Patterns of fat excretion in feces of premature infants fed various preparations of milk. Pediatrics, 25: 375.
22. Zoula, J., V. Melichar, M. Novák, P. Hahn and O. Koldovský 1966 Nitrogen and fat retention in premature infants fed breast milk, "humanized" cow's milk or half skimmed cow's milk. Acta Paediat. Scand., 55: 26.
23. Droese, W., and H. Stolley 1961 Kuhmilchfett und pflanzliches Fett in der Ernährung des jungen, gesunden Säuglings. Deut. Med. Wochenschr., 86: 855.
24. Southgate, D. A. T., E. M. Widdowson, B. J. Smits, W. T. Cooke, C. H. M. Walker and N. P. Mathers 1969 Absorption and excretion of calcium and fat by young infants. Lancet, *1:* 487.

25. Mattson, F. H., and R. A. Volpenhein 1961 The use of pancreatic lipase for determining the distribution of fatty acids in partial and complete glycerides. J. Lipid Res., 2: 58.
26. Van de Kamer, J. H., H. Ten Bokkel Huinink and H. A. Weijers 1949 Rapid method for the determination of fat in feces. J. Biol. Chem., *177:* 347.
27. Renner, R., and F. W. Hill 1961 Factors affecting the absorbability of saturated fatty acids in the chick. J. Nutr., *74:* 254.
28. Tomarelli, R. M., B. J. Meyer, J. R. Weaber and F. W. Bernhart 1968 Effect of positional distribution on the absorption of the fatty acids of human milk and infant formulas. J. Nutr., *95:* 583.
29. Mattson, F. H., and L. W. Beck 1956 The specificity of pancreatic lipase for the primary hydroxyl groups of glycerides. J. Biol. Chem., *219:* 735.
30. Cheng, A. L. S., M. G. Morehouse and H. J. Deuel 1949 The effect of the level of dietary calcium and magnesium on the digestibility of fatty acids, simple triglycerides, and some natural and hydrogenated fats. J. Nutr., *37:* 237.
31. Tadayyon, B., and L. Lutwak 1969 Interrelationship of triglycerides with calcium, magnesium and phosphorus in the rat. J. Nutr., *97:* 246.
32. Hofmann, A. F., and B. Borgström 1964 The intraluminal phase of fat digestion in man: The lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. J. Clin. Invest., *43:* 247.
33. Hofmann, A. F., and D. M. Small 1967 Detergent properties of bile salts: Correlation with physiological function. Annu. Rev. Med., *18:* 333.

Adaptation of the Weanling Rat to Diets Containing Excess Methionine

R. G. DANIEL AND H. A. WAISMAN
Joseph P. Kennedy Jr. Laboratory, Department of Pediatrics, University of Wisconsin Medical Center, Madison, Wisconsin

ABSTRACT High levels of L-methionine in the diet of weanling rats induced a severe growth depression for up to 5 days whereupon weight gain at a near normal rate occurred despite the animals' consumption of the same excess amino acid diet. Alterations in hepatic levels of cystathionine synthase, cystathionase, cysteinesulfinate carboxylase and serine dehydrase occurred concurrent with changes in the growth rate. Alterations in tissue levels of methionine and its metabolites directly paralleled enzymatic changes. From these studies we conclude that the rat adapts to excess dietary methionine by altering its metabolic response to the amino acid.

L-Methionine, when present in excessive amounts in the diet, can have a deleterious effect on growth and development of the weanling rat (1–4). Several inborn errors of methionine metabolism cause accumulations of methionine or its metabolites resulting in severe mental retardation in humans (5–9). These observations have prompted more extensive studies on the toxicity of excess methionine on a molecular level.

When high levels of methionine are fed to rats, pathological lesions develop in the liver, spleen, pancreas, small intestine and kidneys (10–11), but the cause-effect relationship is unknown. We have recently observed that excess methionine can extensively disrupt free amino acid pools of liver and brain of the weanling rat by increasing total free amino acids in liver and decreasing them in brain (12). Other effects of excess methionine on animals have been reported which include a severe disorder in nitrogen metabolism marked by decreased retention of nitrogen (13), decreased blood glucose levels (14), increased iron deposition in liver and spleen (2), increased kidney weight (1) as well as hematological changes (10). The deleterious effects can be alleviated with dietary supplements of arginine and/or glycine (1, 15, 16).

We have observed (4) that the weanling rat after about a week on a diet of sublethal levels of excess methionine, will resume a near-normal growth rate even though consuming the same diet. This report describes some competitive metabolic reactions which occur during this adaptation period.

MATERIALS AND METHODS

All animals [1] were offered from 21 days of age a ground laboratory chow diet containing 24% protein.[2] Some rats were fed ad libitum, others were pair-fed as a group to animals fed diets supplemented with various amounts of L-methionine free of alloisoleucine [3] as shown in figure 1, percentages indicating the excess amino acid added on a total weight basis. Growth conditions and other handling procedures were identical to those previously employed (4). In some experiments, pyridoxal hydrochloride [4] was dissolved in physiological saline and injected intraperitoneally (10 μg in a total volume of 0.25 ml).

Enzyme activities were determined on fresh liver samples obtained at appropriate time intervals from decapitated animals, homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in ice-cold 0.14 M KCl + 0.005 M NaOH buffer (1:10 w/v) and centrifuged at 600 × g. The following enzyme activities were measured in the su-

Received for publication June 9, 1969.
Supported in part by United States Public Health Service grant nos. HD00341 and HDO2301.
[1] Holtzman Rat Company, Madison, Wis.
[2] Ralston Purina Company, St. Louis, Mo.
[3] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[4] California Biochemicals, Los Angeles, Calif.

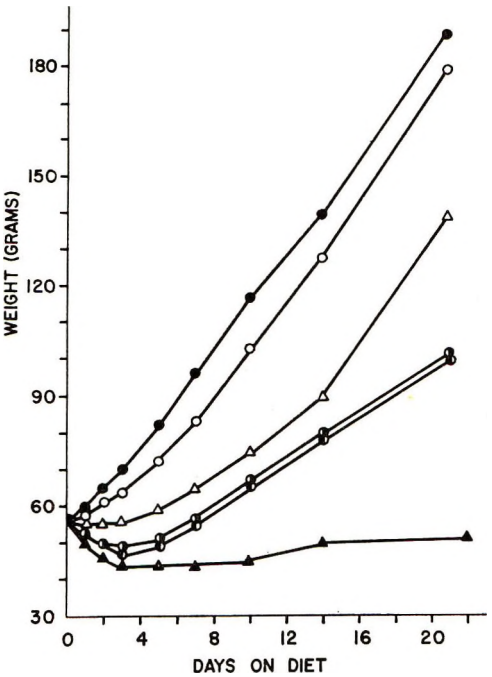

Fig. 1 Growth of the weanling rat on diets containing excess L-methionine. Each point represents the average weight of at least 12 rats. The standard error of the mean was less than 10% in all cases. Day 1 corresponds to the first day of L-methionine supplementation. The animals were 21 days old at the start of the experiment. ●——●. Ad libitum control chow diet. ○——○, Control diet supplemented with 1% L-methionine. ◑——◑, Control diet supplemented with 3% L-methionine. ◐——◐, Control diet supplemented with 3% L-methionine plus intraperitoneal injection of pyridoxal hydrochloride (10 μg daily). △——△, Control diet pair-fed to match daily food consumption of group fed diet supplemented with 3% L-methionine. ▲——▲, Control diet supplemented with 5% L-methionine.

pernatant fraction: methionine activating enzyme (ATP: L-methionine-S-adenosyltransferase, EC. 2.5.1.6) after the method of Cantoni (17); cystathionine synthase [5] after the method of Mudd et al. (18); cystathionase (L-homoserine hydrolyase deaminating EC. 4.2.1.15) after the method of Matsuo and Greenberg (19); serine dehydrase (L-serine hydrolyase, EC. 4.2.1.21) after the method of Selim and Greenberg (20); and cysteinesulfinate carboxylase (EC. 4.1.1.29) according to the method of Bergeret et al. (21). Results were expressed as ratios of the specific activities of the various enzymes from rats fed a diet supplemented with 3% of L-methionine to those of rats fed the ground chow diet ad libitum. Enzyme activities were calculated as units per mg protein. Protein concentrations were determined by the Lowry method (22).

Concentrations of methionine and its metabolites were determined from the soluble tissue extracts described above by the method of Spackman et al. (23). The samples were deproteinized prior to analyses after Gerritsen et al. (24). The results were again expressed as ratios of the values obtained from rats fed the diets supplemented with 3% of L-methionine to those from the control group.

Urine specimens, uncontaminated by food or water, were collected in bottles containing 1 ml of 6 N HCl while the animals were housed in special metabolic cages. Methionine and its metabolites were measured as described above. Urinary inorganic sulfate was determined after the method of Kleeman et al. (25).

RESULTS

Growth depression. Excess methionine induced a concentration-dependent growth inhibition of the weanling rat (fig. 1). This effect was accompanied by a decrease in food intake (1–4), but total growth depression was greater in the excess methionine-fed group than in that fed an equivalent amount of chow diet. Thus the effect was additive and was apparently due both to decreased food intake and to a specific toxic effect of the amino acid itself (4).

Growth was most severely depressed during the first 5 days of feeding the 3%-methionine-supplemented diet after which growth resumed at a nearly normal rate. Because the animals were consuming the same diet during the entire period, it was a clear illustration of adaptation to the excess methionine. The metabolic changes inducing adaptation must occur during this early period. The animals did not adapt to very high amounts of methionine (5% in the diet) and the mortality rate was high. Lowest levels of excess methionine (1% in the diet) had only a slight growth-

[5] Enzyme nomenclature in this paper is in accord with the latest recommendations of the International Union of Biochemistry (40). Cystathionine synthase, although not recommended by the report, is used here for reasons mentioned in the text.

depressing effect. For this reason and also because of the high mortality rate on 5% levels of methionine, the results described below were obtained from animals fed the 3%-methionine-supplemented diets. This level provided experimental animals which were severely affected by the excess amino acid but which all lived and adapted to the diets.

Growth depression was not the result of a deficiency in vitamin B_6 because daily parenteral injection of ten times the minimum daily requirement of pyridoxal hydrochloride (26) to rats fed excess methionine had no significant effect on the growth rate. Furthermore, any vitamin B_6 deficiency would have resulted in a drastic decrease in urinary taurine (27) but results described below show no such decrease. Dietary supplementation of pyridoxal hydrochloride also had no effect on the growth rate.[6]

Alterations in enzyme levels. Excess methionine had variable effects on several enzymes involved in the catabolic breakdown of methionine, and the changes which occurred correlated very closely with the growth rate (fig. 2).

A 3%-methionine-supplemented diet had little significant effect on methionine-activating enzyme over the entire 3-week period but there were alterations in levels of the other enzymes measured. Cystathionine synthase was decreased drastically (about 70%) during the very toxic stages of methionine feeding and remained at low levels when growth resumed. After three weeks, however, the enzyme levels began to show an increase. A very similar pattern occurred with cysteinesulfinate carboxylase; the decreased levels were similar to those observed by Chatagner (28). Cystathionase, however, gradually increased from 1 to 3 weeks and reached levels two-fold higher than controls. Our work confirms a previous report (29) on elevation of cystathionase activity in rats fed or injected with excess L-methionine; however, a more recent report [7] found no such increase when the excess methionine was fed over a short time interval. In our experiments, during the very toxic stages of methionine feeding, the levels of the enzyme were very nearly normal.

Serine dehydrase was elevated markedly during the most toxic stages of methionine intake but as adaptation to the diet occurred, the levels decreased very rapidly to normal values. Several other enzymes, including tryptophan pyrrolase, tyrosine transaminase and glutamic acid dehydrogenase behaved in a similar pattern [8] which, apparently, was much different

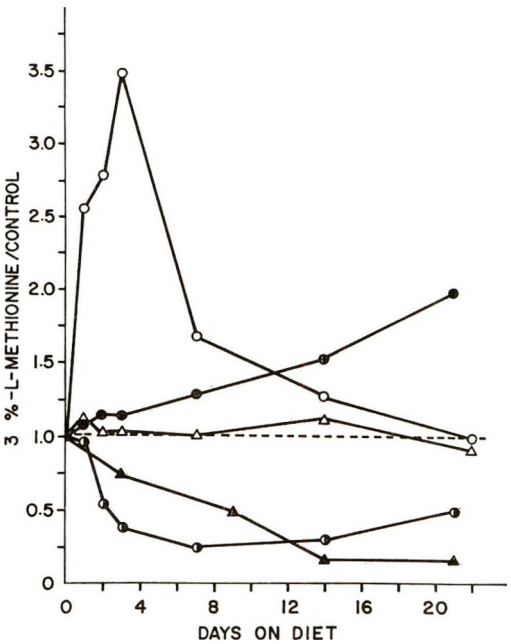

Fig. 2 Alterations in hepatic enzyme levels of weanling rats fed 3%-methionine diets. Plotted is the ratio of the specific enzyme activity of rats on 3%-methionine diets to that of rats on control diets, against days on diet. Each point represents the mean from a minimum of 12 animals. The animals were the same as those of figure 1. Ad libitum control values were identical with those observed in animals pair-fed to methionine-treated animals. △——△, Methionine-activating enzyme; at the start of the experiment the control values were equivalent to 2.25 ± 0.10 μmoles of orthophosphate/mg protein/hour. ○——○, Serine dehydrase; control values equivalent to 7.5 ± 0.44 μmoles pyruvate/mg protein/hour. ◑——◑, Cystathionine synthase; control values equivalent to 285 ± 77 μmoles cystathionine/mg protein/135 minutes. ●——●, Cystathionase; control values equivalent to 12.8 ± 1.33 μmoles α-ketobutyrate/mg protein/hour. ▲——▲, Cysteinesulfinate carboxylyase; control values equivalent to 4.6 ± 0.2 μl CO_2/mg protein/hour.

[6] Daniel, R. G., and H. A. Waisman, unpublished observations.

[7] Sanchez, A., and M. B. Swendseid 1968 Effects of methionine deficiency and excess on amino acid pools and hepatic enzymes of rats. Federation Proc., 27: 613 (abstract).

[8] See footnote 6.

from that observed with the measurement of enzymes from the methionine catabolic pathway. This group of enzymes are known to be governed by adrenal activity (30–33) and responded much more rapidly to environmental changes than we observed with the methionine pathway enzymes. Thus the adaptation phenomenon was much slower, as evidenced by the length of time before growth resumed.

The difference in behavior between cystathionine synthase and serine dehydrase would tend to confirm recent observations (34, 35) of the separate identity of the two enzymes, because previous reports (20, 36, 37) were based on the assumption that the two activities were located on the same protein molecule.

Alterations in methionine metabolite levels. Methionine and its metabolites increased rapidly in liver and urine soon after the weanling rat received the 3% L-methionine-supplemented diet (fig. 3). Depending upon the tissue studied, the levels either remained high or decreased as adaptation to the excess amino acid occurred and growth resumed. Changes in metabolite concentrations paralleled quite closely changes in enzyme levels described above.

Hepatic free methionine was elevated to nearly 15-fold above control levels within 3 days on diet but decreased when the animals resumed growth. Since total methionine intake increased with total food intake during this period (4), the actual decrease in free methionine was significant after 3 weeks (approximately 30% of that at 3 days on a per-gram-food-intake basis). Urinary methionine plus methionine sulfoxide levels showed a different pattern. During the toxic stages of exposure to excess methionine, excretion of methionine plus its sulfoxide was relatively low. As the animal adapted to the diet, this excretion rate increased to high levels (nearly 60-fold above normal by 3 weeks). This drastic increase in methionine excretion was highly significant since these data were calculated on a per-gram-food-intake basis. Thus, coincidental with renewed weight gain, an increased rate of excretion of methionine plus methionine sulfoxide occurred.

Liver taurine levels increased rapidly following excess methionine intake, reaching levels more than 20-fold above normal, but as adaptation began, these levels dropped very rapidly toward normal. Uri-

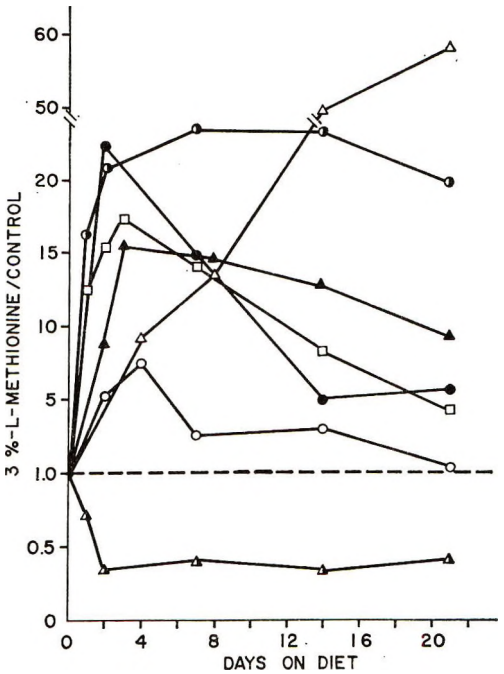

Fig. 3 Alterations in liver and urinary levels of methionine and metabolites of weanling rats fed 3%-methionine diets. The data are plotted as μmoles of compound per gram liver, or total μmoles excreted per gram food intake, of rats on 3%-methionine-supplemented diets divided by that from rats on control diets. The animals were the same as those of figure 1. Each point represents the mean from a minimum of 12 animals. ▲———▲, Liver methionine; at the start of the experiment the control values were equivalent to 0.14 ± 0.01 μmole per gram wet weight tissue. △———△, Urinary methionine + methionine sulfoxide; control values were equivalent to 0.15 ± 0.02 μmole excreted per 24 hours per gram food consumed. ●———●, Liver taurine; control values were equivalent to 0.60 ± 0.13 μmoles per gram wet weight tissue. ○———○, Urinary taurine; control values were equivalent to 3.54 ± 0.46 μmoles excreted per 24 hours per gram food consumed. ▲———▲, Liver serine; control values were equivalent to 1.32 ± 0.21 μmole per gram wet weight tissue. ◑———◑, Liver cystathionine; control values were equivalent to 0.01 μmole per gram wet weight tissue. □———□, Urinary sulfate; control values were equivalent to 0.80 ± 0.04 mg sulfur excreted per 24 hours per gram food consumed.

nary taurine followed the same pattern but peak levels were only about sevenfold above control and after 3 weeks they were back to normal. Likewise, urinary sulfate increased more than 17-fold above controls during the very toxic stages of methionine intake and decreased toward normal levels as growth resumed. Thus, in contrast to free methionine levels, renewed weight gain was accompanied by decreases in liver and urinary free taurine and urinary sulfate.

Free cystathionine levels remained elevated over the entire 3-week period of measurement, reaching peak levels more than 25-fold above normal. In terms of formation from methionine the total amount was quite low, however, since even the peak levels represent only about 0.25 μmole of free cystathionine per gram wet-weight liver. Thus, the cystathionine synthesized from the excess methionine was rapidly catabolized. The relatively low level further indicates the lack of any vitamin B_6 deficiency in our experimental animals because the enzymes responsible for the synthesis and breakdown of cystathionine require vitamin B_6.

Liver serine levels decreased rapidly and remained at about 30% of control. This decrease could be a reflection both of the increase in serine dehydrase activity and of increased methionine metabolism. We have also observed (12) that urinary serine levels of rats fed diets supplemented with excess methionine are elevated.

DISCUSSION

The factors governing the adaptation of the weanling rat to diets containing excess methionine are still obscure. In this study, we have demonstrated that some enzyme activities and metabolites of the catabolic pathway of methionine metabolism are altered during periods of nutritional stress induced by the excess dietary methionine. Furthermore, the changes occurred at specific times and coincided with changes in growth. These metabolic alterations can be summarized briefly. As the weanling rat was given a diet supplemented with 3% of L-methionine, food intake was decreased (4) and the animal began to lose weight. This weight decrease, amounting to about 15% of its initial weight (but greater with increasing amounts of methionine), occurred during the first 5 days. Coincidental with weight loss, alterations in hepatic enzyme levels occurred. Cystathionine synthase and cysteinesulfinate carboxylase began to decrease while serine dehydrase was elevated to high levels. Rapid elevation in hepatic and urinary taurine, hepatic methionine and cystathionine and urinary sulfate occurred. Liver serine was decreased. After 5 to 7 days, growth again resumed at a near normal rate even though the animals were consuming the same diets. The animals gained back the original weight loss rapidly and food intake increased. Cystathionine synthase and cysteinesulfinate carboxylase remained at low levels, serine dehydrase decreased back to normal but cystathionase began to increase rapidly. Coupled with this were decreases in hepatic and urinary taurine and hepatic methionine. Urinary excretion of methionine was rapidly elevated during this period but urinary sulfate excretion was decreased.

Based on these observations, a plausible explanation for the adaptation phenomenon can be made, at least in part. The animals gradually adapted to the excess methionine diet presumably by altering their metabolic response to amino acid by changing levels of key hepatic enzymes. Since cystathionine synthase has been implicated as catalyzing the rate-limiting step in the conversion of methionine to cysteine (37, 38), decreased levels of this enzyme would prevent undue expenditure of energy by limiting the extent to which excess methionine is catabolized. A simultaneous increase of cystathionase and a decrease in cysteinesulfinate carboxylase would channel the methionine which is catabolized, away from taurine formation and into pyruvate and thence a utilizable source of energy resulting in restored growth (fig. 4). Supportive evidence for this supposition is found in the large decrease in liver taurine between days 2 and 14, and also in the decreased urinary excretion of taurine and sulfate during the same period. Since urinary taurine and sulfate are an index of total methionine catabolism, a large increase in the metabolites would indicate a large breakdown of methionine. As urinary methionine in-

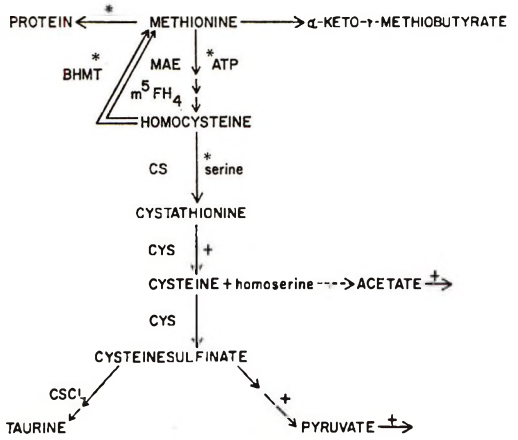

Fig. 4 Schematic diagram of methionine metabolism. Abbreviations: MAE, methionine-activating enzyme; BHMT, betaine-homocysteine methyltransferase; CS, cystathionine synthase; CYS, cystathionase; CSCL, cysteinesulfinate carboxylase; m^5FH_4, 5-methyl-tetrahydrofolic acid; ATP, adenosine-triphosphate.

creased, the levels of taurine and sulfate decreased rapidly, indicating the converse was occurring. Slowdown in the catabolism of excess methionine, reflected by decreased levels of cystathionine synthase, is shown further by a large increase in the urinary excretion of the amino acid from only about fivefold by day 2 to 60-fold by day 14. The rapid drop in serine levels, which remained very low, indicated that a considerable amount of the excess methionine was metabolized, although the increased levels of serine dehydrase could account for much of this decrease.

Thus, the rat adapts to excess dietary methionine by: 1) slowing down the metabolism of the ingested excess amino acid, 2) channeling that which is metabolized into pyruvate rather than taurine and thus providing a source of energy to the animal, and 3) by increasing the urinary excretion of the excess amino acid. Growth depression occurs because there is a time lag before this adaptation mechanism begins, and could be the result of actual metabolism of the excess methionine, rather than of the mere presence of the amino acid.

It is our conclusion from this study, that a large undue expenditure of energy could result from rapid catabolism of excess methionine and this, by itself, could be responsible for decreased growth. Figure 4 illustrates that the initial steps in methionine catabolism are energy consuming (marked with *), whereas the later steps (marked with +) are energy yielding or lead to products which in themselves are energy producing. Activation of methionine requires adenosine triphosphate (ATP), and metabolic transformation of large amounts of the amino acid would presumably require large expenditures of ATP. Recently, Choitz and Kurrie [9] have demonstrated that hepatic levels of ATP from rats fed a diet supplemented with 3% of DL-methionine were 20% below controls, but that total adenine nucleotide levels were similar due to increases in adenosine mono- and diphosphates. Since their levels of excess L-methionine were lower than those we have reported here, a larger decrease in ATP levels would presumably occur with higher amounts of the amino acid. Similarly, the condensation reaction forming cystathionine is an energy consuming step. In addition, serine is removed from the general free amino acid pool and its replacement must come from energy consuming synthetic pathways from glycine and pyruvate precursors. We have demonstrated (12) that hepatic free glycine as well as threonine were considerably reduced in rats fed excess methionine-supplemented diets. A second source of serine or glycine, or both, would be the breakdown of protein and could account for the weight loss and aminoaciduria accompanying excess methionine feeding (12, 13). Reducing the levels of cystathionine synthase would slow down this energy loss, although hepatic *S*-adenosyl-methionine levels have been reported to be sevenfold higher in rats fed excess methionine than those fed normal diets.[10] These results are not conflicting, however, because the methionine-activating enzyme was not depressed and reduced levels of cystathionine synthase would probably result in a buildup of both free methionine and *S*-adenosyl-methionine. On the other hand, increasing cystathionase while decreasing cysteinesulfinate carboxylase

[9] Choitz, H. C., and D. Kurrie 1968 Hepatic levels of ATP and S-adenosyl methionine in rats fed excess methionine. Federation Proc., 27: 613 (abstract).

[10] See footnote 9.

would transfer more of the carbon skeleton of methionine into pyruvate and acetate and thus restore serine and ATP levels promoting macromolecule synthesis and presumably renewed growth. For every molecule of methionine catabolized, at least one ATP and one serine molecule are utilized and one cysteine and one acetate molecule formed. If the cysteine went to taurine or elsewhere, a net energy loss from the methionine would occur. If it went to pyruvate, a net energy gain would be realized since both serine and ATP could be restored and extra ATP made available for synthetic purposes.

Inborn errors of methionine metabolism, leading to severe mental dysfunction in humans, have the genetic defect located after energy consuming steps in methionine metabolism but before energy yielding steps. This line of reasoning holds for all other inborn errors of amino acid metabolism and probably for many others as well. Genetic deletion would then lead only to energy depletion (or to depletion of tricarboxylic acid cycle intermediates) and no energy restoration from the individual excess amino acid would be realized. This is consistent with the thesis recently proposed (39) that mental disease may be the result of disruption in the optimum concentrations of substances normally present in the cellular environment.

LITERATURE CITED

1. Brown, J. H., and J. B. Allison 1948 Effects of excess dietary DL-methionine and/or L-arginine on rats. Proc. Soc. Exp. Biol., 69: 196.
2. Cohen, H. P., H. C. Choitz and C. P. Berg 1958 Response of rats to diets high in methionine and related compounds. J. Nutr., 64: 555.
3. Sauberlich, H. E. 1961 Studies on the toxicity and antagonism of amino acids for weanling rats. J. Nutr., 75: 61.
4. Daniel, R. G., and H. A. Waisman 1968 The effects of excess amino acids on the growth of the young rat. Growth, 32: 255.
5. Gerritsen, T., J. G. Vaughn and H. A. Waisman 1962 The identification of homocystine in the urine. Biochem. Biophys. Res. Commun., 9: 483.
6. Carson, N. A. J., D. C. Cusworth, C. E. Dent, C. M. B. Field, D. W. Neill and R. G. Westall 1963 Homocystinuria: A new inborn error of metabolism associated with mental deficiency. Arch. Dis. Childhood, 38: 425.
7. Frimpter, G. W., A. Haymovitz and M. Horwith 1963 Cystathionuria. N. Eng. J. Med., 268: 333.
8. Hooft, C., J. Timmermans, J. Snoeck, I. Antenu, W. Oyaert and C. H. van den Hende 1965 Methionine malabsorption syndrome. Ann. Paediat., 205: 73.
9. Perry, T. L., D. F. Hartwick, G. H. Dixon, C. L. Dolman and S. Hansen 1965 Hypermethioninemia: A metabolic disorder associated with cirrhosis, islet cell hyperplasia and renal tubular degeneration. Pediatrics, 36: 236.
10. Klavins, J. V., T. D. Kinney and N. Kaufman 1963 Body iron levels and hematologic findings during excess methionine feeding. J. Nutr., 79: 101.
11. Klavins, J. V., and I. L. Peacocke 1964 Pathology of amino acid excess. III. Effects of administration of excessive amounts of sulfur-containing amino acids: Methionine with equimolar amounts of glycine and arginine. Brit. J. Exp. Pathol., 45: 533.
12. Daniel, R. G., and H. A. Waisman 1968 The influence of excess methionine on the free amino acids of brain and liver of the weanling rat. J. Neurochem., *16:* 787.
13. Klain, G. J., D. A. Vaughn and L. N. Vaughn 1963 Some metabolic effects of methionine toxicity in the rat. J. Nutr., *80:* 337.
14. Winitz, M., P. Gullino, J. P. Greenstein and S. M. Birnbaum 1956 Studies on the metabolism of amino acids and related compounds *in vivo*. II. Effect of toxic doses of essential amino acids on blood sugar, liver glycogen and muscle glycogen levels. Arch. Biochem. Biophys., 64: 333.
15. Roth, J. S., and J. B. Allison 1949 The effect of feeding excess glycine, L-arginine, and DL-methionine to rats on a casein diet. Proc. Soc. Exp. Biol. Med., 70: 327.
16. Benevenga, N. J., and A. E. Harper 1967 Alleviation of methionine and homocystine toxicity in the rat. J. Nutr., *93:* 44.
17. Cantoni, G. L. 1953 *S*-Adenosylmethionine: A new intermediate formed enzymatically from L-methionine and adenosinetriphosphate. J. Biol. Chem., *204:* 403.
18. Mudd, S. H., J. D. Finkelstein, F. Irreverre and L. Laster 1964 Homocystinuria: An enzymatic defect. Science, *143:* 1443.
19. Matsuo, Y., and D. M. Greenberg 1958 A crystalline enzyme that cleaves homoserine and cystathionine. I. Isolation procedure and some physicochemical properties. J. Biol. Chem., *230:* 545.
20. Selim, A. S. M., and D. M. Greenberg 1959 An enzyme that synthesizes cystathionine and deaminates L-serine. J. Biol. Chem., 234: 1474.
21. Bergeret, B., F. Chatagner and C. Fromageot 1955 Quelques relations entre le phosphate de pyridoxal et la décarboxylation de l'acide cystéine-sulfinique par divers organes du rat normal ou du rat carence en vitamine B_6. Biochim. Biophys. Acta, *17:* 128.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measure-

ment with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
23. Spackman, D. N., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., *30:* 1190.
24. Gerritsen, T., M. L. Rehberg and H. A. Waisman 1965 On the determination of free amino acids in serum. Anal. Biochem., *11:* 460.
25. Kleeman, C. R., E. Taborsky and F. H. Epstein 1956 Improved methods for determination of inorganic sulfate in biologic fluids. Proc. Soc. Exp. Biol. Med., *91:* 480.
26. Brown, R. A., and M. Sturtevant 1949 The vitamin requirements of the growing rat. Vitamins Hormones, *7:* 171.
27. McAfee, J. W., and M. A. Williams 1962 Effect of cysteine and pyridoxine on taurine excretion of male rats. Proc. Soc. Exp. Biol. Med., *109:* 102.
28. Chatagner, F. 1964 Adaptation métabolique chez les animaux. II. Influence de la teneur en protéines du régime sur la décarboxylase de l'acide 1-cystéine sulfinique dans le foie du rat. Effect de l'éthionine. Biochim. Biophys. Acta, *81:* 400.
29. Trautman, O., and F. Chatagner 1964 Adaptation métabolique de la cystathionase du foie de rat. Bull. Soc. Chim. Biol., *46:* 129.
30. Knox, W. E., and A. H. Mehler 1951 The adaptive increase of tryptophan peroxidase-oxidase system of liver. Science, *113:* 237.
31. Feigelson, P., and O. Greengard 1962 Immunochemical evidence for increased titers of liver tryptophan pyrrolase during substrate and hormonal enzyme introduction. J. Biol. Chem., *237:* 3714.
32. Kenney, F. T. 1962 Induction of tyrosine-α-ketoglutamate transaminase in rat liver. IV. Evidence for an increase in the rate of enzyme synthesis. J. Biol. Chem., *237:* 3495.
33. Nishikawara, M. T., and J. G. Bricker 1966 Relation between glucocorticoid level and hepatic glutamic dehydrogenase activity. Amer. J. Physiol., *210:* 586.
34. Brown, F. C., J. Mallady and J. A. Roszell 1966 The separation of rat liver serine dehydratase and cystathionine synthase. J. Biol. Chem., *241:* 5220.
35. Finkelstein, J. D. 1967 Methionine metabolism in mammals. Effects of age, diet, and hormones on three enzymes of the pathway in rat tissues. Arch. Biochem. Biophys., *122:* 583.
36. Nagabhushanam, A., and D. M. Greenberg 1965 Isolation and properties of a homogenous preparation of cystathionine synthetase-L-serine and L-threonine dehydratase. J. Biol. Chem., *240:* 3002.
37. Kato, A., T. Matsuzawa, M. Suda, H. Nakagawa and J. Ishizuka 1964 Control mechanism in the rat liver enzyme system converting L-methionine to L-cystine. J. Biochem. (Tokyo), *55:* 401.
38. Finkelstein, J. D., and S. H. Mudd 1967 Trans-sulfuration in mammals (The methionine-sparing effect of cystine). J. Biol. Chem., *242:* 873.
39. Pauling, L. 1968 Orthomolecular psychiatry. Science, *160:* 265.
40. "Enzyme Nomenclature: Recommendations 1965 of the International Union of Biochemistry," Elsevier, New York (1965).

Studies of Dietary Effects on Free and Membrane-bound Polysomes in Rat Liver

SANCIA GAETANI, DONATELLA MASSOTTI AND
MARIA ANTONIETTA SPADONI
Istituto Nazionale della Nutrizione, Città Universitaria, Roma, Italia

ABSTRACT Adult rats of the Sprague-Dawley strain were divided into three groups and fed in the following way: group 1 received ad libitum a complete diet, group 2 received ad libitum a protein-free diet and group 3 was pair-fed with group 2 a complete diet. After 10, 20, or 40 days the animals were killed and free and membrane-bound polysomes prepared and analyzed in sucrose gradients. After 20 days of protein starvation the following modifications appear: 1) preferential breakdown of membrane-bound polysomes with increase of light aggregates; 2) shift of free polysomes toward classes heavier than pentamers; 3) increase of monosomes and disomes in both free and membrane-bound polysomes; 4) imbalance between RNase and RNase inhibitor determined by a decrease of RNase inhibitor; 5) increase of the 45S subribosomal particle. These modifications seem to be an adaptation mechanism for survival in adverse conditions like long-term protein deficiency.

It has been demonstrated that, during long-term protein deficiency, the liver develops a compensatory mechanism so that free amino acids are preferentially utilized by this organ for the synthesis of protein mostly concerned in processes of synthesis (1). In the rat and in man the amino-acyl-RNA-synthetases perform an important role in this compensatory mechanism (2, 3).

It has also been shown that in long-term protein deficiency, while there is a loss of total RNA, probably resulting from a modified ratio of ribonuclease to ribonuclease inhibitor, there is an increase in the rate of RNA synthesis (4, 5). Furthermore, in the new equilibrium reached after prolonged protein starvation, there is a modification of the protein population with a preferential synthesis of some and a decrease or loss of others (6–9). The ability of the hepatic cell to synthesize preferentially the proteins indispensable for its functioning is an essential adaptation mechanism for the maintenance of life.

It seems likely that the changes described above are caused by a reorganization of the structures of the protein synthesizing machinery. In particular, in recent years it has been shown that the pattern-size distribution of polysomes, involved in translation, is greatly influenced by dietary protein. It has been reported that in the liver of fasted rats there is a rapid response to a single feeding of protein or of a complete amino acid mixture, as evidenced by a shift of polyribosomes from lighter to heavier aggregates (10). In addition, Munro and co-workers (11, 12) have reported this effect when fasted rats were tube-fed a complete amino acid mixture for a short period of time, but not when they were tube-fed a complete amino acid mixture devoid of tryptophan. More recently, Sidransky et al. (13) have confirmed the effect of tryptophan deprivation on liver polysomal pattern, but were unable to produce similar changes by deleting other essential amino acids from the mixture fed to mice. Wilson and Hoagland (14) have presented evidence that starvation of rats for several days led to a marked decrease of polysomes and accumulation of breakdown products having S values less than 200 S. Refeeding of the starved animals induced a rapid reassembly of polysomes. In experiments where rats were tube-fed for several days a diet lacking threonine or tryptophan, other investigators (15, 16) found a shift of liver polysomes from light toward heavy aggregates. However, it has been demonstrated that some of these modifications induced when nutrition is changed are actually mediated by glucocorticoids (16).

In the present paper, we have studied the influence of protein deficiency on both

Received for publication May 6, 1969.

the free and the membrane-bound liver polysomes. We have also studied the kinetics of these modifications starting from a few days after initiation of the protein-free diet to 40 days when, as has been demonstrated, the compensatory mechanism is well established. In a preceding paper (17) we have already shown that, after 30 days of protein starvation, there is an increase of the heavy in comparison to the light aggregates.

EXPERIMENTAL PROCEDURE

Animals. Male albino rats of the Sprague-Dawley strain weighing 150 g were used in all experiments. The animals were fed a standard laboratory diet. At the beginning of the experiments the animals were divided into three groups.

Group 1 received ad libitum a complete diet, group 2 received ad libitum a protein-free diet, and group 3 received an amount of complete diet equal to the average amount consumed by animals of group 2 (pair-fed rats).

After 10, 20 or 40 days from the beginning of the experiment, the animals were killed and their livers removed and chilled in 0.25 M sucrose solution in TKM buffer (Tris 50 mM, pH 7.6, KCl 25 mM, magnesium acetate 5 mM). For free and membrane-bound polysome preparation, the procedure of Blobel and Potter was followed (18).

Preparation of ribosomes. In this method all the steps are done in the presence of liver postmicrosomal supernatant (S3) which contains the inhibitor of ribonuclease. The livers were homogenized in 2 volumes (w/v) of 0.25 M sucrose-S3 in TKM buffer; the homogenate was centrifuged for 15 minutes at 15,000 × *g*. The postmitochondrial supernatant (PMS) was layered over a two-layer discontinuous gradient (3 ml of 2 M-S3 and 3 ml of 1.38 M-S3) and centrifuged for 24 hours at 40,000 rpm in a 60 rotor of a refrigerated preparative ultracentrifuge (Martin Christ Omega II). The free ribosomes sediment in a pellet at the bottom of the tube, while the membranes with ribosomes still attached remain in the 1.38 M layer. This layer is rehomogenized in the presence of Triton 4% and sodium deoxycholate 1% and applied over 3 ml of a 2 M-S3 sucrose layer and then centrifuged for 24 hours so that ribosomes which were bound to membranes, sediment in a pellet. The two pellets were dissolved in H_2O-S3 and the polysomes fractionated on 27.5 ml of a linear sucrose density gradient 10–34% for 1.15 hours at 27,000 rpm. Gradients were analyzed with an ISCO density gradient fractionator at a constant scanning speed of 2.5 ml per minute using a 1 cm light-path flow cell.

Analysis of ribosomal subunits. Adequate amounts of PMS were layered on a linear sucrose density gradient (10–34% w/v). Some of the samples were treated with antiferritin antiserum in order to precipitate ferritin (19) which sediments between 20 S and 120 S, looks like RNA at 260 mμ and confounds the absorption values unless it is first removed. The gradients were centrifuged for 6 hours at 27,000 rpm and then analyzed as described for polysomes.

Determination of the ribonuclease inhibitor. The activity of the ribonuclease inhibitor was determined according to the method of Quirin-Stricker et al. (5) on liver postmicrosomal supernatant.

RESULTS

Blobel and Potter demonstrated that a quantitative separation of membrane-bound ribosomes is possible only using a postnuclear supernatant (18). With this procedure they found that 25% of the ribosomal population is free and that 75% is bound to the membranes of the endoplasmic reticulum. Under these conditions, however, as Blobel and Potter reported, some breakdown of polysomes occurs, even in the presence of the ribonuclease inhibitor. The use of a postmitochondrial supernatant permits a nearly quantitative recovery only of free ribosomes, and the intactness of both ribosomal fractions is well preserved. On the other hand, when all the experiments are run under strictly controlled conditions, the recovery of polysomes in the two different groups of rats is comparable, and ribosomal aggregates isolated represent the state of aggregation in vivo. Because we were interested in investigating the effect of protein deficiency on the aggregation of polysomes in free and membrane-bound states we thought

that the best source for the isolation of the two polysomal fractions intact, was the postmitochondrial supernatant.

A typical pattern of free and membrane-bound polysomes prepared from the postmitochondrial supernatant of liver of rats fed a standard complete diet is shown in figure 1. Figures 2, 3 and 4 show the effect of protein starvation on ribosomal patterns. Because along with a long-term protein deficiency, a caloric restriction necessarily occurs, ribosomal profiles from liver of protein-starved rats are compared with ribosomal profiles prepared from liver of rats pair-fed with the protein-deficient animals.

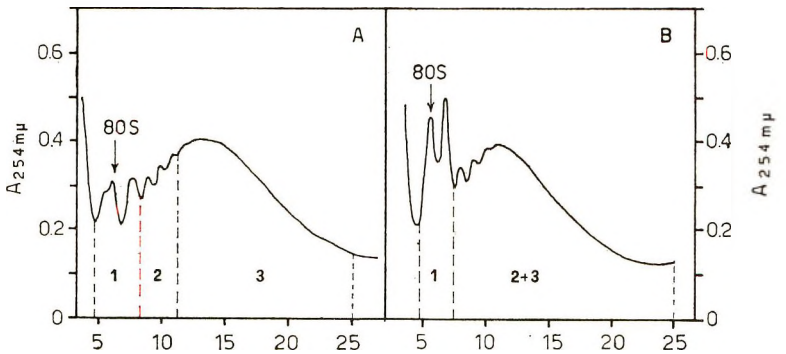

Fig. 1 Sucrose gradient analysis of free (A) and membrane-bound (B) liver ribosomes from rats fed ad libitum a complete diet. Area 1 represents monomers and dimers including also the peak before 80 S; area 2, trimers, tetramers and pentamers; area 3, polysomes higher than pentamers; area 2+3, total polysomes.

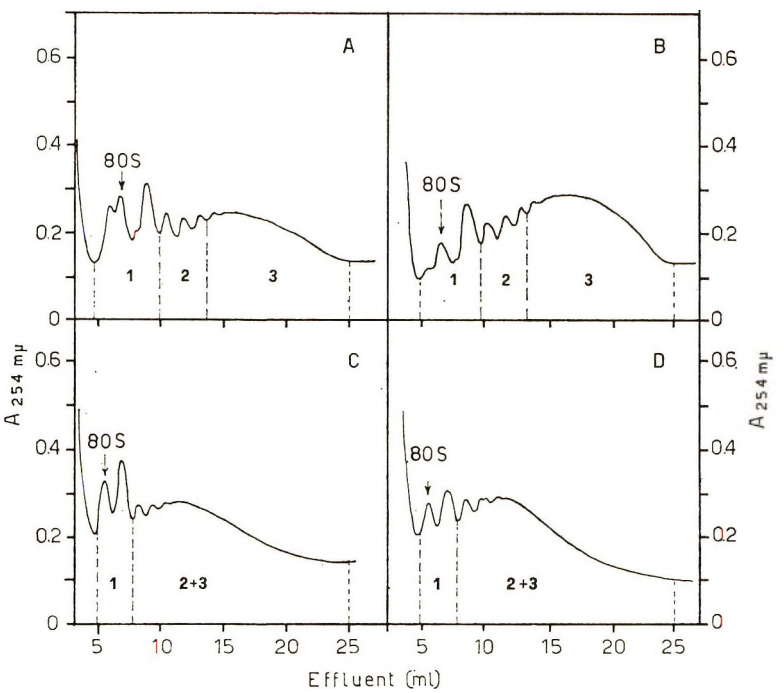

Fig. 2 Sucrose gradient analysis of free (A, B) and membrane-bound (C, D) liver ribosomes prepared from rats fed a protein-free diet for 10 days (A, C), or pair-fed a complete diet (B, D). Polysomal classes are as in figure 1.

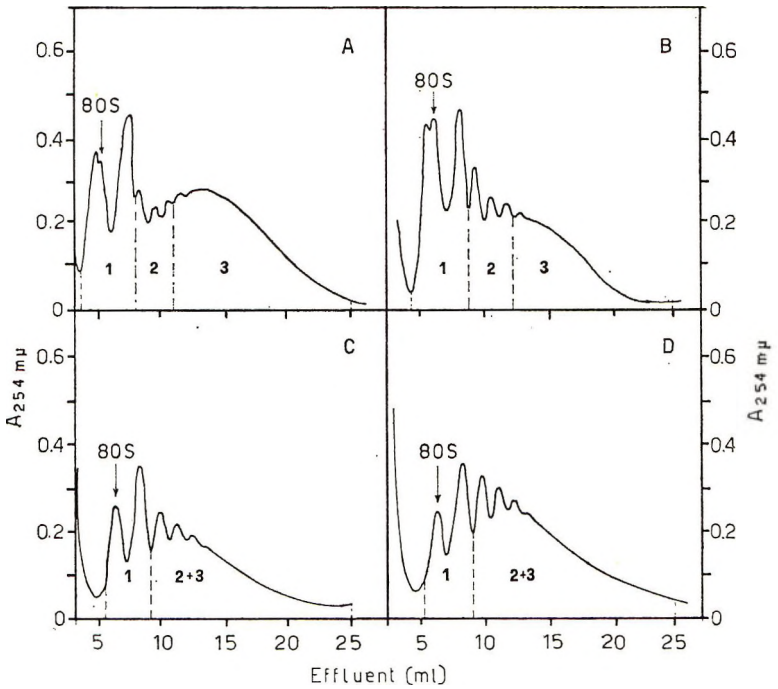

Fig. 3 Sucrose gradient analysis of free (A, B) and membrane-bound (C, D) liver ribosomes prepared from rats fed a protein-free diet for 20 days (A, C), or pair-fed a complete diet (B, D). Polysomal classes are as in figure 1.

The sedimentation profiles of free and membrane-bound ribosomes show modification in the free ribosomal aggregations and in the amount of ribosomes interacting with membranes, as the protein depletion progresses.

Free ribosomes. After 10 days of protein starvation, the free ribosomal population (fig. 2A) shows a higher amount of monosomes and a concomitant decrease of the fraction of polysomes in comparison to free ribosomes prepared from control rats fed a complete diet ad libitum (fig. 1A). The profiles of free ribosomes prepared from the liver of pair-fed animals (fig. 2B) show modifications similar to those observed in protein-starved rats, but less marked.

After 20 days of protein starvation, while monomers and dimers show a further increase (fig. 3A), polysomes begin to display a shift toward heavy aggregates. On the contrary, the breakdown of free polysomes in pair-fed rats (fig. 3B) appears more accentuated.

When the experimental treatment is protracted for 40 days, the differences reported between protein-starved and pair-fed rats are even more accentuated. As figure 4A shows, irrespective of the high proportion of monomers and dimers, the percentage of heavy aggregates as compared to the percentage of small aggregates is further increased in protein-starved rats.

The shift from light toward heavy aggregates is presented numerically in table 1, where, for each point of dietary treatment, is reported the ratio of the area of polysomes heavier than pentamers over the area of trimers, tetramers and pentamers. The data show that this ratio increases significantly starting from day 10 of protein starvation, being maximum after 40 days.

Membrane-bound ribosomes. The behavior of membrane-bound ribosomes is quite different from that of the free aggregates. From day 10 to day 40 of protein starvation there is a progressive breakdown of polysomes toward light aggregates. The process results in a progressive loss of this ribosomal fraction (figs. 2C,

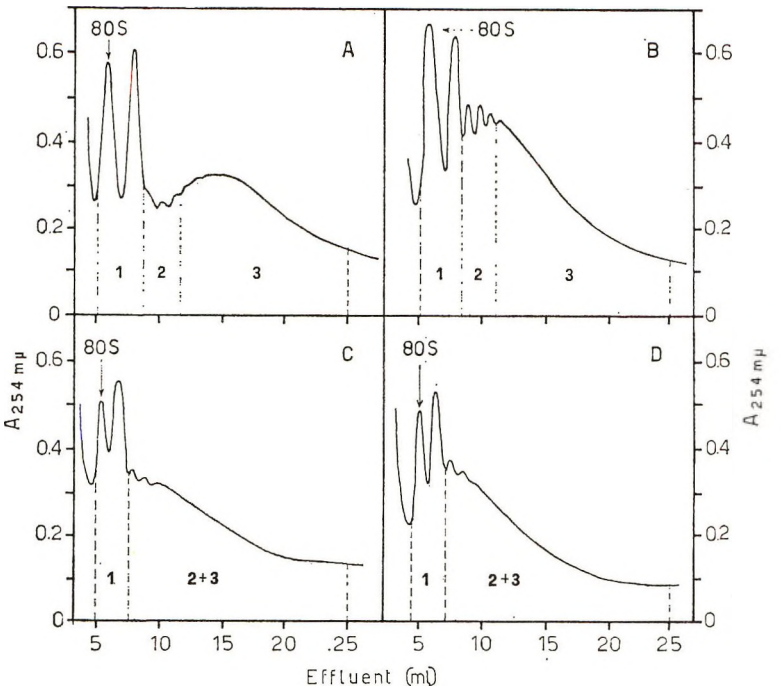

Fig. 4 Sucrose gradient analysis of free (A, B) and membrane-bound (C, D) liver ribosomes prepared from rats fed a protein-free diet for 40 days (A, C) or pair-fed a complete diet (B, D). Polysomal classes are as in figure 1.

TABLE 1

Effect of protein starvation on the aggregation of free ribosomes [1]

Length of dietary treatment	Area of heavy polysomes / Area of light polysomes	
	Protein-starved rats	Pair-fed rats
days		
0		4.1 ± 0.16
10	2.8 ± 0.14 [a]	3.3 ± 0.10 [d]
20	3.5 ± 0.16 [b]	2.1 ± 0.52 [e]
40	4.3 ± 0.18 [c]	2.5 ± 0.25 [f]

[1] Values listed represent the ratio of the area of heavy polysomes (area 3 in figures 1A, 2A,B, 3A,B, 4A,B) to the area of light polysomes (area 2 in the same figures). Each value is the mean ± SEM of experiments done with three different pools of liver; each pool is derived from at least three livers. The probability that the observed difference between groups might occur by chance using Student's *t* test is the following: between a and d $P < 0.05$; between b and e $P < 0.01$; between c and f $P < 0.001$; between a and b $P < 0.01$; between b and c $P < 0.01$.

3C, 4C). This phenomenon is present also, even if less pronounced, in pair-fed animals (figs. 2D, 3D, 4D).

Ribosomal subunits. In the profiles of free ribosomes, there often appears, just before 80 S, a peak having a sedimentation constant of 60 S. This peak is higher in protein-starved and (to a lesser degree) in pair-fed animals than in control rats fed ad libitum. In pair-fed rats the peak appears to be higher only after 20 days of dietary treatment. The sedimentation constant of this fraction corresponds to the large ribosomal subunit, but if ferritin has not been previously removed, the ribosomal peak is covered by the protein. The separation of subunits in the presence of antiferritin antibody demonstrates that this is indeed the case. In figure 5 are recorded typical sedimentation profiles of liver subribosomal particles prepared from rats after 40 days of protein starvation and from their pair-fed control rats. The peak representing the 45 S subribosomal particle compared to the peak of the 60 S particle appears to be higher in protein-depleted than in control rats.

Ribonuclease inhibitor. One of the mechanisms controlling the state of aggregation of ribosomes seems to be represented by the ratio of ribonuclease to

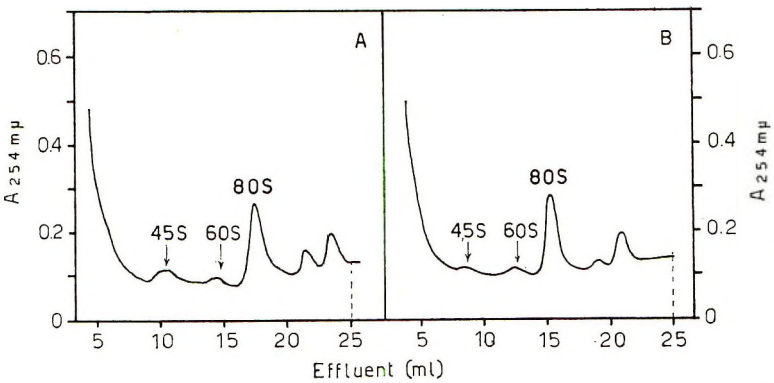

Fig. 5 Sedimentation pattern of ribosomal subunits prepared from liver postmitochondrial supernatant of rats fed for 40 days a protein-free diet (A) or from their pair-fed controls (B) in the presence of antiserum antiferritin (25 μliters per 0.20 ml postmitochondrial supernatant). Sucrose gradient 10–34% w/v. Centrifugation 5 hours at 26,500 rpm.

ribonuclease inhibitor. As Shortman (20) has demonstrated, the amount of ribonuclease inhibitor activity increases in regenerating liver, along with the increase of RNA and of heavy polysomes. Table 2 shows that in the liver of rats maintained for 40 days with a protein-free diet, when an increase of monomers and dimers occurs, there is a concomitant decrease in the ribonuclease inhibitor activity.

This result agrees with the results of Quirin-Stricker et al. (5) in rats protein-starved for 4 weeks.

DISCUSSION

During long-term protein deficiency there is a decrease of total RNA resulting from a loss of ribosomal RNA (21). At the same time the synthesis of messenger RNA is reduced. As our results demonstrate, in addition to these quantitative modifications, there is a redistribution of ribosomes in the free and in the membrane-bound fractions. In particular, there is a great breakdown of polysomes bound to membranes. Besides the modification in the association between membranes and polysomes, starting from day 20 of protein starvation a rearrangement in the free ribosomal fraction takes place with a shift from lighter to heavier classes of polysomes. While this preferential increase of heavy polysomes takes place, the peaks corresponding to monomers and dimers also become progressively higher.

The described reduction in the amount of the ribonuclease inhibitor unaccompanied by a change in the ribonuclease activity, can account for the decrease of the total ribosomal population and for the accumulation of monomers and dimers. In this way monomers, and subunits derived from them, would be made available for the assembly of new polysomes for the syn-

TABLE 2

Effect of protein starvation on liver hepatic ribonuclease inhibitor [1]

Rats	Supernatant	Ribonuclease activity		B/A × 100	Inhibition
		— Supern. A	+ Supern. B		
	ml				%
A [2]	0.025	0.450	0.300	66.6	33.4
A	0.050	0.450	0.163	36.2	63.8
N [2]	0.025	0.450	0.220	48.8	51.2
N	0.050	0.450	0	0	100

[1] Ribonuclease activity, determined according to the method of Quirin-Stricker et al. (5) on postmicrosomal supernatant, is expressed as units of absorption at 260 mμ. The incubation mixture (0.6 ml) contains: 0.2 ml veronal buffer 0.03 M (pH 7.8), 0.2 ml of 1% RNA solution, 0.1 ml postmicrosomal supernatant (1:9 w/v) and 0.1 ml ribonuclease (0.015 mg).

[2] A: rats fed a protein-free diet for 40 days; N, pair-fed control rats.

thesis of protein necessary for the maintenance of the hepatic homeostasis, and after degradation to nucleotides, they would provide building blocks for the synthesis of new messenger RNA strands.

It has been postulated (22) that polysomes bound to membranes synthesize protein for secretion, while free polysomes synthesize protein for intracellular purposes. The increase of the heavy free polysomes after a long period of protein starvation, in conjunction with the demonstration of preferential synthesis of protein involved in processes of intracellular syntheses, supports the hypothesis that those proteins are most likely formed on free polysomes. On the other hand, serum albumin, a typical protein for export, is synthesized only on membrane-bound polysomes (22); furthermore, its synthesis decreases during protein deficiency (23). If this is true, our results indicate that at least under an emergency condition like long-term protein deficiency, the distribution of messenger RNAs (mRNA) is not random between the two kinds of polysomes, and that free and membrane-bound polysomes are not interchangeable in the cytoplasm. Thus the loss of membrane-bound polysomes can be considered as an adaptation mechanism to long-term protein deficiency. However, the possibility should be pointed out that the lower stability of one kind of polysomes as compared to the other could dictate the selection of protein to be synthesized.

Concerning the mechanism of the described modifications it is possible that the preferential loss of membrane-bound polysomes is the consequence of the breakdown of cytoplasmic membranes during protein starvation (24).

The shift of free polysomes toward heavy aggregates can be explained in several ways. One method would be an increased synthesis of selected heavy messenger RNA molecules. This would result in selection of heavy protein molecules. The increase of heavy polysomes obtained after force-feeding of threonine for several days is interpreted by Staehelin et al. (25) as an increase of heavy mRNA molecules, where fewer ribosomes are placed.

Another explanation would be an increased number of ribosomes on any given strand of mRNA which would lead to a more economical utilization of mRNA molecules in a situation of reduced synthesis of these molecules. This higher number of ribosomes could be the result of an increased read-out rate, perhaps due to higher amounts of initiation factors. In this regard it is of interest to note that after a long-term protein deficiency there is, in comparison to pair-fed controls, a higher peak corresponding to the 45 S particle with which, according to several authors, some of the initiation factors are associated. The meaning of the increase of this subunit, however, needs further investigation.

A third possibility to be considered is a change in conformation of polysomal aggregates with increase of sedimentation velocity.

LITERATURE CITED

1. Waterlow, J. C. 1959 Effect of protein depletion on the distribution of protein synthesis. Nature, *184:* 1875.
2. Gaetani, S., A. M. Paolucci, M. A. Spadoni and G. Tomassi 1964 Activity of amino acid-activating enzymes in tissues from protein-depleted rats. J. Nutr., *84:* 173.
3. Stephen, J. M. L., and J. C. Waterlow 1968 Effect of malnutrition on activity of two enzymes concerned with aminoacid metabolism in human liver. Lancet, *1:* 118.
4. Munro, H. N., and C. M. Clark 1960 The nutritional regulation of ribonucleic acid metabolism in the liver cell. Proc. Nutr. Soc., *19:* 55.
5. Quirin-Stricker, C., M. Gross and P. Mandel 1968 Influence d'une carence en protéines sur l'activité des ribonucleases et sur celle de leur inhibiteur dans le foie de rat. Biochim. Biophys. Acta, *159:* 75.
6. Knox, W. E., W. N. Auerbach and E. C. Lin 1956 Enzymatic and metabolic adaptations in animals. Physiol. Rev., *36:* 164.
7. Waterlow, J. C. 1959 Protein nutrition and enzyme changes in man. Federation Proc., *18:* 1143.
8. Pitot, M. C., W. R. Potter and M. P. Morris 1961 Metabolic adaptations in rat hepatomas. I. The effect of dietary protein on some inducible enzymes in liver and hepatoma 5123. Cancer Res., *21:* 1001.
9. Williams, J. N. 1964 Response of the liver to prolonged protein depletion. IV. Protection of succinic oxidase and succinic dehydrogenase by dietary methionine and cystine in a protein-free ration. J. Nutr., *82:* 51.
10. Webb, T. E., G. Blobel and V. R. Potter 1966 Polyribosomes in rat tissues. III. The response of the polyribosomes pattern of rat liver to physiologic stress. Cancer Res., *26:* 253.

11. Fleck, A., J. Shepherd and H. N. Munro 1965 Protein synthesis in rat liver: Influence of amino acids in diet on microsomes and polysomes. Science, *150:* 628.
12. Wunner, W. H., J. Bell and H. N. Munro 1966 The effect of feeding with a tryptophan-free amino acid mixture on rat liver polysomes and ribosomal ribonucleic acid. Biochem. J., *101:* 417.
13. Sidransky, H., D. S. R. Sarma, M. Bongiorno and E. Verney 1968 Effect of dietary tryptophan on hepatic polyribosomes and protein synthesis in fasted mice. J. Biol. Chem., *243:* 1123.
14. Wilson, S. H., and M. B. Hoagland 1967 Physiology of rat-liver polysomes. The stability of messenger ribonucleic acid and ribosomes. Biochem. J., *103:* 556.
15. Sidransky, H., T. Staehelin and E. Verney 1964 Protein synthesis enhanced in the liver of rats force-fed a threonine-devoid diet. Science, *146:* 766.
16. Cammarano, P., G. Chinali, S. Gaetani and M. A. Spadoni 1968 Involvement of adrenal steroids in the changes of polysome organization during feeding of unbalanced amino acid diet. Biochim. Biophys. Acta, *155:* 302.
17. Di Girolamo, A., M. Di Girolamo, S. Gaetani and M. A. Spadoni 1966 Rat liver polysomes in long-term protein deficiency. Proc. 7th Int. Congr. Nutr., *5:* 186.
18. Blobel, G., and V. R. Potter 1967 Ribosomes in rat liver: An estimate of the percentage of free and membrane-bound ribosomes interacting with messenger RNA in vivo. J. Mol. Biol., *28:* 539.
19. Drysdale, J. W., and H. N. Munro 1967 Polysome profiles obtained from mammalian tissues by an improved procedure. Biochim. Biophys. Acta, *138:* 616.
20. Shortman, K. 1962 Studies on cellular inhibitor of ribonuclease. III. The levels of ribonuclease and ribonuclease inhibitor during the regeneration of rat liver. Biochim. Biophys. Acta, *61:* 50.
21. Munro, H. N., and C. M. Clark 1960 The nutritional regulation of ribonucleic acid metabolism in the liver cell. Proc. Nutr. Soc., *19:* 55.
22. Takagi, M., and K. Ogata 1968 Direct evidence for albumin biosynthesis by membrane bound polysomes in rat liver. Biochem. Biophys. Res. Commun., *33:* 55.
23. Chandrasakharam, N., A. Fleck and H. N. Munro 1967 Albumin content of rat hepatic cells at different levels of protein intake. J. Nutr., *92:* 497.
24. Munro, H. N. 1964 In: Mammalian Protein Metabolism, eds., H. N. Munro and I. B. Allison. Academic Press, New York, vol. I, p. 400.
25. Staehelin, T., E. Verney and H. Sidransky 1967 The influence of nutritional change on polyribosomes of the liver. Biochim. Biophys. Acta, *145:* 105.

Muscular Dystrophy in Chicks Fed Crystalline Amino Acid Diets

S. P. NETKE, J. G. VELU, H. W. NORTON AND H. M. SCOTT
Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT Supplementing a crystalline amino acid diet, devoid of sulfur amino acids and lacking vitamin E, with 0.6% DL-methionine maximized chick gain. The incidence of muscular dystrophy was high at all levels of methionine between 0.2% and 0.6%, absent at 0.65% or more and virtually nil at levels less than 0.2%. Adding graded amounts of L-cystine to a dystrophy inducing diet (DD) containing 0.5% methionine, progressively reduced dystrophy lesions but the highest concentration used (0.1%) failed to afford complete protection. When the L-arginine·HCl content of DD was varied over the range 0.48% to 1.57%, growth was depressed and muscle striations were absent so long as L-arginine·HCl continued to be first limiting (0.7% or less). The incidence was high when arginine and methionine were either equally limiting or methionine was first limiting in terms of growth. Comparable results were observed when the lysine content of DD was altered in a similar manner. Imbalancing DD with graded amounts of lysine progressively depressed weight gain and decreased the frequency and severity of the lesions. The growth-promoting ability of the diet appears to be a factor in the etiology of the syndrome.

Following the observation of Dam et al. (1) that chicks developed muscular dystrophy when fed a diet devoid of vitamin E and deficient in sulfur-containing amino acids, numerous investigations have been conducted on various aspects of the myopathy (2–7). Nesheim et al. (3) observed that when their dystrophy-inducing diet was made simultaneously deficient in arginine, chicks grew poorly and did not exhibit striations in the breast muscle so characteristic of muscular dystrophy. However, severe lesions were noted when the arginine-deficient diet was supplemented with this amino acid. They, therefore, concluded that the development of muscular dystrophy on the diet employed depended not only upon dietary levels of vitamin E and sulfur amino acids, but on the concentration of dietary arginine as well. Subsequently, it was shown (5, 6) that the role of arginine in this relationship was not specific for this amino acid.

The present investigation was undertaken to examine the frequency and severity of the dystrophic lesions when the amino acid adequacy of a crystalline amino acid diet was varied over a wide range.

EXPERIMENTAL

Male chicks, 8 days of age and originating from a mating of New Hampshire males to Columbian females, were used throughout these studies. No attempt was made to feed the parent stock a ration low in vitamin E. The chicks were fed an isolated soya protein-glucose purified diet deficient in vitamin E, during the pre-experimental period of 7 days.[1] On day 7, the feeders were removed for 4 hours, replaced for 2 hours and removed thereafter. On the morning of day 8 the chicks were weighed to the nearest gram, wingbanded and so distributed in groups of 10 chicks each. that the average initial starting weight and range in weight were essentially the same for all groups. The groups were housed separately in electrically heated battery brooders with raised wire floors. The various dietary treatments were randomized over the battery compartments. Unless indicated otherwise, two replicates of 10 chicks each were fed each experimental diet. The composition of the basal diet used during the experimental period is given in table 1. Feed and water were

Received for publication May 16, 1969.

[1] When fed the dystrophy-inducing crystalline amino acid diet during the experimental period, muscle striations were first noted by day 13 irrespective of whether the chicks originating from nondepleted hens had been fed the purified diet or a pratical corn-soybean meal chick starting ration during the pre-experimental period. Thus, the depletion of vitamin E stores is not needed to induce early and severe lesions if a dystrophy-inducing crystalline amino acid diet is used during the experimental period.

TABLE 1

Composition of basal diet

	%
Amino acid mixture [1] (variable)	18.58
Stripped lard	4.00
Salt mixture [2]	5.37
Cellulose [3]	3.00
$NaHCO_3$	1.00
Choline chloride	0.20
Vitamins [4]	+
Antioxidant [5] (0.22 g/kg diet)	+
Cornstarch to 100	+
	100

[1] See experimental section and table 2.
[2] Zimmerman, R. A., and H. M. Scott, J. Nutr., 87: 13, 1965.
[3] Solka Floc, Brown Company, Chicago 3, Ill.
[4] Klain, G. J., H. M. Scott and B. C. Johnson. Poultry Sci., 39: 39, 1960, except for omission of α-tocopherol acetate.
[5] Diphenyl-p-phenylene diamine.

supplied ad libitum. Group feed consumption data and individual chick weights were recorded on day 14 and weekly thereafter. At the end of each experiment, all chicks were killed and the skin removed from the breast to assess the incidence and severity of muscular dystrophy. The severity index was obtained by scoring the exposed muscle of afflicted chicks on a scale of 1 to 4 with increasing involvement.

The pattern of crystalline amino acids used in mixtures A, B, C, and D is indicated in table 2. It will be noted that mixture A is devoid of both methionine and cystine. When supplemented with 0.35% DL-methionine and 0.35% L-cystine, this mixture represents the crystalline amino acid reference standard (RS) in use at this laboratory at the time the dystrophy study was initiated. For purposes of growth, mixtures B, C, and D are moderately, and to the same degree, deficient in sulfur-containing amino acids, with cystine as a void. The first limiting amino acid in mixture B is arginine whereas in C the mixture was formulated to be first limiting in lysine. Mixture D contains slightly more lysine than is specified in RS.

RESULTS AND DISCUSSION

In experiment 1 (table 3), muscular dystrophy was detected only when chicks were fed diets containing 0.3 and 0.5% DL-methionine. Since lesions were not noted at dietary levels of 0.1 and 0.7% methionine, it is evident that the point of demarcation between nondystrophic and dystrophic levels of methionine, in the absence of dietary cystine, resides somewhere between 0.1 and 0.3% whereas at the opposite end of the response curve the distinction between the two falls between 0.5 and 0.7% methionine. In experiment 2 (table 3), therefore, supplemental levels of methionine were chosen to encompass the above range. Forty-five percent or more of the chicks fed diets containing 0.2 to

TABLE 2

Composition of crystalline amino acid mixtures (percent of diet)

Amino acids	Amino acid mixtures			
	A	B	C	D
	%	%	%	%
L-Arginine·HCl	1.21	0.48	1.21	1.21
L-Histidine·HCl·H_2O	0.41	0.41	0.41	0.41
L-Lysine·HCl	1.19	1.19	0.44	1.31
L-Tyrosine	0.45	0.45	0.45	0.45
L-Tryptophan	0.15	0.15	0.15	0.15
L-Phenylalanine	0.50	0.50	0.50	0.50
DL-Methionine	—	0.50	0.50	0.50
L-Cystine	—	—	—	—
L-Threonine	0.65	0.65	0.65	0.65
L-Leucine	1.20	1.20	1.20	1.20
L-Isoleucine	0.60	0.60	0.60	0.60
L-Valine	0.82	0.82	0.82	0.82
Glycine	1.20	1.20	1.20	1.20
L-Proline	0.20	0.20	0.20	0.20
L-Glutamic acid	10.00	10.00	10.00	10.00
Total	18.58	18.35	18.33	19.20

TABLE 3

Weight gain and incidence of muscular dystrophy in relation to the dietary concentration of sulfur amino acids (exp. 1, 2 and 3)

Exp. no.		Treatments	Gain/chick /day [1]	Muscular dystrophy	
				Incidence [2]	Severity [3]
			g		
1	1.	Basal [4]	−0.78	0/7	—
	2.	B+0.10% DL-methionine	0.01	0/9	—
	3.	B+0.30% DL-methionine	3.90	8/10	1.62
	4.	B+0.50% DL-methionine	12.92	6/10	2.50
	5.	B+0.70% DL-methionine	13.89	0/10	—
2	1.	Basal [4]+0.10% DL-methionine	< 0.01	4/18	1.75
	2.	B +0.15% DL-methionine	0.24	3/20	2.67
	3.	B +0.20% DL-methionine	0.83	9/20	3.33
	4.	B +0.25% DL-methionine	1.70	14/20	2.85
	5.	B +0.30% DL-methionine	3.23	13/20	2.92
	6.	B +0.50% DL-methionine	9.10	11/20	2.45
	7.	B +0.55% DL-methionine	10.98	7/20	2.57
	8.	B +0.60% DL-mehtionine	12.56	5/20	1.60
	9.	B +0.65% DL-methionine	12.91	0/20	—
	10.	B +0.70% DL-methionine	12.99	0/20	—
3	1.	Basal [5]	11.66	16/20	3.12
	2.	B+0.0125% L-cystine	12.92	17/20	3.00
	3.	B+0.025% L-cystine	13.23	13/20	2.54
	4.	B+0.05% L-cystine	13.42	9/20	2.78
	5.	B+0.10% L-cystine	13.79	7/20	1.29

[1] Average gain for the assay period 8–21 days.
[2] Number of chicks exhibiting lesions of muscular dystrophy over number of surviving chicks.
[3] Average score of chicks showing dystrophy lesions.
[4] Containing amino acid mixture A (table 2).
[5] Containing amino acid mixture A (table 2) supplemented with 0.5% DL-methionine.

0.5% methionine exhibited muscle striations while a concentration of 0.65% or more afforded complete protection. At the two lowest levels of methionine (0.1 and 0.15%) the number of chicks afflicted with dystrophy was low and the lesions were relatively mild.

In experiment 3 (table 3), the dystrophic diet containing 0.5% methionine was supplemented with graded levels of L-cystine. Cystine supplementation reduced the incidence of dystrophy but even the greatest concentration employed (0.1%) failed to protect against the syndrome although the total dietary sulfur amino acid concentration was 0.6%.

The L-arginine·HCl requirement of the chicks fed the mixture of crystalline amino acids representing RS is 1.21%. In experiment 4, the dystrophy-inducing basal diet was formulated to be first limiting in arginine (0.48%) and second limiting in total sulfur amino acids with a complete void of cystine. In the sixth diet, the concentration of L-arginine·HCl exceeded the chicks' requirement by about 30%. As will be noted from the data presented in table 4, muscular dystrophy was not observed so long as arginine was first limiting but the incidence was high on all diets (3 to 6) where arginine was no longer first limiting. It can be assumed that arginine and sulfur amino acids were about equally limiting in the third treatment. There was no evidence to suggest that excess arginine "antagonized" the deficiency of vitamin E as has been postulated (4).

Amino acid mixture C was formulated to be first limiting in lysine and second limiting in sulfur amino acids with a void of cystine. From table 5, it can be seen that lesions of muscular dystrophy were essentially nonexistent so long as lysine remained first limiting.

The effect of imbalancing the dystrophic diet with excess lysine on the occurrence of dystrophy was examined in experiment 6 (table 6). Both weight gain and frequency of dystrophy progressively declined with succeeding increments of the imbalancing amino acid. No doubt the severity of growth depression would have been in-

TABLE 4

Weight gain and incidence of muscular dystrophy in relation to concentration of dietary arginine (exp. 4)

Treatments	Gain/chick /day [1]	Muscular dystrophy	
		Incidence [2]	Severity [3]
	g		
1. Basal [4]	3.15	0/20	—
2. B+0.22% L-arginine·HCl	8.98	0/20	—
3. B+0.44% L-arginine·HCl	10.08	8/20	3.12
4. B+0.65% L-arginine·HCl	10.71	15/20	2.86
5. B+0.87% L-arginine·HCl	9.98	11/20	2.82
6. B+1.09% L-arginine·HCl	10.91	16/20	2.69

[1, 2, 3] See table 3 footnotes.
[4] Containing amino acid mixture B (table 2).

TABLE 5

Weight gain and incidence of muscular dystrophy in relation to concentration of dietary lysine (exp. 5)

Treatments	Gain/chick /day [1]	Muscular dystrophy	
		Incidence [2]	Severity [3]
	g		
1. Basal [4]	2.93	0/20	—
2. B+0.22% L-lysine·HCl	7.07	1/19	1.00
3. B+0.44% L-lysine·HCl	9.75	10/20	2.10
4. B+0.67% L-lysine·HCl	9.35	13/20	2.85
5. B+0.87% L-lysine·HCl	11.25	16/20	2.19
6. B+1.11% L-lysine·HCl	9.60	7/20	3.14

[1, 2, 3] See table 3 footnotes.
[4] Containing amino acid mixture C (table 2).

TABLE 6

Weight gain and incidence of muscular dystrophy in chicks fed a crystalline amino acid diet imbalanced with excess lysine (exp. 6)

Treatments	Gain/chick /day [1]	Muscular dystrophy	
		Incidence [2]	Severity [3]
	g		
1. Basal [4]	9.76	17/20	2.41
2. B+1.11% L-lysine·HCl	7.77	6/20	2.00
3. B+2.22% L-lysine·HCl	7.09	5/20	1.80
4. B+3.33% L-lysine·HCl	6.14	2/20	2.50

[1, 2, 3] See table 3 footnotes.
[4] Containing amino acid mixture D (table 2).

creased had the diet been formulated so as to have methionine and arginine equally limiting (8).

In agreement with the conclusion reached by Jenkins et al. (5, 6) it would appear that arginine is not specifically involved in inducing muscular dystrophy, because diets formulated to be first limiting in an essential amino acid other than methionine with a void of cystine were also nondystrophic but became dystrophic when the deficiency was removed.

The difference in the incidence of dystrophy between treatments within each experiment was found to be highly significant ($P < 0.01$) when examined by the chi-square test. The data for experiments 1 and 2 were pooled since the differences between the two assays were not significant. The incidence of dystrophy was transformed into angles for further analysis. The combined data for experiments 1 and 2 show an approximate quadratic relation of dystrophy incidence to methionine level, with the maximum incidence being about 70% at about 0.36% methionine. In experiment 3 the incidence of dystrophy decreased as cystine increased, and in experi-

ment 4 the incidence increased as arginine increased. In experiments 1, 2, 3, and 4, partial regressions on weight gain were not significant. The data of experiments 5 and 6 agree in indicating that weight gain is the relevant variable, not lysine level, and are in agreement as to the slope and intercept of the regression of angle on gain.

The evidence presented herein would support the view that growth per se is involved in inducing muscle degeneration. Whenever growth is less than that which could be supported by the concentration of dietary methionine with a void of cystine, the diet is markedly less dystrophic. Presumably, under these conditions, the diet contains more methionine than is needed to maximize gain and the excess could then function to prevent the occurrence of dystrophy.

LITERATURE CITED

1. Dam, H., I. Prange and E. Sondergaard 1952 Muscular degeneration (white striation of muscles) in chicks reared on vitamin E deficient, low fat diets. Acta Pathol. Microbiol. Scand., *31:* 172.
2. Machlin, L. J., and W. T. Shalkop 1956 Muscular degeneration in chickens fed diets low in vitamin E and sulfur. J. Nutr., 60: 87.
3. Nesheim, M. C., C. C. Calvert and M. L. Scott 1960 Effect of arginine deficiency on nutritional muscular dystrophy in the chick. Proc. Soc. Exp. Biol. Med., *104:* 783.
4. Scott, M. L., and C. C. Calvert 1962 Evidence of a specific effect of cystine in the prevention of nutritional muscular dystrophy in vitamin E-deficient chicks. J. Nutr., *77:* 105.
5. Jenkins, K. J., D. C. Hill, L. M. Hutcheson and H. D. Branion 1962 Relation of arginine and sulfur amino acids to the development of muscular dystrophy in the chick. Poultry Sci., *41:* 61.
6. Jenkins, K. J., D. C. Hill and L. M. Hutcheson 1964 Nonspecific role of arginine in the promotion of muscular dystrophy in the chick. J. Nutr., *82:* 415.
7. Hathcock, J. N., and M. L. Scott 1966 Alterations of methionine to cysteine conversion rates and nutritional muscular dystrophy in chicks. Proc. Soc. Exp. Biol. Med., *121:* 908.
8. Huston, R. L., and H. M. Scott 1968 Effect of varying the composition of a crystalline amino acid mixture on weight gain and pattern of free amino acids in chick tissue. Federation Proc. 27: 1204.

Biosynthesis of Folate Coenzymes in Riboflavin-deficient Rats

CARLA BOVINA, LAURA LANDI, PETRONIO PASQUALI AND MARIO MARCHETTI

Istituto di Chimica Biologica e di Biochimica Applicata, Università di Bologna, Bologna, Italy

ABSTRACT The effects of riboflavin deficiency on folic acid metabolism, particularly on distribution of liver folate coenzymes and on enzymic activities involved in the synthesis of these structures, were studied. In the liver of riboflavin-deficient rats the storage of folate derivatives, and in particular of the reduced forms, is lower than that in control rats. Furthermore in the same experimental conditions, H_4 folate dehydrogenase, the enzymic activity responsible for the overall conversion of folate to H_4 folate and HCO-H_4 folate synthetase, are markedly decreased. On the other hand, no significant differences were observed in the liver levels of serine hydroxymethyl transferase and 5,10-CH_2-H_4 folate dehydrogenase. The results demonstrate the influence of riboflavin on the utilization of folic acid for the biosynthesis of coenzymic derivatives. Such influence occurs mainly in the reduction steps responsible for the conversion of folate to H_4 folate.

Nelson et al. (1) observed that congenital malformations induced in the offspring of pregnant rats fed a riboflavin-deficient diet had some features in common with those induced by folate deficiency. Since Miller et al. (2) showed a marked decrease of liver folate and citrovorum factor (CF) in riboflavin-deficient mothers, it can be thought that teratogenic effects of these two deficiencies are a consequence of a common biochemical lesion, such as tissue depletion of folate coenzymes. In fact, these coenzymes are involved in the reactions in which one-carbon units are utilized for the biosynthesis of purines (3, 4), pyrimidines (5) and amino acids (6, 7), and therefore carry on an important role during organogenesis.

The exact mechanism by which riboflavin deficiency influences CF content in the liver is unknown. Therefore, it seemed worthwhile to study more directly the effect of riboflavin deficiency in this metabolic area. For this reason the liver distribution of various folate derivatives was studied. Furthermore, in order to know the mechanism by which this deficiency can influence liver concentration of these compounds, enzymic activities involved in the synthesis of folate coenzymes were studied. In particular, H_4 folate [1] dehydrogenase (EC. 1.5.1.3),[2] the enzymic activity responsible for the overall conversion of folate to H_4 folate, HCO-H_4 folate synthetase (EC. 6.3.4.3), 5, 10-CH_2-H_4 folate dehydrogenase (EC. 1.5.1.5), and serine hydroxymethyltransferase (EC. 2.1.2.1), were determined.

EXPERIMENTAL

Weanling male albino rats were divided into two groups, housed in cages with wire bottoms, and fed ad libitum a riboflavin-deficient diet, and the same diet supplemented with 30 mg riboflavin per kilogram of diet, respectively.

The percentage composition of the deficient diet was: vitamin-free casein, 18; sucrose, 66; peanut oil, 8; shark oil fortified with α-tocopherol (250 mg/liter), 2; vitamin mixture, 2; and salt mixture IV, 4%. The vitamin mixture provided the following per kilogram of diet: (in mg) thiamine·HCl, 5; niacin, 20; *p*-aminobenzoic acid, 10; pyridoxine·HCl, 5; Ca pantothenate, 50; inositol, 400; biotin, 0.3; folic acid,

Received for publication May 19, 1969.

[1] The following abbreviations are used: H_2 folate: dihydrofolate; H_4 folate: tetrahydrofolate; 5(10)—HCO—H_4 folate: 5(10) formyltetrahydrofolate; 5,10—CH_2—H_4 folate: 5,10-methylenetetrahydrofolate: 5,10—CH=H_4 folate: methylidynetetrahydrofolate; 5(10)—HCO—H_4 Pte Glu_n: 5(10) formyl derivatives of tetrahydropteroylpolyglutamic acid; 5(10)—HCO Pte Glu_n: 5(10) formyl-derivatives of polyglutamic acid.

[2] Nomenclature and classification of enzymes recommended by International Union of Biochemistry (1964).

5.5; cyanocobalamin, 0.03; menadione, 5; and choline·HCl, 1000.

Animals were killed after 60 days of this dietary treatment and the livers removed for analysis. Flavins were determined fluorometrically by the method of Bessey et al. (8). To determine the distribution of folate derivatives, liver acetone powders were extracted in 1% (w/v) potassium ascorbate solution, pH 6.0 at 75° for 30 minutes. Measured portions of the clear extract were chromatographed on DEAE-cellulose-Hyflo Super Cel (1:1.25 w/v) columns with an ascorbate-phosphate eluent.

The eluted fractions, after suitable dilutions, were assayed for folic acid activities with *Pediococcus cerevisiae* ATCC 8081, *Streptococcus faecalis* R ATCC 8043, and *Lactobacillus casei* ATCC 7469. The media employed were those described by Bakerman (9), using calcium leucovorin as a reference standard. The components of folate activities were differentiated from each other using the criteria previously described (10). For assaying H_4 folate dehydrogenase, the livers were homogenized in 4 volumes of 0.01 M tris-HCl buffer, pH 7.0 and centrifuged at 20,000 $\times g$ for 10 minutes at 4°. The enzyme was determined in the supernatant, measuring the decrease in the absorbance at 340 mμ caused by the conversion of NADPH to $NADP^+$ and of H_2 folate to H_4 folate (11). In the same supernatant the conversion of folate to H_4 folate was assayed (12). The diazotizable amine, *p*-aminobenzoylglutamic acid, quantitatively formed from H_4 folate, was measured by the Bratton-Marshall method (13).

In order to verify FAD influence on the two enzymic activities, supernatants of deficient animals were preincubated with 2 μg of FAD for 20 minutes. After this period the other components of the reaction mixtures were added and the two enzymic activities were assayed as previously described.

For assaying the other enzyme activities, the livers were homogenized in 9 volumes of 0.05 M tris-HCl buffer, pH 7.5 and centrifuged at 10,000 $\times g$ for 30 minutes at 4°. In the supernatant, serine hydroxymethyl transferase was estimated measuring colorimetrically free HCHO and the bound HCHO in 5,10—CH_2—H_4 folate with the acetylacetone reagent (14). The 5,10—CH_2—H_4 folate dehydrogenase was assayed by determining spectrophotometrically at 355 mμ the 5,10—CH=H_4 folate formed in the system (15). HCO—H_4 folate synthetase was assayed in the supernatant partially purified with protamine sulfate and solid ammonium sulfate, measuring the 5,10—CH=H_4 folate formed (16). Protein was determined by the colorimetric method of Lowry et al. (17) with crystalline bovine plasma albumin as the standard.

RESULTS

The liver content of flavins, FAD, and free riboflavin + FMN in deficient rats is remarkably reduced as compared with control animals ($P < 0.001$) (table 1). The data of table 2 concerning the microbiological assays of folate activities give indicative results of the distribution of these substances in the two groups of animals.

In riboflavin-deficient rats, values obtained with *P. cerevisiae* which measures only the amount of monoglutamate derivatives of folate reduced to tetrahydro level, were lower than in control rats ($P < 0.01$).

TABLE 1

Body weight and liver flavins of riboflavin-deficient rats

Group	Animals in experiment	Final body wt	Flavins	
			Free riboflavin + FMN	FAD
		g	*μg/g tissue*	*μg/g tissue*
1	Control	306±28 (27) [1]	59±7.1 (6)	946±20 (6)
2	Riboflavin-deficient	105±14 (25)	27±3.2 (6)	529±21 (6)

[1] Mean ± SEM; number of animals in parentheses.

Smaller but significant differences are also observed in the values obtained with *S. faecalis* which tests the monoglutamate derivatives of folate and its reduced forms ($P < 0.05$).

No significant difference was found between values obtained with *L. casei* which responds to all folate activities as mono- and polyglutamyl derivatives of reduced and oxidized forms.

The data of table 3 show in detail the different distributions of folate derivatives in the liver of riboflavin-deficient rats and of control rats. A considerable reduction of the amounts of 10—HCO—H_4 folate, H_4 folate and 5—HCO—H_4 Pte Glu_n was observed in deficient animals, while compounds such as 10—HCO—H_2 folate and 10—HCO folate were increased. No significant difference was noted when the other compounds were considered.

From the data of table 4, it appears that the H_4 folate dehydrogenase was significantly lower in the liver of riboflavin-deficient rats when compared with control rats ($P < 0.001$). In the same animals a marked decrease of the enzymic activity which converts folate to H_4 folate was observed ($P < 0.001$). No significant increase of H_4 folate dehydrogenase and of

TABLE 2

Folate activities in the liver of riboflavin-deficient rats

Group	Animals in experiment	Folate activity assay		
		P. cerevisiae	*L. casei*	*S. faecalis*
		µg/g tissue	*µg/g tissue*	*µg/g tissue*
1	Control	4.05 ± 0.35 [1]	30.69 ± 3.8	9.40 ± 1.05
2	Riboflavin-deficient	2.43 ± 0.30	26.65 ± 3.5	6.56 ± 0.95

[1] Mean ± SEM of 6 determinations on different animals.

TABLE 3

Distribution of various folate derivatives in the liver of riboflavin-deficient rats

Compounds	Liver concentration of folate derivatives	
	Control rats	Riboflavin-deficient rats
	µg/g tissue	*µg/g tissue*
10—HCO—H_4 folate	2.93 ± 0.19 [1]	1.68 ± 0.13
10—HCO—H_2 folate and 10—HCO—folate	3.06 ± 0.23	5.51 ± 0.61
5—HCO—H_4 folate	0.76 ± 0.05	0.62 ± 0.08
10—HCO—H_4 Pte Glu_n	8.94 ± 1.03	7.74 ± 0.95
H_4 folate	0.66 ± 0.04	0.25 ± 0.01
10—HCO Pte Glu_n	3.92 ± 0.41	3.14 ± 0.36
5—HCO—H_4 Pte Glu_n	2.43 ± 0.21	1.58 ± 0.23
5—HCO Pte Glu_n	0.98 ± 0.17	1.46 ± 0.20

[1] Mean ± SEM of 6 determinations on different animals.

TABLE 4

H_4 folate dehydrogenase and enzymic activity which converts folate to H_4 folate in the liver of riboflavin-deficient rats

Group	Animals in experiment	H_4 folate dehydrogenase	Enzymic activity which converts folate to H_4 folate
		mµmoles H_2 folate reduced/min/mg protein	*mµmoles H_4 folate formed/40 min/mg protein*
1	Control	5.30 ± 0.19 (10) [1]	1.73 ± 0.07 (10)
2	Riboflavin-deficient	4.12 ± 0.13 (7)	1.11 ± 0.10 (7)

[1] Mean ± SEM; number of animals in parentheses.

TABLE 5

Serine hydroxymethyl transferase, HCO—H_4 folate synthetase and 5,10—CH_2—H_4 folate dehydrogenase in the liver of riboflavin-deficient rats

Group	Animals in experiment	Serine hydroxymethyl transferase	HCO—H_4 folate synthetase	5,10—CH_2—H_4 folate dehydrogenase
		mμmoles of HCHO utilized/ 20 min/mg protein	*mμmoles of 5,10—CH=H_4 folate formed/ 20 min/mg protein*	*mμmoles of 5,10—CH=H_4 folate formed/ 20 min/mg protein*
1	Control	1,128 ± 73 (7) [1]	1,179 ± 84 (6)	481 ± 25 (9)
2	Riboflavin-deficient	993 ± 71 (9)	634 ± 20 (7)	428 ± 22 (10)

[1] Mean ± SEM; number of animals in parentheses.

TABLE 6

Effect of the addition of FAD on H_4 folate dehydrogenase and on enzymic activity which converts folate to H_4 folate derived from liver homogenates of riboflavin-deficient rats

Reaction mixture	H_4 folate dehydrogenase	Enzymic activity which converts folate to H_4 folate
	mμmoles H_2 folate reduced/ min/mg protein	*mμmoles H_4 folate formed/ 40 min/mg protein*
Without addition of FAD	4.12 ± 0.13 [1]	1.11 ± 0.10
With addition of FAD	4.23 ± 0.09	1.16 ± 0.13

[1] Mean ± SEM of 6 determinations.

activity which converts folate to H_4 folate was observed when liver supernatants of deficient rats were preincubated with suitable quantities of FAD (table 5).

The data concerning the other enzymes responsible for the synthesis of one-carbon derivatives of H_4 folate (table 6) showed that the liver levels of serine hydroxymethyl transferase, and of 5,10—CH_2—H_4 folate dehydrogenase do not present significant variations in deficient animals when compared with the controls. A marked decrease of HCO—H_4 folate synthetase, however, was observed in riboflavin deficient rats ($P < 0.001$).

DISCUSSION

The data obtained in the present research confirm the relationships between riboflavin and folate, demonstrated indirectly by Nelson et al. (1) and by Miller et al. (2). With respect to the nature of these relationships, our results show that riboflavin is involved in the biosynthesis of folate coenzymes. The fact that in riboflavin-deficient rats the reduced forms are more affected by deficiency, demonstrates that damage occurs especially in the reduction steps of this process. This hypothesis is also supported by the data concerning the effect of deficiency on the enzymic activities involved in the synthesis of folate coenzymes. The decrease of the enzymic activity which converts folate to H_4 folate, and of H_4 dehydrogenase observed in riboflavin-deficient rats explains the lower levels of reduced forms of folate. The addition of suitable amounts of FAD to the reaction mixtures with enzymic preparations from deficient rats does not enhance significantly the activities studied (table 5). Therefore, it is conceivable that the decreased activities of these enzymes cannot be ascribed to the lower coenzyme levels.

LITERATURE CITED

1. Nelson, M. M., C. W. Scott and H. M. Evans 1952 Production of multiple congenital abnormalities in young by maternal pteroylglutamic acid deficiency during gestation. J. Nutr., *48:* 61.
2. Miller, Z., I. Poncet and E. Takacs 1962 Biochemical studies on experimental congenital malformations: flavin nucleotides and folic acid in fetuses and livers from normal and riboflavin-deficient rats. J. Biol. Chem., *237:* 968.
3. Hartman, S. C., and J. M. Buchanan 1959 Biosynthesis of the purines. XXVI. The identification of the formyl donors of the trans-

formylation reactions. J. Biol. Chem., *234:* 1812.

4. Buchanan, J. M., and C. H. Hartman 1959 Enzymic reaction in the synthesis of the purines. Advan. Enzymol., *21:* 199.
5. Friedkin, M., and A. Kornberg 1957 The Chemical Basis of Heredity. Johns Hopkins Press, Springfield, Illinois.
6. Huennekens, F. M., and M. J. Osborn 1959 Folic acid coenzymes and one-carbon metabolism. Advan. Enzymol., *21:* 369.
7. Nakao, A., and D. M. Greenberg 1958 Studies of the incorporation of isotope from formaldehyde-C^{14} and serine-3-C^{14} into the methyl group of methionine. J. Biol. Chem., *230:* 603.
8. Bessey, O. A., O. H. Lowry and R. H. Love 1949 The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. J. Biol. Chem., *180:* 755.
9. Bakerman, H. A. 1961 A method for measuring the microbiological activity of tetrahydrofolic acid and other labile reduced folic acid derivatives. Anal. Biochem., *2:* 558.
10. Marchetti, M., P. Pasquali and L. Landi 1965 Relationships between biotin and vitamin B_{12}. Effects of biotin and vitamin B_{12} on folic acid metabolism. Biochem. J., *94:* 763.
11. Mathews, C. K., K. G. Scrimgeour and F. M. Huennekens 1963 Dihydrofolic reductase. Methods Enzymol., *6:* 364.
12. Zakrzewski, S. F. 1960 Purification and properties of folic acid reductase from chicken liver. J. Biol. Chem., *235–II:* 1776.
13. Bratton, A. C., and E. K. Marshall 1939 A new coupling component for sulfanilamide determination. J. Biol. Chem., *128:* 537.
14. Scrimgeour, K. G., and F. M. Huennekens 1962 Serine hydroxymethylase. Methods Enzymol., *5:* 838.
15. Scrimgeour, K. G., and F. M. Huennekens 1963 N^5, N^{10}-Methylene-tetrahydrofolic dehydrogenase. Methods Enzymol., *6:* 368.
16. Rabinowitz, J. C., and W. E. Pricer, Jr. 1963 Formyltetrahydrofolate synthetase. Methods Enzymol., *6:* 375.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem., *193:* 265.

Effect in Man of the Addition of Tryptophan or Niacin to the Diet on the Excretion of Their Metabolites

ITSIRO NAKAGAWA, TETSUZO TAKAHASHI, TAKESHI SUZUKI AND YOUKO MASANA

Department of Nutrition and Biochemistry, Institute of Public Health, Tokyo, Japan

ABSTRACT Two experiments, each lasting 16 days, were carried out, the second experiment 1 month after the first one finished. Six healthy college women served as experimental subjects. The basal diet contained natural amino acid mixture, cornstarch, corn oil, minerals, and vitamin mixture, including 0.25 g of tryptophan and 0 mg of niacin. In experiment 1, tryptophan was added to the basal diet at different levels (0.50, 0.75, 1.25, and 1.75 g), keeping the amount of niacin at zero. In the second experiment, niacin was added to the basal diet at different levels (8.3, 12.5, 20.8, and 29.2 mg), keeping the amount of tryptophan at a constant level. Urinary excretion of total nitrogen, urea, creatinine, *N*-methyl-2-pyridone-5-carboxamide, N^1-methylnicotinamide, nicotinic acid, kynurenic acid, and xanthurenic acid was determined and, in the second experiment, the levels of NAD and NADP in blood plasma were also measured. All the subjects were in good health and their body weight was at a constant level throughout the experiment, nitrogen equilibrium being maintained. In experiment 1, excretion of niacin metabolites increased markedly on the addition of 1.25 g of tryptophan to the basal diet, and in the second experiment, excretion of these metabolites increased on the addition of 8.3 mg of niacin to the basal diet. The amount of niacin metabolites excreted on the addition of tryptophan or niacin was compared with that excreted during the basal diet period. The relationship between the amount of tryptophan added and three niacin metabolites seemed to be linear from 1.25 to 1.75 g of tryptophan added, and that between the amount of niacin added and niacin metabolites was also linear from 8.3 to 29.2 mg of niacin. The ratio of the amount (in mg) of tryptophan to that (in mg) of niacin added by which the same excretion of niacin metabolites appeared was calculated, i.e., how much tryptophan is convertible to niacin: this ratio decreased from about 122 to about 75 as the amounts of added tryptophan and niacin increased. The levels of NAD and NADP in blood plasma did not change significantly.

Goldsmith et al., and Horwitt et al., about 10 years ago, studied the minimal niacin requirement and relative efficacy of tryptophan in substituting for niacin, and the data reported by them have generally been used since. Goldsmith et al. (1–4) created niacin deficiency in experimental subjects by feeding them a semipurified diet containing 190 mg of tryptophan and 4.7 mg of niacin for 80 to 100 days, and the deficiency was recovered by ingestion of 2 mg or more of niacin. In another experiment, various amounts of tryptophan or niacin were added to a diet containing 200 mg of tryptophan, and niacin requirement and the amount of tryptophan convertible to niacin were determined by measuring the main excretory products of niacin-tryptophan metabolism, N^1-methylnicotinamide (*N*-MNA), *N*-methyl-2-pyridone-5-carboxamide (pyridone), and quinolinic acid. It was noted that 8 to 10 mg of niacin produced a marked increase of niacin metabolites and, therefore, man requires 8 to 10 mg of niacin when his diet contains 200 mg of tryptophan. In fact, 1 mg of niacin was equivalent to 55.8 mg of tryptophan on the average, varying from 34 to 86 mg (4).[1] Horwitt et al. (5) reported that comparison of the amounts of *N*-MNA excreted at different levels of niacin and tryptophan intake showed that approximately 60 mg of tryptophan is equivalent to 1 mg of niacin, although there was an individual variation, but they did

Received for publication May 5, 1969.

[1] Goldsmith, G. A., O. N. Miller and W. G. Unglaub 1956 Efficiency of tryptophan as a niacin precursor. Federation Proc., *15*: 553 (abstract). In this preliminary version of reference 4, the quantitative results differ only slightly from those given in the later paper (4).

TABLE 1

Age, height, weight and energy intake of subjects

Subject	Age	Body height	Body wt	Avg daily energy intake [1]
	years	*cm*	*kg*	*kcal/kg/day*
O.Z.	23	159.0	60.0	35
F.D.	21	159.0	49.0	35
S.D.	23	158.0	50.0	34
F.Z.	23	157.0	53.5	35
K.Z.	21	153.5	47.0	37
S.J.	22	158.5	55.0	27

[1] Calories derived from the amino acid mixture are not included.

TABLE 2

Composition of experimental basal diet

	Daily intake	Calories
	g	*kcal*
Cornstarch	160	640
Sucrose	82	328
Butter	42	378
Corn oil	135	1215
Salt mixture [1]	4.7	
Vitamin mixture [2]	1.0	
Baking powder	7.0	
Sodium chloride	13	
Cellulose powder (carboxymethyl cellulose)	12	
Candy	13	52
Total		2613

[1] In grams: Ca $(H_2PO_4)_2 \cdot H_2O$, 4.00 (including 2.86 derived from baking powder); $CaCO_3$, 1.00; $KHCO_3$, 1.30; $MgSO_4 \cdot 7H_2O$, 0.65; $FeC_6H_5O_7 \cdot 6H_2O$, 0.10; and: (in milligrams) $MnSO_4 \cdot 4H_2O$, 3; $CuSO_4 \cdot 5H_2O$, 8; KI, 0.4; $ZnCl_2$, 5; $CoCl_2 \cdot 6H_2O$, 0.2; $(NH_4)_3PO_4 \cdot 12MoO_3 \cdot 6H_2O$, 0.2.

[2] Thiamine, 5.0 mg; riboflavin, 2.0 mg; vitamin B_6, 0.5 mg; folic acid, 0.5 mg; Ca p-pantothenate, 0.5 mg; ascorbic acid, 40 mg; retinyl palmitate, 4000 IU; vitamin D_2, 400 IU. Biotin and vitamin B_{12} were considered unnecessary in this type of comparatively short-term experiment.

TABLE 3

Composition and daily intake of amino acid mixture

	Daily intake	Nitrogen content
	g	*g*
Essential amino acids		
Isoleucine	1.40	0.150
Leucine	2.20	0.236
Lysine	1.60	0.306
Methionine	2.20	0.206
Phenylalanine	2.20	0.186
Threonine	1.00	0.118
Tryptophan	0.25	0.034
Valine	1.60	0.192
Nonessential amino acids		
Alanine	7.85	1.234
Arginine	9.64	3.101
Aspartic acid	9.23	0.972
Glutamic acid	9.32	0.887
Sodium glutamate	10.62	0.879
Glycine	5.00	0.929
Histidine	0.62	0.168
Proline	3.92	0.478
Serine	5.88	0.783
Total (essential + nonessential)		10.859

not measure the urinary excretion of pyridone. More pyridone than *N*-MNA is excreted in the urine; thus, pyridone is an important end product of niacin metabolism.

EXPERIMENTAL PROCEDURE

The present study consists of two experiments. In experiment 1, six healthy college women served as experimental subjects (table 1). Composition of their diet is shown in table 2. A natural amino acid mixture was used as the source of protein (table 3) and one-third of this mixture was weighed for each subject for each meal. After the subjects had consumed the basal diet (tryptophan 0.25 g and niacin 0 mg) for 6 days, they were divided into two groups of three each. Group 1 was given the basal diet supplemented with 0.50 g of tryptophan for 5 days and with 1.25 g for the next 5 days. Group 2 was given the basal diet supplemented with 0.75 g of tryptophan for 5 days and with 1.75 g for the next 5 days. Total nitrogen in both groups was maintained at a constant level by the substitution of isonitrogenous glycine. Determinations were made of the 24-hour urinary excretion of total nitrogen, urea, creatinine (6), pyridone (7, 8), *N*-MNA, free nicotinic acid,[2] xanthurenic

[2] Miyazawa, S. 1952 Determination of vitamin by use of microorganisms. Annual Report of Takamine Laboratory, 4: 115. Free nicotinic acid was obtained from the biological assay of nonhydrolyzed urine samples.

acid (9), and kynurenic acid (9). Urine samples were collected in dark bottles containing 5 ml of glacial acetic acid, and creatinine determination ascertained completeness of urine collection.

Experiment 2 was carried out on the same subjects except one (S.J.), 1 month after the first experiment finished. The same basal diet was again fed for 6 days, and then the five subjects were divided into two groups of three and two, respectively; group 1 was given the basal diet supplemented first with 8.3 mg of niacin for 5 days, then with 20.8 mg for 5 days, and group 2 was given 12.5 mg of niacin for 5 days followed by another 5 days with 29.2 mg. The amount of niacin fed during each period was designed to correspond to 1 mg of niacin for 60 mg of supplemental tryptophan. The duration of each period in experiment 2 was the same as in experiment 1.

EXPERIMENTAL RESULTS

The results obtained in experiment 1 are presented in table 4. Nitrogen in the feces was not determined. Even though this nitrogen was estimated to be 1 g per day, all of the subjects maintained a nitrogen equilibrium within ± 5%. Nitrogen equilibrium is defined, according to Leverton et al. (10), as the zone in which the difference between the intake and excretion does not exceed 5%, i.e., excretion is within 95 to 105% of the intake. Excretion of urea-nitrogen paralleled the amount of total nitrogen. On addition of 0.5 g of tryptophan to the basal diet in group 1, excretion of pyridone increased to only a slight degree in one subject (O.Z.), but not in the other two subjects (F.D. and S.D.). On addition of 1.25 g of tryptophan, however, rapid increase was seen in all the subjects. Following the basal diet period, 0.75 g of tryptophan was added to the diet in group 2 (F.Z., K.Z. and S.J.) but excretion of pyridone did not increase. However, it increased rapidly on the addition of 1.75 g of tryptophan. Excretion of *N*-MNA paralleled that of pyridone, but the increase was not as pronounced. Excretion of nicotinic acid increased on the addition of 0.50 or 0.75 g of tryptophan, but not so rapidly as the excretion of pyridone or *N*-MNA, even by further addition of tryptophan. It is natural that the excretion of kynurenic acid and xanthurenic acid increased on the addition of tryptophan (fig.

TABLE 4

Urinary excretion of total nitrogen, creatinine, N-MNA, pyridone and nicotinic acid at different levels of tryptophan intake (niacin 0, experiment 1)

Subject	Daily tryptophan intake	Daily N intake	Daily urinary N	Creatinine N	*N*-MNA	Pyridone	Nicotinic acid
	g	*g*	*g*	*mg*	*mg*	*mg*	*mg*
Group 1							
O.Z.	0.25	11.52	9.02	1201	0.97	1.43	0.35
	0.75	11.51	10.63	1165	1.29	2.20	0.64
	1.50	11.46	8.35	1173	3.45	5.79	0.71
F.D.	0.25	11.51	10.43	1138	3.52	4.40	0.41
	0.75	11.47	10.68	1069	2.06	3.27	0.60
	1.50	11.45	11.18	1149	4.55	9.27	0.68
S.D.	0.25	11.40	10.21	971	3.25	4.07	0.34
	0.75	11.47	9.35	1082	2.12	2.40	0.55
	1.50	11.44	8.72	865	3.03	5.03	0.53
Group 2							
F.Z.	0.25	11.46	10.68	1001	3.95	6.76	0.44
	1.00	11.41	11.09	1158	2.74	4.58	0.66
	2.00	11.37	9.01	1053	8.93	21.02	0.68
K.Z.	0.25	11.45	10.40	949	2.49	4.30	0.41
	1.00	11.41	8.74	809	2.82	3.77	0.45
	2.00	11.36	9.56	878	5.25	17.68	0.51
S.J.	0.25	11.43	10.10	1017	2.40	3.25	0.45
	1.00	11.35	9.91	1024	3.40	3.65	0.46
	2.00	11.29	9.53	1049	5.10	15.61	0.70

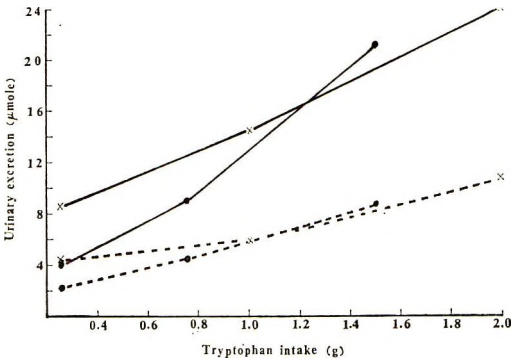

Fig. 1 Excretion of kynurenic acid and xanthurenic acid at different levels of tryptophan intake (niacin 0, experiment 1): ——, kynurenic acid; – – –, xanthurenic acid.

1). All the subjects were in good health and body weight changes were relatively small during the entire period of the experiment.

Results obtained in experiment 2 are summarized in table 5. The intake of tryptophan was kept at a level of 0.25 g per day during this experiment, and nitrogen equilibrium was maintained in all the subjects. Data with respect to the excretion of creatinine and urea were similar to those in experiment 1. On addition of 8.3 mg of niacin (if 60 mg of tryptophan is assumed to be equivalent to 1 mg of niacin, 0.5 g of tryptophan will be equivalent to 8.3 mg of niacin) to the basal diet, excretion of pyridone tended to increase; when niacin was supplemented to the level of 20.8 mg (equivalent to 1.25 g of tryptophan), it increased markedly. In group 2, it increased considerably at a level of 12.5 mg of niacin (equivalent to 0.75 g of tryptophan), and another large increase was observed on the addition of 29.2 mg of niacin (equivalent to 1.75 g of tryptophan). Excretion of *N*-MNA again paralleled that of pyridone, but the increase was not so rapid. Excretion of nicotinic acid did not increase significantly. Excretion of kynurenic acid and xanthurenic acid did not increase as was observed on the addition of tryptophan: rather, it tended to decrease (fig. 2). The levels of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) in blood plasma did not show any definite tendency. All subjects were healthy and kept a constant body weight during the experiment, as in experiment 1.

DISCUSSION

The experimental subjects maintained a nitrogen equilibrium throughout the experimental period at the level of 0.25 g of tryptophan without having niacin. Addition of 1.25 g of tryptophan produced an

TABLE 5

Urinary excretion of total nitrogen, creatinine, N-MNA, pyridone and nicotinic acid at different levels of niacin intake (tryptophan 0.25 g, experiment 2)

Subject	Daily niacin intake	Daily N intake	Daily urinary N	Creatinine N	*N*-MNA	Pyridone	Nicotinic acid
	mg	*g*	*g*	*mg*	*mg*	*mg*	*mg*
Group 1							
O.Z.	0	11.54	9.40	1123	2.45	2.83	0.46
	8.3	11.40	8.60	1108	2.32	4.20	0.50
	20.8	11.37	9.59	1153	4.63	10.67	0.29
F.D.	0	11.36	10.56	1002	1.48	2.39	0.40
	8.3	11.32	10.32	892	2.19	3.19	0.41
	20.8	11.29	9.96	987	5.66	13.16	0.51
S.D.	0	11.37	9.25	854	1.29	1.44	0.29
	8.3	11.31	9.66	828	1.66	2.78	0.35
	20.8	11.27	8.94	965	4.32	11.98	0.45
Group 2							
F.Z.	0	11.37	9.83	975	2.62	2.90	0.35
	12.5	11.31	9.60	996	3.40	7.24	0.33
	29.2	11.31	9.00	1007	7.26	21.31	0.51
K.Z.	0	11.26	9.23	865	1.11	1.28	0.30
	12.5	11.29	10.18	753	2.73	5.85	0.32
	29.2	11.26	8.80	774	5.75	19.61	0.35

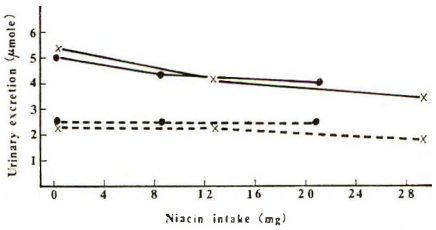

Fig. 2 Excretion of kynurenic acid and xanthurenic acid at different levels of niacin intake (tryptophan 0.25 g, experiment 2): ——, kynurenic acid; – – –, xanthurenic acid.

increase in total amount of three main excretory products of niacin-tryptophan metabolism (*N*-MNA, pyridone, and nicotinic acid) but 0.75 g of tryptophan did not. On the contrary, when niacin was added in stepwise increases to the basal diet, excretion of these three metabolites increased markedly on the addition of 8.3 mg of niacin. At the point where a rapid increase in the excretion of these metabolites occurred, the amount of niacin intake to that of tryptophan was 8.3 to 1000 mg., i.e., 1:120 (by interpolation between the tryptophan data, see fig. 3).

The relationship between the amounts of tryptophan or niacin ingested and the increase in amount of the three metabolites excreted is plotted in figure 3. Despite the fact that the amounts of tryptophan and niacin added during each period were designed to be 60:1, the two curves neither overlap nor intersect. From this graph, the ratio of the amounts of tryptophan and niacin producing the same excretion of

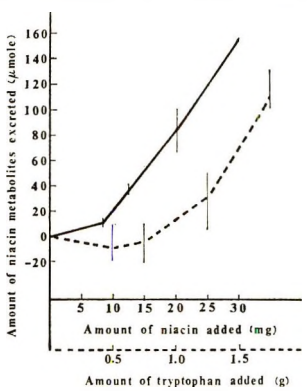

Fig. 3 The relationship between niacin metabolites excreted and the amount of tryptophan or niacin added: ——, niacin-supplemented diet; – – –, tryptophan-supplemented diet.

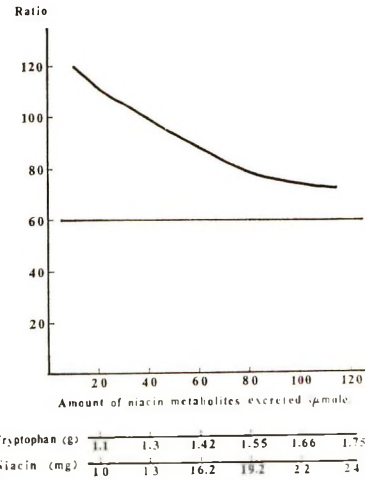

Fig. 4 The ratio of the amounts of added tryptophan and niacin by which the same quantity of niacin metabolites was excreted.

niacin metabolites was calculated, i.e., how much tryptophan is convertible into niacin (fig. 4). For example, it is necessary to add 1,000 mg of tryptophan or 8.2 mg of niacin to the basal diet for excretion of 10 μmoles of niacin metabolic products: hence the efficacy of niacin to tryptophan is 8.2: 1,000 or 1:122. Likewise, in order to excrete 50 μmoles of niacin products, 1,360 mg of tryptophan or 14.6 mg of niacin must be added to the basal diet, hence the ratio is 1:93; and for 100 μmoles of these products, 1,650 mg of tryptophan or 22.2 mg of niacin must be added, hence the ratio is 1:75. The concentrations of NAD and NADP in blood plasma did not change significantly. Thus, the conversion ratio of tryptophan to niacin appears to vary between 122 and 75 under the conditions of our experiments.

LITERATURE CITED

1. Goldsmith, G. A., H. P. Sarett, U. D. Register and J. Gibbens 1952 Studies of niacin requirement in man. I. Experimental pellagra in subjects on corn diets low in niacin and tryptophan. J. Clin. Invest., *31:* 533.
2. Rosenthal, H. L., G. A. Goldsmith and H. P. Sarett 1953 Excretion of N^1-methylnicotinamide and the 6-pyridone of N^1-methylnicotinamide in urine of human subjects. Proc. Soc. Exp. Biol. Med., *84:* 208.
3. Goldsmith, G. A., H. L. Rosenthal, J. Gibbens and W. G. Unglaub 1955 Studies of niacin requirement in man. II. Requirement on

wheat and corn diets low in tryptophan. J. Nutr., 56: 371.
4. Goldsmith, G. A., O. N. Miller and W. G. Unglaub 1961 Efficiency of tryptophan as a niacin precursor in man. J. Nutr., 73: 172.
5. Horwitt, M. K., C. C. Harvey, W. S. Rosenthal, J. L. Cutler and D. Hafferson 1956 Tryptophan-niacin relationships in man. Studies with diets deficient in riboflavin and niacin, together with observations on the excretion of nitrogen and niacin metabolites. J. Nutr., 60, Supplement I.
6. Peters, J. H. 1942 The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146:* 179.
7. Price, J. M. 1954 The determination of *N*-methyl-2-pyridone-5-carboxamide in human urine. J. Biol. Chem., *165:* 293.
8. Walters, C. J., R. R. Brown, M. Kaihara and J. M. Price 1955 The excretion of *N*-methyl-2-pyridone-5-carboxamide by man following ingestion of several known potential precursors. J. Biol. Chem., *217:* 489.
9. Price, J. M., R. R. Brown and N. Yess 1956 Testing the functional capacity of the tryptophan-niacin pathway in man by analysis of urinary metabolites. III. Quantitative methods for tryptophan metabolites. A. Kynurenic acid and xanthurenic acid. (Cited from Levine, R., and R. Luft 1965 Advan. Metabol. Disord., 2: 166).
10. Leverton, R. M., M. R. Gram, M. Chaloupka, E. Brodovsky and A. Mitchell 1956 The quantitative amino acid requirements of young women. I. Threonine. J. Nutr., 58: 59.

Tissue Lactic Dehydrogenase, Glutamic-oxalacetic Transaminase, and Peroxidase Changes of Selenium-deficient Myopathic Lambs [1,2]

P. D. WHANGER, P. H. WESWIG, O. H. MUTH AND J. E. OLDFIELD
Departments of Agricultural Chemistry, Veterinary Medicine and Animal Science, Oregon State University, Corvallis, Oregon

ABSTRACT The concentrations of lactic dehydrogenase (LDH), glutamic-oxalacetic transaminase (GOT), and peroxidase were determined in heart, liver, kidney, spleen, and skeletal muscles (semitendinosus and longissimus dorsi) of normal and selenium-deficient myopathic lambs. There were no differences in the concentrations of these enzymes in the heart, liver (except LDH), kidney, and spleen of normal and myopathic lambs, but there was a significant reduction in the concentration of these enzymes in the skeletal muscles. Severely affected myopathic lambs had a significantly lower LDH in the livers. The concentration of LDH, GOT, and peroxidase in the skeletal muscles was correlated with the severity of damage to these muscles. Two of the lysosomal enzymes, aryl sulfatase and β-glucuronidase, were significantly higher in skeletal muscles of myopathic lambs. There were no differences in the concentrations of LDH, peroxidase, or aryl sulfatase in hearts from lambs with heart lesions as compared to those without these lesions.

The lysosomal enzymes have been shown to increase in the muscle of myopathic rabbits (1), chicks (2), and lambs (3). Because the lysosomal enzymes possess hydrolytic activity (4), they are probably associated with the increased proteolytic and autolytic activity of skeletal muscle from myopathic animals (5). Also, the activity of an enzyme similar to the lysosomal enzymes, 5′-nucleotidase, has been shown to be elevated in muscle of myopathic lambs (6).

Serum glutamic-oxalacetic transaminase (GOT) (7–9) and lactic dehydrogenase (LDH) (7, 9, 10) are elevated in myopathic lambs. If the hypothesis that the increase in these serum enzymes is due to their release from damaged tissues into the blood is correct, there should be enzyme activity changes in the affected tissue of myopathic (white-muscle diseased, WMD) lambs. In support of this reasoning, the LDH concentration has been shown to be lower in longissimus dorsi muscle (10), and the diaphragm, tongue and gluteus medius of myopathic lambs fed an artificial diet [3] (10). Also, a relationship between peroxidation and "muscular dystrophy" has been proposed (11). Therefore, this investigation was undertaken to compare the LDH, GOT and peroxidase levels in various tissues of normal and myopathic lambs fed natural diets.

METHODS

Tissues were taken from six-week-old lambs whose dams were fed a comparatively high-selenium alfalfa hay (0.23 ppm Se), low-selenium alfalfa hay (0.01 ppm Se), or the low-selenium alfalfa hay plus various sulfur sources as previously described (9). In some cases, however, tissues for enzyme assays were taken from lambs on the basis of their plasma enzyme levels. Because previous analyses of forages have shown no direct relationship between tocopherol content and WMD incidence (12), the lambs in the present study were considered normal with respect to vitamin E status. Blocks of tissues were routinely selected, processed, examined histologically (13), and rated with a numerical score from 1 to 3, according to the degree of tissue degeneration. Tissues for enzyme

Received for publication May 7, 1969.

[1] Published with the approval of the Director of the Oregon State University Agricultural Experiment Station as Technical paper 2657.

[2] Supported in part by Public Health Service Research grant no. NB 07413-01 from the National Institute of Neurological Diseases and Blindness.

[3] Paulson, G. D. 1967 I. Lactic dehydrogenase isoenzymes in normal and dystrophic lamb tissues. II. Selenium metabolism in the sheep. PhD Thesis, University of Wisconsin, Madison, Wisc.

assays were collected and frozen (−10°) until the assays could be performed. A preliminary trial indicated that the activities of the enzymes under study were similar in fresh and in frozen tissues.

The tissues were homogenized with a "Virtis" [4] homogenizer (medium speed) for 1 minute in either 0.2 M KCl or 0.25 M sucrose (10% homogenate) and the debris spun down at 8,000 × *g* for 10 minutes. The supernatant was used for the enzyme assays. LDH was assayed according to the procedure of Wroblewski and LaDue (14). GOT was assayed by a colorimetric [5] or spectrophotometric [6] method and aryl sulfatase and β-glucuronidase were assayed according to the methods of Roy (15) and Gianetto and DeDuve (16), respectively. Peroxidase, in a volume of 3 ml, was assayed with 30 μM phosphate buffer (pH 5.7), 2 μM hydrogen peroxide, 5 mg *O*-dianisidine and tissue homogenate (0.1 to 0.3 ml). The rate of color development was measured at 460 mμ. The LDH, peroxidase and GOT (spectrophotometric) activities were determined using a spectrophotometer [7] with a Gilford attachment (17). Protein was determined on the tissue homogenates by the method of Lowry et al. (18), using bovine serum albumin as the protein standard.

The data were subjected to statistical analysis (19).

RESULTS

There was no significant difference in the LDH or in the peroxidase activities of the heart, liver, kidney, spleen or in the peroxidase activity in the longissimus dorsi between the lambs from dams fed 0.23 ppm or 0.01 ppm selenium diets (table 1). However, adequate or deficient levels of selenium resulted in a significant difference in the levels of LDH ($P < 0.001$) in the semitendinosus and longissimus dorsi muscles and in the levels of peroxidase ($P < 0.005$) in the semitendinosus muscle.

In the next study, tissues were selected from lambs with high plasma levels of LDH (average 9,390 units/ml plasma) and GOT (average 4,130 units/ml plasma) and from lambs with low plasma levels of LDH (average 1,760 units/ml plasma) and GOT (average 150 units/ml plasma). All of the lambs in the high plasma group had a muscle damage score of 2 or 3, whereas those lambs with low plasma levels either had no muscle damage or a score of 1 (four normal and five with score of 1). Between these two groups there was no significant difference in the levels of GOT, LDH, or peroxidase in the heart, kidney, spleen, or liver, except that LDH was significantly higher ($P < 0.05$) in the high plasma enzyme group. LDH, GOT, and peroxidase were significantly lower in the semitendinosus and longissimus dorsi muscles

[4] "Virtis 45," The Virtis Company, Inc., Gardiner, New York.

[5] Colorimetric determination of glutamic-oxalacetic and glutamic-pyruvic transaminases at 505 mμ. Sigma Technical Bulletin no. 505. Sigma Chemical Company, St. Louis, Mo.

[6] The determination of glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase at 340 mμ. Sigma Technical Bulletin no. 410. Sigma Chemical Company, St. Louis, Mo.

[7] Beckman DU Spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

TABLE 1

Lactic dehydrogenase and peroxidase activities of heart, liver, kidney, spleen, semitendinosus, and longissimus dorsi of normal and myopathic lambs

Tissue	Specific activity [1] of lactic dehydrogenase		Statistical significance	Specific activity [2] of peroxidase		Statistical significance
	0.23 ppm Se	0.01 ppm Se	*P*	0.23 ppm Se	0.01 ppm Se	*P*
Heart	3.54 ± 0.32 [3]	3.28 ± 0.40	N.S.	23.0 ± 2.10	21.0 ± 2.0	N.S.
Liver	0.86 ± 0.08	0.85 ± 0.06	N.S.	1.75 ± 0.60	1.50 ± 0.14	N.S.
Kidney	0.19 ± 0.04	0.16 ± 0.02	N.S.	1.50 ± 0.30	1.41 ± 0.20	N.S.
Spleen	0.46 ± 0.07	0.43 ± 0.06	N.S.	34.9 ± 2.50	39.6 ± 2.8	N.S.
Semitendinosus	12.26 ± 0.62	7.80 ± 0.95	< 0.001	16.0 ± 1.50	11.0 ± 0.6	< 0.005
Longissimus dorsi	9.68 ± 0.70	7.98 ± 0.59	< 0.001	22.0 ± 1.70	22.0 ± 1.6	N.S.

[1] One unit of LDH is defined as that amount which will convert one μmole of pyruvate to lactate per minute at 25°. Specific activity is the number of units per mg protein.

[2] One unit of peroxidase activity is that amount of enzyme decomposing 1 μmole of peroxide per minute at 25°. Specific activity is the number of units per mg protein times 10^3.

[3] Mean ± SE.

TABLE 2

Lactic dehydrogenase, glutamic-oxalacetic transaminase, and peroxidase of heart, liver, kidney, spleen, semitendinosus, and longissimus dorsi of lambs with high and low plasma levels of lactic dehydrogenase and glutamic-oxalacetic transaminase

Tissue	GOT [1]			LDH [1]			Peroxidase [1,2]		
	High [3]	Low [3]	P	High	Low	P	High	Low	P
Heart	1250 ± 100 [4]	1360 ± 76	N.S.	2.35 ± 0.21	2.29 ± 0.13	N.S.	45.0 ± 1.9	52.0 ± 2.3	N.S.
Liver	236 ± 15	260 ± 13	N.S.	0.44 ± 0.04	0.34 ± 0.02	< 0.05	0.45 ± 0.05	0.77 ± 0.16	N.S.
Kidney	172 ± 8	168 ± 6	N.S.	1.05 ± 0.09	1.08 ± 0.03	N.S.	1.49 ± 0.1	1.27 ± 0.12	N.S.
Spleen	116 ± 6	112 ± 5	N.S.	0.27 ± 0.04	0.30 ± 0.05	N.S.	26.0 ± 1.0	31.1 ± 1.9	N.S.
Semitendinosus	609 ± 35	758 ± 52	< 0.05	4.14 ± 0.54	9.98 ± 0.62	< 0.001	3.8 ± 1.0	10.4 ± 1.8	< 0.001
Longissimus dorsi	540 ± 49	733 ± 44	< 0.01	6.80 ± 0.97	9.25 ± 0.56	< 0.05	16.0 ± 2.3	30.5 ± 2.5	< 0.001

[1] Units of enzyme per mg protein. Definition of units for LDH and peroxidase is same as in table 1. The activity of GOT is expressed as Sigma Frankel units (text footnote 5).
[2] Specific activity times 10^3.
[3] The average plasma levels of GOT and LDH were, respectively, 150 and 1770 units per ml in the low plasma group and 4,130 and 9,390 units per ml in the high plasma group.
[4] Mean ± SE.

of lambs with high plasma enzyme levels. There was no difference in the liver LDH between lambs with ewes fed diets with adequate and deficient levels of selenium (table 1), but when livers from lambs with low plasma enzyme levels were compared to those from lambs with high plasma enzyme levels, there was a significant difference (table 2). This observation is in agreement with the work of Paulson,[8] who reported no difference in the LDH in livers of controls and of lambs with "early dystrophy," but a lower LDH in livers of lambs with "advanced dystrophy." The significant difference in the LDH levels in the muscles of normal and affected lambs also confirms the work of Paulson.[9]

The concentration of LDH was greatest in the muscle, followed by the heart, liver, kidney, and spleen (table 1 and 2), which is in agreement with a previous report.[10] Peroxidase, however, was highest in the heart and spleen, followed by the muscles, and lowest in the liver and kidney. The reason for high peroxidase levels in the spleen is unknown and deserves further investigation. GOT was highest in heart, followed by muscles, liver, kidney, and spleen. This relative concentration of GOT between the various organs is in agreement with other reports (20, 21).

The results from the spectrophotometric method also indicated that GOT was significantly lower ($P < 0.05$) in the semitendinosus muscle of myopathic lambs (table 3). The magnitude of difference was greater between normal lambs and severely affected lambs (high plasma vs. low plasma) than between normal and deficient lambs (0.23 ppm vs. 0.01 ppm Se).

Two of the lysosomal enzymes, aryl sulfatase and β-glucuronidase, had a different trend than encountered with the previous enzymes in that they were significantly higher in the semitendinosus muscle of myopathic lambs (table 4). Again, a trend similar to LDH and GOT was noted; the magnitude of difference was greater between normal and severely affected lambs than between normal and deficient lambs. The increase of lysosomal enzymes in mus-

[8] See footnote 3.
[9] See footnote 3.
[10] See footnote 3.

TABLE 3

Glutamic-oxalacetic transaminase of semitendinosus muscle from lambs with high or low plasma GOT, and from lambs whose dams were fed rations containing 0.23 or 0.01 ppm selenium

Diet or plasma levels	Specific activity [1]	Statistical significance P
0.23 ppm selenium	746 ± 37 [2]	
		< 0.05
0.01 ppm selenium	659 ± 76	
High plasma GOT [3]	591 ± 40	
		< 0.01
Low plasma GOT [3]	753 ± 71	

[1] Units per mg protein. Activity was determined by a spectrophotometric method (text footnote 6). One unit is equal to a change in OD of 0.001 per minute which is equal to the formation of 4.82×10^{-4} μM of glutamate per minute at 25°.
[2] Mean ± SE.
[3] GOT of semitendinosus muscle, determined by a colorimetric method, is given in table 2.

cles of myopathic lambs is in agreement with a previous report (3).

Since these results suggested a relationship between the enzyme content and severity of muscle damage, the data from this study and other similar studies were combined and the enzymatic activity of the semitendinosus muscle plotted against the degree of damage to this muscle (fig. 1). The levels of LDH ($r = -0.69$), GOT ($r = -0.62$), and peroxidase ($r = -0.56$) were correlated with the severity of muscle damage. The LDH concentration in this muscle of lambs with myopathic scores of 2 and 3 was significantly lower ($P < 0.001$) than in normal lambs. The LDH concentration in semitendinosus muscle of lambs with a myopathic score of 3 was significantly lower ($P < 0.01$) than those with a score of 2. The GOT concentration was significantly lower ($P < 0.05$) in semitendinosus muscle from lambs with a myopathic score of 2 than those with a score of 1, and those with a myopathic score of 3 were significantly lower ($P < 0.01$) than either normal lambs or those with a score of 1. The peroxidase levels were significantly lower ($P < 0.01$) in semitendinosus muscle of lambs with myopathic scores of 2 or 3 than in this muscle from normal lambs.

When the enzyme concentration of the longissimus dorsi was plotted against the severity of damage to the semitendinosus muscle (fig. 2), a trend similar to that noted in figure 1 was evident. The assumption is made that the damage to the longissimus dorsi is correlated with the damage to the semitendinosus muscle. This may not be entirely correct, thus accounting for a lesser degree of correlation between enzyme concentration and muscle damage. The peroxidase concentration of the longissimus dorsi from lambs with a myopathic score of 2 was significantly lower ($P < 0.05$) than in normal lambs. The GOT levels were significantly lower ($P < 0.01$) in this muscle from lambs with a myopathic score of 2 or 3 than in normal lambs. The only significant difference ($P < 0.05$) in the LDH concentration in the longissimus dorsi was between those lambs with scores of 1 and 3. A plot of the enzyme (GoT, LDH, or peroxidase) concentration in the liver against the severity of damage of the semitendinosus muscle did not reveal any trend, thus indicating no correlation between the levels of the liver enzymes and muscle damage.

TABLE 4

Aryl sulfatase and β-glucuronidase of semitendinosus muscle from lambs with high or low plasma levels of GOT and LDH, and from lambs whose dams were fed rations containing 0.23 or 0.01 ppm selenium

Diet or plasma levels	Specific activity [1]			
	Aryl sulfatase	P	β-Glucuronidase	P
0.23 ppm selenium	1.8 ± 0.02 [2]		7.9 ± 0.08	
		< 0.05		< 0.01
0.01 ppm selenium	2.6 ± 0.04		23.5 ± 0.37	
Low plasma GOT and LDH	2.1 ± 0.06		6.4 ± 0.08	
		< 0.01		< 0.01
High plasma GOT and LDH	5.1 ± 0.07		44.3 ± 1.20	

[1] Specific activity is the number of micrograms of product formed per hour per mg protein as 37°.
[2] Mean ± SE.

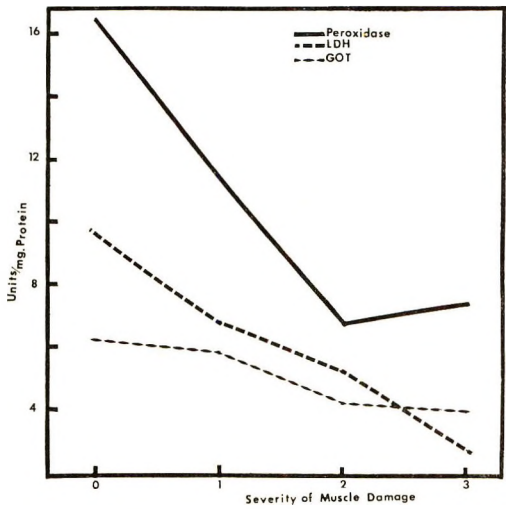

Fig. 1 Specific activity (units/mg protein) of peroxidase, glumatic-oxalacetic transaminase, and lactic dehydrogenase in semitendinosus muscle in relation to the severity of damage to this muscle. The activity of peroxidase is expressed as the specific activity times 10^3, the activity of glutamic oxalacetic transaminase as the specific activity times 10^{-2}, and the activity of lactic dehydrogenase as indicated in table 1.

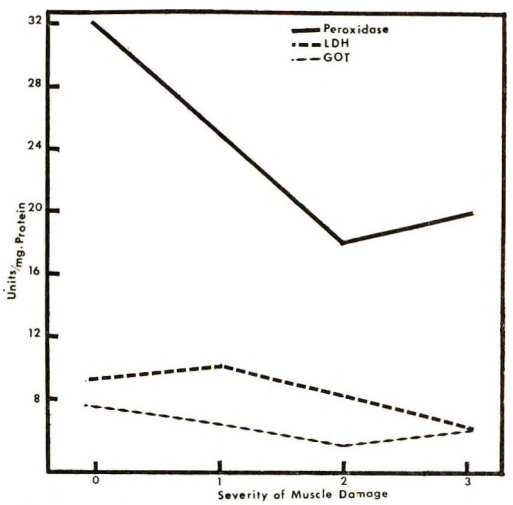

Fig. 2 Specific activity (units/mg protein) of peroxidase, glutamic-oxalacetic transaminase, and lactic dehydrogenase in longissimus dorsi muscle in relation to the severity of damage to the semitendinosus muscle. The activities of the enzymes are expressed as indicated in figure 1.

There was no significant difference in the levels of aryl sulfatase, LDH, or peroxidase in the hearts from lambs with and without heart lesions (table 5), with one exception: there was a slightly lower LDH concentration in hearts from lambs with heart lesions as compared to those without these lesions.

TABLE 5

Aryl sulfatase, lactic dehydrogenase, and peroxidase activities in hearts of lambs with and without heart lesions

Enzyme	Specific activity [1]	
	Lambs with heart lesions	Lambs without heart lesions
Aryl sulfatase	3.8 ±0.14 [2]	3.6 ±0.05
Lactic dehydrogenase	2.47±0.28	2.75±0.30
Peroxidase [3]	49.3 ±2.0	50.8 ±2.4

[1] Specific activity for aryl sulfatase is the same as indicated in table 4, and for LDH and peroxidase as indicated in table 1.
[2] Mean ± SE.
[3] Specific activity times 10^3.

DISCUSSION

Only in the skeletal muscles (longissimus dorsi and semitendinosus) was there a significant reduction in the concentration of LDH, GOT, and peroxidase between normal and myopathic lambs. Other workers have shown that LDH is significantly lower in skeletal muscles of WMD lambs[11] (10) and of mice with hereditary muscular dystrophy (22). There is also a redistribution of the LDH isoenzymes in skeletal muscle of myopathic lambs (10). These results substantiate the histopathologic data indicating that WMD is primarily a disorder of the muscle (12, 13, 23–25). In severe cases, however, the LDH levels in the liver are significantly reduced, which is in agreement with a previous report.[12] These data and those implicating the kidney in WMD (26) suggest that although the muscles are most severely injured there are other tissues affected in selenium-deficient myopathic lambs.

The plasma levels of GOT in the same lambs used for tissue enzyme studies are correlated ($r = 0.68$) with the severity of muscle damage, but the plasma LDH levels were not found to be correlated with this myopathy, as reported previously (9). The levels of both of these enzymes in the tissue, however, were significantly correlated

[11] See footnote 3.
[12] See footnote 3.

with the severity of the myopathy. The present data, indicating a reduction in LDH concentration in the liver of severely affected lambs, suggest the liver may also be releasing this enzyme into the blood system. This offers a possible explanation for the lack of correlation between plasma LDH levels and muscle damage.

Desai (3) reported a 35-fold increase of aryl sulfatase, whereas we found only a two- to threefold increase. We found the same magnitude of difference as reported by Desai, however, in β-glucuronidase levels between normal and myopathic lambs. Desai (3) used tissues from lambs fed a diet which contained an anti-vitamin E factor [13] (27), whereas our tissues were from selenium-deficient lambs receiving adequate vitamin E. These procedural differences may account for the apparent disagreement.

No significant differences in the concentration of LDH, aryl sulfatase, or peroxidase were noted in hearts from lambs with heart lesions as compared to those without these lesions (table 5). Preliminary work from our laboratory also indicated that there are no differences in the levels of β-glucuronidase or acid phosphatase in hearts from these respective lambs. Apparently the lysosomal enzymes are not associated with heart lesions as has been shown for muscle degeneration in WMD (3). Therefore, the biochemical alterations involved in producing heart lesions do not appear to be the same as those related in the production of muscle lesions (WMD). Sulfate appears to be related to heart lesions, because its presence in the diet increases the number of lambs with these lesions (9). This effect of sulfate, however, is prevented by additional selenium.[14]

Peroxidative conditions and irradiation have been shown to damage lysosomes, releasing the lysosomal enzymes into the media (28, 29). The lysosomal enzymes apparently are not directly implicated in the cause of nutritional myopathies, but their increase (1, 2, 3, 4, 11, 30) is probably due to damage to the lysosomes and they are involved in the hydrolytic breakdown and removal of degradative products from the tissues (11).

Selenium compounds have been postulated to function as biological antioxidants or free radical scavengers (31). In light of these reports, it is reasonable to postulate the following sequence of biochemical events in WMD. Due to conditions in the cells, possibly peroxidative or other related conditions, the lysosomes rupture releasing their enzymes into the cytoplasm. The hydrolytic nature of these enzymes (4, 11) results in destruction of the tissue cells releasing the cellular enzymes into the blood. This apparently is the reason for the frequently observed increase of plasma enzyme activity in myopathic animals and for the reduced enzyme concentration (LDH, GOT, and peroxidase) in muscles of WMD lambs. It appears, therefore, that the problem to be solved is: how does selenium maintain the integrity of the cell or, more precisely, of the lysosomes?

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mrs. Susan Carnegie for her competent technical assistance, and to Mr. H. W. Pendell for his assistance with the statistical analysis.

LITERATURE CITED

1. Zalkin, H., A. L. Tappel, K. A. Caldwell, S. Shibko, I. D. Desai and T. A. Holliday 1962 Increased lysosomal enzymes in muscular dystrophy of vitamin E deficient rabbits. J. Biol. Chem., 237: 2678.
2. Desai, I. D., C. C. Calvert, M. L. Scott and A. L. Tappel 1964 Peroxidation and lysosomes in nutritional muscular dystrophy of chicks. Proc. Soc. Exp. Biol. Med., *115*: 462.
3. Desai, I. D. 1966 Activity of lysosomal enzymes in white muscle disease. Nature, 209: 1349.
4. Tappel, A. L., P. L. Sawant and S. Shibko 1963 Lysosomes: Distribution in animals, hydrolytic capacity and other properties. In: Ciba Foundation Symposium on Lysosomes. Little, Brown and Co., Boston, Mass., p. 78–113.
5. Koszalka, T. R., K. E. Mason and G. Krol 1961 Relation of vitamin E to proteolytic and autolytic activity of skeletal muscle. J. Nutr., 73: 78.
6. Arnold, M. A., P. H. Weswig, O. H. Muth and J. E. Oldfield 1965 Relationship of 5′-nu-

[13] Houge, D. E., and H. F. Hintz 1964 Anti-vitamin E activity of kidney beans in nutritional muscular dystrophy in the chick. Federation Proc., 23: 395 (abstract).

[14] Whanger, P. D., P. H. Weswig, O. H. Muth and J. E. Oldfield. Selenium and white muscle disease: Effect of sulfate and energy level on plasma enzymes and rumen microbes. Submitted to Amer. J. Vet. Res.

cleotidase activity in lamb muscle to selenium levels administered to their dams. Proc. Soc. Exp. Biol. Med., *118:* 75.
7. Boyd, J. W. 1964 Serum enzyme changes in lambs with experimentally-induced acute muscular dystrophy. Res. Vet. Sci., 5: 419.
8. Blincoe, C., and D. M. Marble 1960 Blood enzyme interrelationships in white muscle disease. Amer. J. Vet. Res., *21:* 866.
9. Whanger, P. D., O. H. Muth, J. E. Oldfield and P. H. Weswig 1969 Influence of sulfur on incidence of white muscle disease in lambs. J. Nutr., 97: 553.
10. Paulson, G. D., A. L. Pope and C. A. Baumann 1966 Lactic dehydrogenase isoenzymes in tissue and serum of normal and dystrophic lambs. Proc. Soc. Exp. Biol. Med., *122:* 321.
11. Desai, I. D., C. C. Calvert and M. L. Scott 1964 A time-sequence study of the relationship of peroxidation, lysosomal enzymes, and nutritional muscular dystrophy. Arch. Biochem. Biophys., *108:* 60.
12. Schubert, J. R., O. H. Muth, J. E. Oldfield, and L. F. Remmert 1961 Experimental results with selenium in white muscle disease of lambs and calves. Federation Proc., 20: 689.
13. Muth, O. H., J. E. Oldfield, J. R. Schubert and L. F. Remmert 1959 White muscle disease (myopathy) in lambs and calves. VI. Effects of selenium and vitamin E on lambs. Amer. J. Vet. Res., 20: 231.
14. Wroblewski, F., and J. S. LaDue 1955 Lactic dehydrogenase activity in blood. Proc. Soc. Exp. Biol. Med., *90:* 210.
15. Roy, A. B. 1953 The sulphatase of ox liver. I. The complex nature of the enzyme. Biochem. J., 53: 12.
16. Gianetto, R., and C. DeDuve 1955 Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. Biochem, J., 59: 433.
17. Wood, W. A., and S. R. Gilford 1961 A system for automatic recording of absorbancy and its application to enzyme-catalyzed reactions. Anal. Biochem., 2: 589.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
19. Snedecor, G. W. 1946 Statistical Methods. The Iowa State College Press, Ames, Iowa.
20. Cornelius, C. E., J. Bishop, J. Switzer and E. A. Rhode 1959 Serum and tissue transaminase activities in domestic animals. Cornell Vet., *49:* 116.
21. Wroblewski, F., and J. S. LaDue 1956 Serum glutamic pyruvic transaminase in cardiac and hepatic disease. Proc. Soc. Exp. Biol. Med., *91:* 569.
22. McCowan, W. M. 1963 Enzyme studies of skeletal muscle in mice with hereditary muscular dystrophy. Amer. J. Physiol., 205: 897.
23. Muth, O. H. 1955 White muscle disease (myopathy) in lambs and calves. I. Occurrence and nature of the disease under Oregon conditions. J. Amer. Vet. Med. Ass., *126:* 355.
24. Proctor, J. F., D. E. Hogue and R. G. Warner 1958 Selenium, vitamin E and linseed oil meal as preventatives of muscular dystrophy in lambs. J. Anim. Sci., *17:* 1183.
25. Hartley, W. J., and A. B. Grant 1961 A review of selenium responsive diseases of New Zealand livestock. Federation Proc., 20: 679.
26. Wise, W. R., P. H. Weswig, O. H. Muth and J. E. Oldfield 1968 Dietary interrelationship of cobalt and selenium in lambs. J. Anim. Sci., 27: 1462.
27. Hogue, D. E., J. F. Proctor, R. G. Warner and J. K. Loosli 1962 Relation of selenium, vitamin E and an unidentified factor to muscular dystrophy (stiff-lamb or white-muscle disease) in lambs. J. Anim. Sci., 21: 25.
28. Desai, I. D., P. L. Sawant and A. L. Tappel 1964 Peroxidative and radiation damage to isolated lysosomes. Biochim. Biophys. Acta, 86: 277.
29. Wills, E. D., and A. E. Wilkinson 1966 Release of enzymes from lysosomes by irradiation and the relation of lipid peroxide formation to enzyme release. Biochem. J., *99:* 657.
30. Desai, I. D., C. C. Calvert, M. L. Scott and A. L. Tappel 1964 Peroxidation and lysosomes in nutritional muscular dystrophy of chicks. Proc. Soc. Exp. Biol. Med., *115:* 462.
31. Tappel, A. L., and K. A. Caldwell 1967 Redox properties of selenium compounds related to biochemical function. In: Selenium in Biomedicine, eds., O. H. Muth, J. E. Oldfield and P. H. Weswig. The AVI Publishing Co., Westport, Conn., Chapter 21.

Significance of Adipose Tissue and Liver as Sites of Fatty Acid Synthesis in the Pig and the Efficiency of Utilization of Various Substrates for Lipogenesis [1]

EUGENE KEVIN O'HEA AND GILBERT A. LEVEILLE
Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois at Urbana-Champaign, Urbana, Illinois

ABSTRACT A combination of in vivo and in vitro techniques as well as the assay of the activity of several enzymes presumably involved in lipogenesis have been employed to study the relative importance of liver and adipose tissue in overall fatty acid synthesis in the pig. Both the in vivo and in vitro results indicate that when glucose-U-^{14}C is used as substrate, virtually all the newly synthesized fatty acids are formed in the adipose tissue. The incorporation of acetate-1-^{14}C into liver fatty acids was, however, much greater than that of glucose-U-^{14}C, suggesting that, if acetate was freely available in vivo, the contribution of liver to overall lipogenesis may be appreciable. Data on the activity of citrate cleavage enzyme and of three NADPH-generating dehydrogenase enzymes in liver and adipose tissue complement the results obtained in the lipogenic studies. The hepatic capacity for the production of cytoplasmic acetyl CoA from mitochondrially derived citrate is insignificant, as is its ability to generate NADPH required in the reductive synthesis of fatty acids. Collectively the results indicate that the adipose tissue plays a major, if not a nearly exclusive role in fatty acid synthesis in the pig. Nonsaponifiable lipid synthesis in the liver requires acetate rather than glucose as a starting substrate.

Even though many body organs and tissues possess the ability to synthesize fatty acids from simple carbohydrate precursors, it is now well established that, excluding the mammary gland, liver and adipose tissue are the principal sites (1). The biosynthetic pathway is apparently similar in both tissues, but because of differences in hormonal response and in enzyme content, the control and extent of lipogenesis in each site may be markedly different. In view of this, several authors have investigated the relative contribution made by liver and adipose tissue to overall lipogenesis. In the mouse (1, 2) and rat (2, 3), adipose tissue accounts for at least 50% of the newly synthesized fatty acids; however, hepatic tissue is the major if not the only site of fatty acid formation in the pigeon (4) and chicken (5). In ruminant animals such as the cow and sheep, where acetate is the chief precursor of fatty acids, both liver and adipose tissue appear to be important sites of synthesis (6).

This paper presents the results of experiments designed to investigate the relative contribution made by liver and adipose tissue to overall lipogenesis in the pig. A combination of in vitro and in vivo techniques was employed and, in addition, the activity of several lipogenic enzymes was studied.

EXPERIMENTAL

Animals and diets. Male uncastrated pigs of the Yorkshire and Duroc breeds were used in all experiments. The final body weights are indicated in the tables of results. The composition of the diet fed has been described previously (7). Feed and water were supplied ad libitum.

In vitro procedures. For the enzymatic and in vitro lipogenic studies, liver and adipose tissue samples were obtained immediately after slaughter and placed in 0.9% NaCl during transportation to the laboratory. Liver samples were chilled on

Received for publication June 6, 1969.
[1] Supported in part by grants from the Eli Lilly Research Laboratories, Indianapolis, Indiana, and the Moorman Manufacturing Company, Quincy, Illinois.

ice whereas adipose tissue samples were maintained at room temperature. Observations in this laboratory have shown that if adipose tissue is chilled prior to incubation, the in vitro lipogenic rate is significantly impaired. Slices (100 to 150 mg) were prepared using a Stadie-Riggs hand microtome. Incubations were carried out at 37° in 3 ml of Krebs-Ringer bicarbonate buffer (Ca^{2+}-free), pH 7.4, under 95% O_2 and 5% CO_2 in a metabolic shaker (90 strokes per minute) for 2 hours. Labeled substrates (glucose-U-^{14}C, acetate-1-^{14}C, pyruvate-2-^{14}C, DL-lactate-2-^{14}C or propionate-2-^{14}C) were added at a rate of 0.3 μCi per ml of buffer and the insulin [2] concentration was 0.1 unit per ml. All buffers contained 5 μmoles of glucose per milliliter and in addition 10 μmoles of the specific substrate used (sodium salt of acetate, pyruvate, propionate or L-lactate).

Analytical procedures. Carbon dioxide released during the incubations was collected and the various lipid fractions were isolated and counted as previously described (8).

In vivo procedures. Trace amounts (see tables of results) of glucose-U-^{14}C or sodium acetate-1-^{14}C were dissolved in 0.9% NaCl and injected into the vena cava. The animals were killed after specific periods, between 15 and 40 minutes as denoted in tables 4 and 5. In one experiment blood was obtained from the eye vein prior to slaughter. After slaughter (by exsanguination) the liver was removed, weighed and a sample stored at −20° until analyzed (within 1 week). Adipose tissue samples obtained from various sites were similarly treated. The blood was centrifuged and the serum collected and stored. The lipid fractions of all samples were isolated and prepared for liquid scintillation counting as already described (8).

Enzymes studies. Liver and adipose tissue homogenates were prepared in 0.15 M KCl immediately after the samples were obtained. The 100,000 × *g* supernatant was used for all liver enzyme assays and for adipose tissue citrate cleavage enzyme (EC. 4.1.3.6) assay. A 20,000 × *g* supernatant was used for assaying other adipose tissue enzymes. Glucose-6-phosphate dehydrogenase (EC.1.1.1.49) and 6-phosphogluconate dehyrogenase (EC. 1.1.1.44) were assayed by the method of Glock and McLean (9). NADP-malate dehydrogenase (EC. 1.1.1.40) was assayed as described by Ochoa (10) and citrate cleavage enzyme according to the method of Srere (11). The protein content of the homogenates was determined by the method of Lowry et al. (12).

Calculations. From the results obtained in the in vivo studies (tables 4 and 5), estimates of the overall contribution of the liver and adipose tissue to fatty acid synthesis have been made. In all experiments total liver weight was recorded and adipose tissue mass was assumed to be 10% of body weight (13). Using these values the total radioactivity in liver and adipose tissue was calculated and the percentage contribution of each derived.

RESULTS

In vitro experiments. In table 1 are summarized results showing the relative utilization of glucose-U-^{14}C by pig liver and adipose tissue slices. The incorporation of acetate-1-^{14}C into fatty acids and nonsaponifiable lipids by liver slices was manyfold greater than that of glucose-U-^{14}C, the incorporation of which was insignificant. Similarly, the oxidation of glucose-U-^{14}C to $^{14}CO_2$ was only a fraction of that of acetate-1-^{14}C, and its incorporation into glyceride-glycerol was also quite low. Results of the second experiment confirm these findings and indicate that pig liver has a limited capacity to convert glucose to CO_2 and lipid. The results also show that the ability of adipose tissue slices to convert acetate-1-^{14}C to fatty acids is about 10 times that of liver slices when expressed on an equal weight basis.

In a third experiment (table 2) a range of ^{14}C-labeled substrates were employed and their metabolism by pig liver and adipose tissue slices evaluated. All substrates were readily incorporated into adipose tissue fatty acids, whereas incorporation into liver fatty acids was very low, especially in the case of glucose-U-^{14}C and propionate-2-^{14}C. These data also reveal that the amount of glucose-U-^{14}C oxidized to $^{14}CO_2$ by liver slices was only a fraction

[2] The insulin was generously supplied by Dr. R. Chance, Eli Lilly Research Laboratories, Indianapolis, Indiana.

TABLE 1

Utilization of glucose-U-^{14}C and acetate-1-^{14}C by pig liver and adipose tissue slices [1]

Experiment	Tissue	Metabolite measured	Substrate used	
			Glucose-U-^{14}C	Acetate-1-^{14}C
1	Liver	$^{14}CO_2$	32.00 ± 3.70 [2]	1189.2 ± 258.0
		Fatty acids	0.16 ± 0.02	38.2 ± 7.4
		Nonsaponifiable lipids	0.15 ± 0.03	31.7 ± 11.0
		Glyceride-glycerol	6.60 ± 0.40	—
2	Liver	$^{14}CO_2$	39.60 ± 2.30 [3]	1179.0 ± 20.0
		Fatty acids	3.40 ± 0.60	101.0 ± 22.0
		Nonsaponifiable lipids	0.60 ± 0.20	49.0 ± 3.4
	Adipose tissue	$^{14}CO_2$	—	649.0 ± 149.0
		Fatty acids	—	1032.0 ± 122.0

[1] Results are expressed as nanomoles of substrate converted to the product indicated/100 mg tissue per 2 hours.
[2] Mean ± SEM for 6 pigs (average weight 30 kg).
[3] Mean ± SEM for 6 pigs (average weight 12 kg).

TABLE 2

Relative rates of utilization of various ^{14}C-labeled substrates by pig liver and adipose tissue slices [1]

Tissue	Substrate	Metabolite measured			
		$^{14}CO_2$	Fatty acids	Nonsaponifiable lipids	Glyceride-glycerol
Liver	Acetate-1-^{14}C	843 [2]	213	42	116
	Pyruvate-2-^{14}C	319	35	5	46
	DL-Lactate-2-^{14}C [3]	748	79	25	66
	Glucose-U-^{14}C	62	2	0	13
	Propionate-2-^{14}C	373	8	7	62
Adipose tissue	Acetate-1-^{14}C	160	1690	—	186
	Pyruvate-2-^{14}C	74	768	—	75
	DL-Lactate-2-^{14}C [3]	38	2753	—	150
	Glucose-U-^{14}C	694	963	—	191
	Propionate-2-^{14}C	401	381	—	76

[1] Results are expressed as in table 1.
[2] Mean of duplicate samples from 2 pigs.
[3] Based on equal utilization of D- and L-lactate-2-^{14}C. If D-lactate is not utilized by pig liver then the values must be increased by a factor of 2.

of that of any other substrate. Glyceride-glycerol contained appreciable radioactivity from all substrates except in the case of liver slices incubated with glucose-U-^{14}C in which the incorporation of ^{14}C was quite low.

Enzyme data. Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-malate dehydrogenase enzymes were assayed in homogenates of adipose tissue from three different sites and also in liver homogenates. Citrate cleavage enzyme was also assayed in liver and back fat tissue (table 3). In the case of glucose-6-phosphate dehydrogenase and NADP-malic enzyme the activity in kidney fat was higher than back fat which in turn was higher than abdominal fat; however, these differences failed to reach statistical significance ($P > 0.05$). In contrast to adipose tissue, the activity of all three enzymes in liver homogenates was extremely low. Likewise citrate cleavage enzyme, presumably involved in the production of acetyl CoA in the cytoplasm, appears to have minimal activity in pig liver homogenates.

In vivo experiments. Since the in vitro experiments and the results on enzymatic activity indicated that pig liver was a relatively inactive lipogenic tissue, three in vivo experiments were performed to obtain more precise data on the relative lipogenic potential of liver and adipose tissue. The results of the first two in vivo experiments are presented in table 4. Individual data

TABLE 3

Activity of the pentose pathway dehydrogenases, NADP-malate dehydrogenase and citrate cleavage enzyme in pig liver and adipose tissue homogenates [1]

	Back fat	Abdominal fat	Kidney fat	Liver
Glucose-6-P-dehydrogenase	289±49(6) [2]	239±17(5)	334±36(5)	9±1.0(6)
6-P-gluconate dehydrogenase	81±4 (6)	61±3 (5)	72±10(5)	12±2.0(6)
NADP-malate dehydrogenase	312±33(6)	271±44(5)	383±41(5)	3±0.1(6)
Citrate cleavage enzyme	15±3 (2)	—	—	< 1 (2)

[1] Activity expressed as nanomoles substrate converted/minute per milligram protein.
[2] Mean ± SEM for the number of animals shown in parentheses. The animals used for the citrate cleavage enzyme assay weighed 13 kg each; all other animals weighed approximately 30 kg.

TABLE 4

In vivo incorporation of acetate-1-^{14}C into fatty acids by pig liver and adipose tissue

Time of killing [1]	Animal [2] no.	Liver wt	Adipose tissue [3]	Total counts		Contribution	
				Liver	Adipose tissue	Liver	Adipose tissue
min		*g*	*g*	*10^3 dpm*		*%*	
			Experiment 1				
20	1	312	1305	1679	6543	20	80
	2	242	950	215	500	30	70
	3	292	1205	278	906	24	76
	Avg					25	75
40	4	307	1200	115	1410	8	92
	5	267	1160	341	561	38	62
	6	300	1125	285	316	47	53
	Avg					31	69
			Experiment 2				
15	1	275	1404	1342	2370	36	64
	2	394	1404	798	3955	17	83
	3	470	1860	746	1362	35	65
	4	333	1359	2083	6883	23	77
	Avg					28	72

[1] Time elapsed between the administration of sodium acetate-1-^{14}C (in 0.9% saline) and killing of animals.
[2] In experiment 1, animals weighed 11.6 ± 0.5 kg and the dose rate was 20 μCi/pig. In experiment 2, animals weighed 15.0 ± 1.2 kg and the dose rate was 50 μCi/pig.
[3] Assumed to be 10% of body mass.

are presented because of the high degree of variation among animals. It is apparent from these results that in the case of each animal the adipose tissue was responsible for a greater percentage contribution to overall fatty acid synthesis than was liver. Counts in serum fatty acids (results not presented) were extremely low, suggesting that mobilization from either tissue was minimal during the periods investigated. Collectively the results of these two experiments suggest that when acetate-1-^{14}C is used as substrate, the adipose tissue accounts for 70 to 75% and the liver for 25 to 30% of the newly synthesized fatty acids.

In the monogastric animal, glucose rather than acetate is the more physiological precursor of fatty acids. An experiment was therefore performed to investigate the in vivo incorporation of glucose-U-^{14}C into fatty acids. Data from this experiment are summarized in table 5. As had been observed in the in vitro experiments (tables 1 and 2), the radioactivity found in liver fatty acids was insignificant, whereas adipose tissue fatty acids were highly labeled. When the contribution of liver and adipose tissue to overall fatty acid synthesis is calculated, the adipose tissue in all cases accounts for more than 99%.

DISCUSSION

Before a proper understanding of the control of fatty acid synthesis in any species can be attained, it is necessary to

TABLE 5

In vivo incorporation of glucose-U-^{14}C into fatty acids by pig liver and adipose tissue

Time of killing [1]	Animal [2] no.	Liver wt	Adipose tissue [3]	Total counts		Contribution	
				Liver	Adipose tissue	Liver	Adipose tissue
min		*g*	*g*	*10^3 dpm*		*%*	
20	1	402	1360	33	11986	0.3	99.7
	2	400	1410	53	8429	0.6	99.4
	3	462	1590	79	22133	0.4	99.5
	4	463	1590	31	12741	0.3	99.7
	Avg					0.4	99.6
40	5	384	1310	45	18602	0.2	99.8
	6	405	1220	41	17460	0.2	99.8
	7	485	1540	35	7714	0.6	99.4
	8	413	1720	42	16240	0.3	99.7
	Avg					0.3	99.7

[1] Time elapsed between the administration of 50 μCi of glucose-U-^{14}C (in 1.0 ml saline) and killing of animals.
[2] Animals weighed 14.7 $\pm$ 0.6 kg.
[3] Assumed to be 10% of body mass.

identify the chief synthetic sites and, if possible, the relative importance of each. Once this basic information is available, enzymatic and hormonal regulatory mechanisms can be more meaningfully investigated. In the experiments reported, both in vivo and in vitro techniques as well as the study of several enzyme systems have been employed to determine the lipogenic importance of porcine liver and adipose tissue. The results presented are in full accord with our previous speculation (14) that adipose tissue plays a major role in fatty acid synthesis in the pig. In fact, when glucose-U-^{14}C was used as substrate, the data from in vivo and in vitro experiments suggest that hepatic synthesis may be minute and that more than 99% of de novo synthesis occurs in the adipose tissue. When other substrates such as acetate, pyruvate, lactate and propionate were tested in vitro, all were incorporated into liver fatty acids to a greater extent than glucose. Even in vivo, acetate-1-^{14}C was well utilized for hepatic fatty acid synthesis and the indications were that, once supplied with acetate, the liver may account for 25 to 30% of newly synthesized fatty acids. In this context, it is of interest to note that organic acids such as acetic, propionic and lactic are produced in the digestive tract of pigs (15, 16), appear in the blood stream (17, 18) and are removed by the liver (18). It is conceivable, therefore, that these acids may serve as substrates for hepatic fatty acid synthesis; however, the quantitative significance of such a contribution remains to be established.

Despite these speculations, it is important not to overemphasize the results obtained by using acetate-1-^{14}C as substrate. Favarger (1) has indicated the advisability of using glucose rather than acetate in investigations on the quantitative aspects of lipid synthesis in various tissues. Furthermore, since the diets normally fed to pigs may be composed of up to 80% starch, glucose is likely the predominant substrate absorbed and presented to lipogenic tissues. Thus it is likely that the results obtained by using glucose-U-^{14}C, i.e., that over 99% of newly synthesized fatty acids are formed in adipose tissue, more truly reflect the actual situation.

Regarding the synthesis of nonsaponifiable lipids in the pig, an earlier report from this laboratory (19) showed that little synthesis takes place in the adipose tissue. The present results indicate that, in vitro, pig liver slices cannot convert glucose to nonsaponifiable lipid. Similar results were obtained in the in vivo experiment using glucose-U-^{14}C (not presented). It therefore appears likely that for nonsaponifiable lipid synthesis in the pig liver, free acetate is required. Pig liver possesses an active acetyl CoA synthetase enzyme (20) which could activate acetate of endogenous or exogenous origin and make it available for the synthesis of nonsaponifiable lipid components such as cholesterol. In the fasted pig the acetate and

acetyl CoA pools would be expected to increase. It is tempting to suggest that this increased availability of acetate and acetyl CoA may, in part, be responsible for the dramatic increase in serum cholesterol observed in pigs subjected to a 7-day fast (21). In view of the fact that a similar increase in serum cholesterol occurs in humans fasted for several days (22), further investigations into the source of the immediate precursors of cholesterol in human subjects would appear warranted.

Reducing equivalents in the form of NADPH, required for the reductive biosynthesis of fatty acids, are produced mainly by the pentose pathway dehydrogenase enzymes and NADP-malic enzyme (23-25). Since the activity of these enzymes in pig liver is insignificant, the low lipogenic activity observed may, in part, be due to its limited capacity to generate NADPH. In contrast, the activity of the three dehydrogenase enzymes in adipose tissue is high, in accord with its high fatty acid synthetic capacity. Malic enzyme is often the rate-limiting enzyme in the "NADH-$NADP^+$ transhydrogenation cycle" proposed by Ballard and Hanson (25). In view of the high activity of this enzyme in pig adipose tissue it is likely that such a transhydrogenation cycle also operates in this tissue.

The key role played by citrate cleavage enzyme in the lipogenic process is now well documented (26). In monogastric animals where glucose is the chief precursor of fatty acids, citrate cleavage enzyme is essential for the production of cytoplasmic acetyl CoA. The almost complete absence of citrate cleavage enzyme in pig liver is undoubtedly an important factor contributing to its low capacity for fatty acid synthesis from glucose. Thus pig liver resembles bovine and ovine liver and adipose tissue (6) in that it lacks an active citrate cleavage enzyme and is unable to convert glucose to fatty acids.

The data presented in this and previous publications (14, 19) show that pig adipose tissue has all the attributes of an active lipogenic tissue. Results in the following paper (27) show that the lipogenic and enzymatic machinery of this tissue is also highly adaptive to dietary manipulation—a further indication of the central role it plays in fatty acid synthesis in the pig.

ACKNOWLEDGMENTS

We are indebted to Dr. B. G. Harmon for supplying the animals used in these experiments and to Mr. L. Nash for care of the animals.

LITERATURE CITED

1. Favarger, P. 1965 Relative importance of different tissues in the synthesis of fatty acids. In: Handbook of Physiology, section 5: Adipose tissue, eds. A. E. Renold and G. F. Cahill, Jr. American Physiological Society, Washington, D.C., p. 19.
2. Jansen, G. R., C. F. Hutchison and M. E. Zanetti 1966 Studies of lipogenesis in vivo. Effect of dietary fat or starvation on conversion of ^{14}C-glucose into fat and turnover of newly synthesized fat. Biochem. J., *99:* 333.
3. Leveille, G. A. 1967 In vivo fatty acid synthesis in adipose tissue and liver of meal-fed rats. Proc. Soc. Exp. Biol. Med., *125:* 85.
4. Goodridge, A. G., and E. G. Ball 1967 Lipogenesis in the pigeon: in vivo studies. Amer. J. Physiol., *213:* 245.
5. O'Hea, E. K., and G. A. Leveille 1969 Lipid biosynthesis and transport in the domestic chick (Gallus domesticus). Comp. Biochem. Physiol., *30:* 149.
6. Ballard, F. J., R. W. Hanson and D. S. Kronfeld 1969 Gluconeogenesis and lipogenesis in tissue from ruminant and non-ruminant animals. Federation Proc., 28: 218.
7. Baker, D. H., D. E. Becker, A. H. Jensen and B. G. Harmon 1968 Effect of dietary dilution on performance of finishing swine. J. Anim. Sci., *27:* 1332.
8. O'Hea, E. K., and G. A. Leveille 1968 Lipogenesis in isolated adipose tissue of the domestic chick (Gallus domesticus). Comp. Biochem. Physiol., *26:* 111.
9. Glock, G. E., and P. McLean 1953 Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J., *55:* 400.
10. Ochoa, S. 1955 In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 739.
11. Srere, P. A. 1962 Methods in Enzymology, vol. 5, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 641.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 256.
13. Agricultural Research Council, London 1967 Nutrient Reuirements of Farm Livestock. 3. Pigs.
14. O'Hea, E. K., and G. A. Leveille 1969 Influence of feeding frequency on lipogenesis and enzymatic activity of adipose tissue and on the performance of pigs. J. Anim. Sci., *28:* 336.

15. Friend, D. W., H. M. Cunningham and J. W. G. Nicholson 1963 Volatile fatty acids and lactic acid in the alimentary tract of the young pig. Can. J. Anim. Sci., *43:* 174.
16. Friend, D. W., H. M. Cunningham and J. W. G. Nicholson 1963 The production of organic acids in the pig. II. The effect of diets on the levels of volatile fatty acids and lactic acid in sections of the alimentary tract. Can. J. Anim. Sci., *43:* 156.
17. Barcroft, J. R., R. A. McAnally and A. T. Phillipson 1944 Absorption of volatile acids from the alimentary tract of sheep and other animals. J. Exp. Biol., *20:* 120.
18. Friend, D. W., J. W. G. Nicholson and H. M. Cunningham 1964 Volatile fatty acid and lactic acid content of pig blood. Can. J. Anim. Sci., *44:* 303.
19. O'Hea, E. K., and G. A. Leveille 1968 Lipid metabolism in isolated adipose tissue of the domestic pig (Sus domesticus). Comp. Biochem. Physiol., *26:* 1081.
20. Baldwin, R. L., M. Ronning, C. Radanovics and G. Plange 1966 Effect of carbohydrate and fat intakes upon the activities of several enzymes in rats, guinea piglets, piglets and calves. J. Nutr., *90:* 47.
21. Kornegay, E. T., E. R. Miller, B. E. Brent, C. H. Long, D. E. Ullrey and J. A. Hoefer 1964 Effect of fasting and refeeding on body weight, rectal temperature, blood volume and various blood constituents in growing swine. J. Nutr., *84:* 295.
22. Ende, N. 1960 Serum cholesterol in acute starvation. J. Nutr., *71:* 85.
23. Young, J. W., E. Shrago and H. A. Lardy 1964 Metabolic control of enzymes involved in lipogenesis and gluconeogenesis. Biochemistry, *3:* 1687.
24. Ball, E. G. 1966 Regulation of fatty acid synthesis in adipose tissue. In: Advances in Enzyme Regulation, vol. 4, ed., G. Weber. Pergamon Press, New York, p. 3.
25. Ballard, F. J., and R. W. Hanson 1967 The citrate cleavage pathway and lipogenesis in rat adipose tissue: Replenishment of oxaloacetate. J. Lipid Res., *8:* 73.
26. Goodwin, T. W., ed. 1968 Metabolic Roles of Citrate. Academic Press, New York.
27. O'Hea, E. K., and G. A. Leveille 1969 The influence of fasting and refeeding on lipogenesis and enzymatic activity of pig adipose tissue. J. Nutr., *99:* 345.

Influence of Fasting and Refeeding on Lipogenesis and Enzymatic Activity of Pig Adipose Tissue [1]

EUGENE KEVIN O'HEA AND GILBERT A. LEVEILLE
Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois at Urbana-Champaign, Urbana, Illinois

ABSTRACT Fatty acid synthesis and $^{14}CO_2$ production from glucose-U-^{14}C were virtually abolished in biopsy adipose tissue samples obtained from pigs subjected to a 4-day fast. Refeeding for 2 days fully restored lipogenesis to the prefasting level, and refeeding for 4 days was associated with a twofold overshoot in the capacity for glyceride-glycerol synthesis. No overshoot in fatty acid synthesis could be detected after 4, 6, or 12 days of refeeding. When pigs were refed diets high in protein or fat after a 4-day fast, the restoration of the lipogenic capacity of the adipose tissue was limited to about 50% of that observed in animals refed a high carbohydrate diet. The nonparallel behavior of citrate cleavage enzyme activity and lipogenesis discounts any regulatory role for this enzyme in fatty acid synthesis. Among the NADPH-generating dehydrogenase enzymes studied, malic enzyme appeared more adaptive than the pentose pathway enzymes. The rate of lipogenesis was more closely correlated with the activity of acetyl CoA carboxylase than with any other enzyme assayed. The regulatory implications of these findings in relation to lipogenic control in pig adipose tissue are discussed.

The fatty acid synthetic process in an active lipogenic tissue is generally highly adaptive to dietary manipulations. The replacement of dietary carbohydrate by protein or fat depresses fatty acid synthesis in liver and adipose tissue of the laboratory rat (1–6) and hepatic lipogenesis in the chicken (7, 8). On the other hand, lipogenesis is markedly enhanced in periodically-fed as contrasted to ad libitum-fed animals (9–13). Food deprivation for varying periods of time has repeatedly been shown to depress lipogenesis (1, 14–17), whereas refeeding following a fast rapidly restores lipid synthesis to the normal fed level and frequently induces an "overshoot" (9, 16, 17). Parallel changes in the activity of several hepatic enzymes have also been well documented (17–22). The numerous studies relating to the influence of dietary factors on lipogenesis have been reviewed and the contribution of these investigations to our knowledge of the factors involved in the control of fatty acid synthesis and of the regulatory role of certain enzyme systems have been considered in detail (23, 24).

In a previous report (25) we showed that the rate of fatty acid synthesis and the activity of three NADPH-generating enzymes in pig adipose tissue was not altered by feeding a single daily meal. This lack of response was surprising in view of the marked effect of "meal-feeding" in the rat (12) and the fact that adipose tissue is the chief lipogenic site in the pig (26). These data might be interpreted to suggest that lipogenesis in pig adipose tissue does not respond to dietary stimuli as does tissue of the rat. However, such a conclusion would seem unlikely in view of the known capacity of the pig to synthesize and store lipid (27). Consequently, further investigations on the adaptability of lipogenesis in pig adipose tissue appeared warranted.

The present studies were undertaken to investigate the effects of various dietary manipulations on adipose tissue metabolism in the pig. The effect of fasting and refeeding on the rate of lipid synthesis in this tissue was investigated and, in addition, the activity of the pentose pathway dehyrogenases, malic enzyme, citrate cleavage enzyme and acetyl CoA carboxylase were studied.

EXPERIMENTAL

Animals and diets. Male uncastrated pigs of the Yorkshire and Duroc breeds

Received for publication June 6, 1969.

[1] Supported in part by grants from the Moorman Manufacturing Company, Quincy, Illinois, and the Eli Lilly Research Laboratories, Indianapolis, Indiana.

were used. Body weights at various experimental stages are shown in tables 2 and 3. The composition of the diet fed in experiment 1 has been described (28). The diets fed in experiment 3 are shown in table 1.

Adipose tissue samples. Biopsy samples (approximately 2 g) were obtained as previously described (25) and were used in all experiments. An initial sample was obtained at the start of each experiment when the animals were in the fed state, and subsequent samples were obtained at various stages of the fasting and refeeding periods. Thus each animal served as its own control throughout the study. The design of each experiment is indicated in the tables of results.

In vitro procedures. Duplicate adipose tissue slices were prepared using a Stadie-Riggs hand microtome. The incubation conditions have been described in the previous paper (26). The incubation medium contained, per ml: 5 μmoles of glucose, 0.3 μCi glucose-U-^{14}C and 0.1 unit of insulin.[2]

Enzyme studies The adipose tissue remaining after the preparation of slices was homogenized as previously described (26) for enzyme assay. Glucose-6-phosphate dehydrogenase (EC. 1.1.1.49), 6-phosphogluconate dehydrogenase (EC. 1.1.1.44), NADP-malate dehydrogenase (EC.1.1.1.40) and citrate cleavage enzyme (EC.4.1.3.6) were assayed as previously described (26). Acetyl CoA carboxylase (EC. 6.4.1.2) was assayed by the method of Chang et al. (29). Both citrate and Mg^{2+} were omitted from the blank tube in accord with the findings of Greenspan and Lowenstein (30). The concentrations of $MgCl_2$, K citrate and ATP in the assay tube were 20, 20 and 4 mM, respectively. The reaction was allowed to proceed for 10 minutes.

Plasma free fatty acids. Blood was obtained from the vena cava using a heparinized syringe and the plasma separated by centrifugation. Plasma free fatty acids were determined by the method of Ko and Royer (31).

RESULTS

Table 2 shows the effects of a 7-day fast and a subsequent 4-day refeeding period on body weight, adipose tissue metabolism and plasma free fatty acid level. Fasting for 7 days virtually eliminated the ability of adipose tissue to convert glucose-U-^{14}C to fatty acids. Conversion of this substrate to glyceride-glycerol and $^{14}CO_2$ was also drastically diminished by fasting. Refeeding for 4 days restored the fatty acid synthetic capacity to the fed value; glyceride-glycerol synthesis showed an almost twofold stimulation ($P < 0.01$) over values observed in the fed state. The activity of 6-phosphogluconate dehydrogenase and malic enzyme was significantly ($P < 0.05$) reduced by fasting, and although the pattern of change was similar, the reduction in the activity of glucose-6-phosphate dehydrogenase failed to reach statistical significance ($P > 0.05$). Refeeding for 4 days

[2] The insulin was generously supplied by Dr. R. Chance, Eli Lilly Research Laboratories, Indianapolis, Indiana.

TABLE 1

Composition of diets fed in experiment 3

Component	Diet			
	High carbohydrate	High protein	High fat	High protein + fat
	%	%	%	%
Ground yellow corn	35.5	35.5	35.5	35.5
Cornstarch	32.0	—	5.0	2.5
Corn oil	—	—	12.0	6.0
Soybean meal (50% protein)	26.0	62.0	26.0	44.0
Cellulose	4.0	—	19.0	9.5
Micronutrients [1]	2.5	2.5	2.5	2.5
		Estimated nutrient content		
Digestible energy (kcal/kg)	3309	3300	3309	3307
Protein (%)	16.3	34.3	16.3	25.3
Fat %	1.5	1.7	13.5	7.6

[1] The mineral and vitamin mix used was as described by Baker et al. (28).

TABLE 2

Effects of fasting and refeeding on body weight, adipose tissue metabolism and enzyme activity and on plasma free fatty acid levels

Aspect investigated	Nutritional state		
	Fed	Fasted (7 days)	Refed (4 days)
Body weight, kg	22.7 ± 0.6 [1]	18.8 ± 0.8	23.7 ± 0.9
	Adipose tissue metabolism [2]		
$^{14}CO_2$	519 ± 57	12 ± 1	558 ± 69
Fatty acids	561 ± 82	< 1	662 ± 109
Glyceride-glycerol	77 ± 7	18 ± 1	131 ± 8
	Adipose tissue enzymes [3]		
Glucose-6-P-dehydrogenase	84 ± 16	56 ± 6	85 ± 27
6-P-gluconate dehydrogenase	50 ± 3	39 ± 2	43 ± 6
NADP-malate dehydrogenase	56 ± 9	25 ± 2	85 ± 29
Plasma free fatty acids [4]	220 ± 12	948 ± 136	182 ± 18

[1] Mean ± SEM for 5 pigs.
[2] Nanomoles of substrate converted to the product indicated/100 mg tissue per 2 hours.
[3] Activity expressed as nanomoles substrate converted/minute per milligram protein.
[4] Values in μEq/liter.

restored the activity of all enzymes to the fed level, with a nonsignificant ($P > 0.05$) overshoot observed in malic enzyme activity. The trend in plasma free fatty acids was as expected: a highly significant ($P < 0.01$) increase occurred with fasting and a return to the normal fed level upon refeeding for 4 days.

A second experiment was conducted to define more clearly the time course of the changes in pig adipose tissue metabolism in relation to fasting and refeeding. The results (table 3) indicate that even after only 2 days of fasting, fatty acid synthesis was significantly ($P < 0.01$) reduced as were $^{14}CO_2$ production ($P < 0.01$) and the synthesis of glyceride-glycerol ($P < 0.05$). Fatty acid synthesis after 4 days of starvation was virtually abolished. Refeeding for as little as 2 days was sufficient to return all three parameters to the initial fed level. Glyceride-glycerol synthesis was the only component which surpassed the normal fed value; the overshoot observed amounted to a twofold increase and was, as in the first experiment, highly significant ($P < 0.01$). The activity of citrate cleavage enzyme did not closely parallel the lipogenic profile. Even after 4 days of fasting about 30% of the initial fed activity was still present. Refeeding for 2 days failed to stimulate the enzyme activity; on the contrary, a slight decrease was detected. Refeeding for 4 days produced an increase in activity which, however, was still less than 50% of the initial activity.

A third experiment was undertaken to investigate the effects of feeding diets high in protein, fat or both protein and fat upon the refeeding adaptation of adipose tissue. Eight pigs were fed a high carbohydrate diet for several weeks and, following a 4-day fast, were divided into four groups of two animals each. The paired groups were then refed, ad libitum, the four diets described in table 1.

As observed in the previous experiments, a 4-day fast virtually abolished lipogenesis (table 4). Refeeding the high carbohydrate diet for 2 or 6 days restored fatty acid synthesis to the level observed in the fed animals. In contrast, the ingestion of the high protein, high fat or the combination of both prevented, in part, the refeeding adaptation observed in the animals fed the high carbohydrate diet. This reduction, which in all cases exceeded 50%, was most evident in the animals refed the high fat diet. The results obtained for glyceride-glycerol synthesis and $^{14}CO_2$ production from these animals are presented in tables 5 and 6. Precisely the same trend as was observed for fatty acid synthesis is apparent. As a consequence of refeeding for 6 or 12 days the high protein, high fat and high protein-high fat diets, the synthesis of glyceride-glycerol and production of $^{14}CO_2$ were less than half those observed in animals refed the high carbohydrate diet. The overshoot in glyceride-glycerol synthesis, consistently observed in the previous experi-

TABLE 3

Effects of fasting and refeeding on body weight, adipose tissue metabolism and on citrate cleavage enzyme activity

Aspect investigated	Nutritional state				
	Fed	Fasted (2 days)	Fasted (4 days)	Refed (2 days)	Refed (4 days)
Body weight, kg	36.8 ± 1.1 [1]	33.4 ± 0.8	32.0 ± 0.8	38.7 ± 0.8	39.3 ± 0.7
			Adipose tissue metabolism [2]		
$^{14}CO_2$	288 ± 17	84 ± 26	18 ± 2	250 ± 38	235 ± 25
Fatty acids	368 ± 37	67 ± 35	7 ± 2	370 ± 55	246 ± 34
Glyceride-glycersol	70 ± 3	33 ± 10	20 ± 2	69 ± 9	142 ± 12
Citrate cleavage enzyme [3]	36.6 ± 10.0	16.1 ± 3.4	12.7 ± 2.4	9.2 ± 1.7	15.2 ± 2.8

[1] Mean ± SEM for 5 pigs.
[2] Nanomoles of substrate converted to the product indicated/100 mg tissue per 2 hours.
[3] Activity expressed as nanomoles substrate converted/minute per milligram protein.

TABLE 4

Effect of high protein and fat diets on fatty acid synthesis by adipose tissue of fasted-refed pigs [1]

Nutritional state	Diet fed			
	High carbohydrate	High protein	High fat	High protein + fat
Fed [2]	356 ± 34	—	—	—
Fasted (4 days) [2]	< 1	—	—	—
Refed (2 days) [3]	330	137	48	148
Refed (6 days) [3]	381	174	115	150
Refed (12 days) [3]	290	46	57	35

[1] Values are nanomoles of glucose-U-^{14}C converted to fatty acids/100 mg tissue per 2 hours.
[2] Mean ± SEM for 8 pigs.
[3] Mean for 2 pigs.

TABLE 5

Effects of high protein and fat diets on the adipose tissue glyceride-glycerol synthesis in fasted-refed pigs [1]

Nutritional state	Diet fed			
	High carbohydrate	High protein	High fat	High protein + fat
Fed [2]	56 ± 3	—	—	—
Fasted (4 days) [2]	14 ± 1	—	—	—
Refed (2 days) [3]	37	29	24	29
Refed (6 days) [3]	99	53	36	50
Refed (12 days) [3]	65	20	24	33

[1] Values are nanomoles of glucose-U-^{14}C converted to glyceride-glycerol/100 mg tissue per 2 hours.
[2] Mean ± SEM for 8 pigs.
[3] Mean for 2 pigs.

ments occurred only in those animals refed the high carbohydrate diet. Acetyl CoA carboxylase activity (table 7) was reduced approximately 66% following a 4-day fast ($P < 0.02$). This activity was more than recovered in the adipose tissue of animals refed the diet high in carbohydrate. A diminished activity was, however, still evident in the adipose tissue of animals refed the other three diets.

DISCUSSION

The primary objective of these experiments was to obtain information on the adaptive nature of the lipogenic machinery of pig adipose tissue consequent to fasting and refeeding. In addition, the likelihood that dietary protein and fat may modify any refeeding adaptations was also tested.

Fasting for a period of 7 days virtually abolished in vitro fatty acid synthesis by

TABLE 6

Effect of high protein and fat diets on the adipose tissue $^{14}CO_2$ production of fasted-refed pigs [1]

Nutritional state	Diet fed			
	High carbohydrate	High protein	High fat	High protein + fat
Fed [2]	225 ±21	—	—	—
Fasted (4 days) [2]	10.8± 0.4	—	—	—
Refed (2 days) [3]	193	95	42	85
Refed (6 days) [3]	208	92	64	102
Refed (12 days) [3]	168	39	42	39

[1] Values are nanomoles of glucose-U-^{14}C converted to $^{14}CO_2$/100 mg tissue per 2 hours.
[2] Mean ± SEM for 8 pigs.
[3] Mean for 2 pigs.

TABLE 7

Effect of high protein and fat diets on the adipose tissue acetyl CoA carboxylase activity of fasted-refed pigs [1]

Nutritional state	Diet fed			
	High carbohydrate	High protein	High fat	High protein + fat
Fed [2]	4.6±0.7	—	—	—
Fasted (4 days) [2]	1.6±0.3	—	—	—
Refed (6 days) [3]	6.3	3.5	2.0	1.7

[1] Values are nanomoles substrate carboxylated/minute per milligram protein.
[2] Mean ± SEM for 8 pigs.
[3] Mean for 2 pigs.

adipose tissue. Even a 4-day fast was found to be sufficient to accomplish this. Fasting also reduced severely the overall metabolic activity of adipose tissue as indicated by the low levels of $^{14}CO_2$ after a 4- or 7-day fast. Refeeding for as little as 2 days, however, restored all the metabolic parameters of the adipose tissue to the unfasted values. These results indicate that pig adipose tissue is metabolically highly adaptive and that the lipogenic process, largely confined to this tissue, is readily altered. The major lipogenic tissues of other species already examined appear to respond in a similar manner to fasting and refeeding. The adipose tissue of the starved rat and mouse largely loses the ability to synthesize fatty acids and refeeding for short periods restores this ability (16). Goodridge (18) and Leveille (17) have published similar observations for chick liver.

The metabolic significance of the overshoot in the synthesis of glyceride-glycerol observed following refeeding is not clear, especially since there was no evidence of an overshoot in fatty acid synthesis. Fasting produces a marked increase in circulating free fatty acids in the pig (32), and the results in table 2 further substantiate this finding. Because epinephrine and norepinephrine administered in vivo also increase circulating free fatty acids in pigs (33) it is likely that the increase noted during fasting is catecholamine-mediated. Hence the lipolytic capacity of adipose tissue would be enhanced after a period of fasting and this may be manifested as a greater turnover during refeeding. This would account for the increased synthesis of glyceride-glycerol during refeeding if we assume that the glycerokinase enzyme, known to be absent in rat adipose tissue (34), is also missing in pig adipose tissue.

The enzymes assayed in these experiments are known to be intimately involved in various aspects of fatty acid synthesis. The three dehydrogenase enzymes generate NADPH required for the reductive synthesis of fatty acids (35). In monogastric animals, citrate cleavage enzyme produces cytoplasmic acetyl CoA and oxaloacetate from citrate derived from the mitochondria (36). This acetyl CoA is then activated to malonyl CoA by acetyl CoA carboxylase.

Even though a diminished activity of all enzymes was observed during fasting and a complete or partial recovery of activity upon refeeding, the overall changes in activity were much less pronounced than the corresponding lipogenic changes. The activities of glucose-6-phosphate dehydrogenase and malic enzyme were reduced by 33% and 55%, respectively, after a 7-day fast which is in accord with the reported 40% and 55% reduction in the activity of these two enzymes in the adipose tissue of rats fasted for 4 days (37). Anderson and Hollifield (38) have suggested that such changes in enzyme activity may be largely responsible for changes in lipogenic activity. As pointed out by Masoro (23), however, the evidence that NADPH availability could control lipogenesis is slight. Evidence that increased lipogenesis is not dependent upon antecedent increases in the activity of NADPH-generating enzymes has been provided by Leveille (39).

Even though a number of authors (21, 40, 41) have drawn attention to the parallel behavior of citrate cleavage enzyme activity and fatty acid synthesis in lipogenic tissues, a regulatory role for this enzyme in fatty acid synthesis has not been established. The results presented show that even though a greater than 60% decrease in citrate cleavage enzyme activity occurred following a 4-day fast, there was no relationship between fatty acid synthesis and the enzyme activity upon refeeding. Whereas 2 days of refeeding fully restored fatty acid synthesis to the fed level, the activity of the enzyme did not begin to increase until the fourth day of refeeding. These data agree with the results of Foster and Srere (42) for rat liver, and of Goodridge (18) for chick liver and suggest that changes in the activity of citrate cleavage enzyme in pig adipose tissue are not responsible for alterations in the rate of fatty acid synthesis.

Several investigators have proposed a key regulatory role for acetyl CoA carboxylase in fatty acid synthesis, and the pertinent literature has been reviewed recently (24). The results presented reveal a definite relationship between the rate of fatty acid synthesis and the activity of the enzyme. Following a 4-day fast, the activity of acetyl CoA carboxylase was depressed by almost 66%, whereas lipogenesis fell by more than 99%. Korchak and Masoro (22) found that a 50% reduction in the carboxylase activity accompanied a 99% depression in fatty acid synthesis in the livers of fasted rats. As in the case of the dehydrogenase enzymes, however, the cause-and-effect relationships between the activity of acetyl CoA carboxylase and the rate of lipogenesis are currently the subject of controversy.

The mechanism whereby dietary fat or protein causes a reduction in lipogenesis when fed in place of carbohydrate calories is still obscure. The possibility that these dietary components may have specific effects on fat synthesis has been discussed (8). Long-chain acyl-CoA derivatives may reduce fatty acid synthesis through an inhibitory effect on acetyl CoA carboxylase activity (43, 44). The concentration of long chain acyl CoA derivatives is increased by fasting and fat feeding (44, 45) and these treatments may exert their inhibitory effects on fatty acid synthesis by the above mechanism. It is apparent, however, that regulatory mechanisms other than the inhibitory effects of fatty acyl CoA derivatives on the enzyme, also operate; the feeding of the high protein diet depressed lipogenesis to about the same extent as the high fat diet, and also considerably reduced the activity of the carboxylase enzyme.

Yeh and Leveille (8) have suggested that the major factor limiting hepatic fat synthesis in chicks fed a high protein diet is the lack of reducing equivalents. In pig adipose tissue the activity of the NADPH-generating enzymes is diminished by the feeding of a high protein diet [3] and thus a lack of reducing equivalents might limit fatty acid synthesis under these conditions. In chick liver, malic enzyme is the chief, if not the only, source of reducing equivalents necessary for lipid synthesis (46, 47), whereas in pig adipose tissue the pentose pathway dehydrogenases also appear to be of importance (48), as in the present report. Thus the supply of reduc-

[3] O'Hea, E. K., and G. A. Leveille 1969 Lipogenesis and enzyme activity of pig adipose tissue as influenced by dietary protein and fat. Federation Proc., 28: 687 (abstract).

ing equivalents may be a more important regulatory factor in chick liver than in pig adipose tissue.

The overall results indicate that the loss of enzymatic activity in pig adipose tissue is a result rather than a cause of reduced lipogenesis following a dietary change. Hence, further experiments are necessary to study very early changes in the rate of fatty acid synthesis and of the factors, enzymatic or otherwise, involved in these changes.

ACKNOWLEDGMENTS

We are indebted to Dr. B. G. Harmon for supplying the animals used in these experiments and to Mr. L. Nash for care of the animals.

LITERATURE CITED

1. Masoro, E. J., I. L. Chaikoff, S. S. Chernick and J. M. Felts 1950 Previous nutritional state and glucose conversion to fatty acids in liver slices. J. Biol. Chem., *185:* 845.
2. Whitney, J. E., and S. Roberts 1955 Influence of previous diet on hepatic glycogenesis and lipogenesis. Amer. J. Physiol., *181:* 446.
3. Hill, R., J. M. Linazasoro, F. Chevallier and I. L. Chaikoff 1958 Regulation of hepatic lipogenesis: the influence of dietary fats. J. Biol. Chem., 233: 305.
4. Cohen, A. M., and A. Teitelbaum 1966 Effect of different levels of protein in sucrose and starch diets on lipid synthesis in the rat. Israel J. Med. Sci., 2: 727.
5. Leveille, G. A. 1967 Influence of dietary fat level on the enzymatic and lipogenic adaptations in adipose tissue of meal-fed rats. J. Nutr., *91:* 267.
6. Leveille, G. A. 1967 Influence of dietary fat and protein on metabolic and enzymatic activities in adipose tissue of meal-fed rats. J. Nutr., *91:* 25.
7. Nishida, T., A. Ueno and F. A. Kummerow 1960 Effect of dietary protein on the metabolism of sodium acetate-1-^{14}C in chicks. J. Nutr., *71:* 379.
8. Yeh, Yu-Yan, and G. A. Leveille 1969 Effect of dietary protein on hepatic lipogenesis in the growing chick. J. Nutr., *98:* 356.
9. Tepperman, J., and H. H. Tepperman 1958 Effects of antecedent food intake pattern on hepatic lipogenesis. Amer. J. Physiol., *193:* 55.
10. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. 1. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 245.
11. Stevenson, J. A. F., V. Feleki, A. Szlavko and J. R. Beaton 1964 Food restriction and lipogenesis in the rat. Proc. Soc. Exp. Biol. Med., *116:* 178.
12. Leveille, G. A., and R. W. Hanson 1965 Influence of periodicity of eating on adipose tissue metabolism in the rat. Can. J. Physiol. Pharmacol., *43:* 857.
13. Fabry, P. 1967 Metabolic consequences of the pattern of food intake. In: Handbook of Physiology, section 6: Alimentary Canal, eds., C. F. Code and W. Heidel. American Physiological Society, Washington, D. C. p. 31.
14. Boxer, G. E., and D. Stetten, Jr. 1944 The role of thiamine in the synthesis of fatty acids from carbohydrate precursors. J. Biol. Chem., *153:* 607.
15. Leveille, G. A. 1966 In vitro lipogenesis in adipose tissue of fed and fasted rats: mechanism of pyruvate stimulation. Life Sci., 5: 421.
16. Jansen, G. R., C. F. Hutchison and M. E. Zanetti 1966 Studies on lipogenesis in vivo. Effect of dietary fat or starvation on conversion of ^{14}C-glucose into fat and turnover of newly synthesized fat. Biochem. J., *99:* 323.
17. Leveille, G. A. 1969 In vivo fatty acid and cholesterol synthesis in fasted and fasted-refed chicks. J. Nutr., *98:* 367.
18. Goodridge, A. G. 1968 The effect of starvation and starvation followed by feeding on enzyme activity and the metabolism of [U-^{14}C] glucose in liver from growing chicks. Biochem. J., *108:* 667.
19. Tepperman, H. M., and J. Tepperman 1958 The hexosemonophosphate shunt and adaptive hyperlipogenesis. Diabetes, 7: 478.
20. Tepperman, H. M., and J. Tepperman 1964 Pattern of dietary and hormonal induction of certain NADP-linked liver enzymes. Amer. J. Physiol., 206: 357.
21. Kornacker, M. S., and L. M. Lowenstein 1965 Citrate and the conversion of carbohydrate into fat. The activities of citrate-cleavage enzyme and acetate thiokinase in livers of starved and refed rats. Biochem. J., *94:* 209.
22. Korchak, H. M., and E. J. Masoro 1962 Changes in the level of fatty acid synthesizing enzymes during starvation. Biochim. Biophys. Acta, 58: 354.
23. Masoro, E. J. 1962 Biochemical mechanisms related to the homeostatic regulation of lipogenesis in animals. J. Lipid Res., 3: 149.
24. Zakim, D., and R. H. Herman 1969 Regulation of fatty acid synthesis. Amer. J. Clin. Nutr., 22: 200.
25. O'Hea, E. K., and G. A. Leveille 1969 Influence of feeding frequency on lipogenesis and enzymatic activity of adipose tissue and on the performance of pigs. J. Anim. Sci., 28: 336.
26. O'Hea, E. K., and G. A. Leveille 1969 Significance of adipose tissue and liver as sites of fatty acid synthesis in the pig and the efficiency of utilization of various substrates for lipogenesis. J. Nutr., *99:* 338.

27. Wierzuchowski, M., and S. M. Ling 1925 Animal calorimetry. On fat production in a young hog. J. Biol. Chem., *64:* 697.
28. Baker, D. H., D. E. Becker, A. H. Jensen and B. G. Harmon 1968 Effect of dietary dilution on performance of finishing swine. J. Anim. Sci., *27:* 1332.
29. Chang, H., I. Seidman, G. Teebor and M. D. Lane 1967 Liver acetyl CoA carboxylase and fatty acid synthetase: relative activities in the normal state and in hereditary obesity. Biochem. Biophys. Res. Commun., *28:* 682.
30. Greenspan, M. D., and J. M. Lowenstein 1968 Effect of magnesium ions, adenosine triphosphate, palmitoylcarnitine, and palmitoyl coenzyme A on acetyl coenzyme A carboxylase. J. Biol. Chem., *243:* 6273.
31. Ko, H., and M. E. Royer 1967 A submicromolar assay for nonpolar acids in plasma and depot fat. Anal. Biochem., *20:* 205.
32. Machlin, L., M. Horino, F. Hertelendy and D. M. Kipnis 1968 Plasma growth hormone and insulin levels in the pig. Endocrinology, *82:* 369.
33. Cunningham, H. M., and D. W. Friend 1965 Influence of epinephrine, norepinephrine and nicotine on blood levels of glucose, free fatty acids and amino nitrogen in pigs. J. Anim. Sci., *24:* 41.
34. Margolis, S., and M. Vaughan 1962 α-Glycerophosphate synthesis and breakdown in homogenates of adipose tissue. J. Biol. Chem., *237:* 44.
35. Ball, E. G. 1966 Regulation of fatty acid synthesis in adipose tissue. In: Advances in Enzyme Regulation, vol 4, ed., G. Weber. Pergamon Press, New York. p. 3.
36. Srere, P. A., and A. Bhaduri 1962 Incorporation of radioactive citrate into fatty acids. Biochim. Biophys. Acta, *59:* 487.
37. Young, J. W., E. Shrago and H. A. Lardy 1964 Metabolic control of enzymes involved in lipogenesis and gluconeogenesis. Biochemistry, *3:* 1687.
38. Anderson, J., and G. Hollifield 1966 The effects of starvation and refeeding on hexosemonophosphate shunt enzyme activity and DNA, RNA and nitrogen content of rat adipose tissue. Metab. Clin. Exp., *15:* 1098.
39. Leveille, G. A. 1966 Glycogen metabolism in meal-fed rats and chicks and the time sequence of lipogenic and enzymatic adaptive changes. J. Nutr., *90:* 449.
40. Brown, J., and P. McLean 1965 Effect of alloxan-diabetes on the activity of citrate cleavage enzyme in adipose tissue. Nature, *207:* 407.
41. Howanitz, P. J., and H. R. Levy 1965 Acetyl-CoA carboxylase and citrate cleavage enzyme in the rat mammary gland. Biochim. Biophys. Acta, *106:*430.
42. Foster, D. W., and P. A. Srere 1968 Citrate cleavage enzyme and fatty acid synthesis. J. Biol. Chem., *243:* 1926.
43. Bortz, W. M., and F. Lynen 1963 The inhibition of acetyl-CoA carboxylase by long chain acyl-CoA derivatives. Biochem. Z., *337:* 505.
44. Tubbs, P. K., and P. B. Garland 1963 Fatty acyl thioesters of Co enzyme A: Inhibition of fatty acid synthesis in liver of normal, fasted, and fat or sugar fed rats. Biochem. J., *89:* 25.
45. Bortz, W. M., and F. Lynen 1963 Elevation of long chain acyl-CoA derivatives in liver of fasted rats. Biochem. Z., *339:* 77.
46. O'Hea, E. K., and G. A. Leveille 1968 Lipogenesis in isolated adipose tissue of the domestic chick (Gallus domesticus). Comp. Biochem. Physiol., *26:* 111.
47. Goodridge, A. G. 1968 Citrate-cleavage enzyme, 'malic' enzyme and certain dehydrogenases in embryonic and growing chicks. Biochem. J., *108:* 663.
48. O'Hea, E. K., and G. A. Leveille 1968 Lipid metabolism in isolated adipose tissue of the domestic pig (Sus domesticus). Comp. Biochem. Physiol., *26:* 1081.

Effect of Intestinal Microflora on Calcium, Phosphorus and Magnesium Metabolism in Rats [1]

BANDARU S. REDDY, J. R. PLEASANTS AND B. S. WOSTMANN
Division of Biochemistry and Nutrition, Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana

ABSTRACT The effect of intestinal microflora on the absorption, excretion, retention and tissue distribution of calcium, phosphorus and magnesium was investigated. Weanling germfree and conventional rats were fed a steam-sterilized semipurified diet or a Millipore-filtered liquid diet until 115 days, and balance studies were carried out for 10 days. Neither germfree status nor the diets used appreciably influenced serum calcium, phosphorus and magnesium levels. Both germfree conditions and feeding of liquid diet increased apparent absorption of calcium and magnesium. There was no difference in phosphorus absorption between germfree and conventional animals, but feeding of liquid diet resulted in higher absorption. Urinary excretion and net retention of both calcium and magnesium were increased in germfree rats. Urinary phosphorus excretion was decreased in germfree rats causing an increase in retention values. The results also indicate a higher calcium, phosphorus and magnesium concentration of the femur in germfree rat, but the ratios among these minerals were similar. No difference in the mineral composition of kidneys was observed between germfree and conventional rats. These data clearly suggest that the presence of microflora in the gut influences the metabolism of calcium, phosphorus and magnesium.

It has been shown in our laboratory that germfree rabbits utilized iron more efficiently from a natural source than from a mineral supplement, whereas conventional rabbits utilized either source equally efficiently (1). Because the amount of iron present in the mineral supplement was adequate for the conventional rabbits, its availability for germfree animals must have been influenced by the absence of an intestinal microflora. In another study, it has been observed that the calcium uptake was higher in germfree than in conventional chickens (2). Gustafsson and Norman (3) found a high incidence of urinary calculi, and an increase in urinary concentration of calcium and citrate, but a low urinary phosphate concentration in germfree rats. These symptoms disappeared when the germfree rats were conventionalized by bringing them outside the isolator and subsequently introducing intestinal contents from conventional rats per rectum. Germfree rats raised in our laboratory on certain water-soluble formulas showed a mild form of hydronephrosis, and calcification, degeneration and necrosis of the kidney tubules. None of these symptoms were observed in conventional rats fed similar diets. These changes, together with a "sudden death" syndrome observed at about 70 days of age in germfree mice reared on the same water-soluble formulas, were suggestive of magnesium deficiency (4) although the diets contained more magnesium than recommended by the National Research Council (5). In other studies magnesium deficiency and calcification of kidneys of conventional rats were produced either by the addition of calcium or phosphorus or both to low magnesium diets (6), or by increasing phosphorus in the diet containing normal amounts of Mg (7).

The above mentioned observations point to the possibility of basic differences in the metabolism of calcium, phosphorus and magnesium between germfree and conventional rats which could possibly lead to pathological conditions. As yet, there is no direct evidence that these syndromes are the consequences of changes in the pattern of absorption, excretion and depo-

Received for publication April 4, 1969.

[1] Supported by Public Health Service Research Grant no. AM-11300 from the National Institute of Arthritis and Metabolic Diseases and no. HD-00855 from the National Institute of Child Health and Human Development.

sition of the minerals in the germfree state. Thus, the present investigation was designed to study the effect of the intestinal microflora on the absorption, retention and tissue distribution of calcium, phosphorus and magnesium. Futhermore, the data would not only help to formulate calcium, phosphorous and magnesium requirements of the rat under germfree conditions and thereby produce an experimental animal free of the above mentioned pathology, but would also enhance our scant knowledge of the influence of the viable intestinal microflora on mineral metabolism in general. Because of the importance of chemically defined, water-soluble diet (antigen-free) in our previous nutritional and immunological studies (8), this investigation was extended to germfree and conventional rats fed this diet in addition to those fed semipurified diet.

MATERIALS AND METHODS

The male germfree and conventional rats of Wistar origin (Lobund strain) used in this investigation were genetically closely related. All rats were housed individually in stainless steel cages with raised wire bottom. Germfree animals were maintained in the Trexler flexible plastic isolators and conventional rats in the temperature and humidity controlled room as described in our earlier report (9). The germfree status of rats was verified at biweekly intervals and also at the termination of the experiment in our Bacteriology Laboratory by the routine procedures (10). All cultures from the germfree animals and isolators were negative. Following weaning, the rats were assigned randomly to one of the experimental groups, and fed the following experimental diets (table 1) with deionized water ad libitum: a Millipore-filtered, chemically defined, water-soluble diet (L-479E9) based on amino acids and glucose with added ethyl linoleate; and a steam-sterilized, semipurified diet (L-474E12) based on purified casein, starch and corn oil (11). Water-soluble diet was provided in overhead bottles. Care was taken to eliminate sources of mineral contamination in the environment as well as in the diet. All animals were fed these diets until about 115 days of age. Body weights were recorded weekly.

In order to conduct mineral balance studies, at 85 to 90 days of age, germfree rats were transferred to individual plastic metabolism cages with stainless steel screen bottoms, and the conventional rats were transferred to similar plastic metabolism cages kept in the same room where the germfree rats were located. After a 1-week precollection adjustment period, feces and urine were collected daily, and feed intake was measured daily for 10 days. During this time, daily body weights were recorded. At the conclusion of the balance trial, the rats were killed after 2 hours of fasting with free access to water: they were anesthetized with ether, blood was collected directly from the heart whereupon they were decapitated.

In the case of the germfree and conventional rats fed semipurified diet, the femurs, kidneys and intestinal tract were excised immediately. Femurs and kidneys were freed of extraneous material and

TABLE 1

Composition of diets [1]

	Water-soluble diet (L-479 E9)	Semipurified diet (L-474 E12)
	g/100 g solids	
Amino acid mixture	22.2	—
Casein	—	24.0
DL-Methionine	—	0.3
Glucose	70.5	—
Starch	—	60.4
Cellophane spangles	—	5.0
i-Inositol	—	0.1
Corn oil	—	3.0
Ladek-55 (fat-soluble vitamins)	—	2.0
Ethyl linoleate and fat-soluble vitamins [2]	—	—
Vitamin B mixture	0.32	0.5
Mineral mixture [3]	7.00 [4]	4.7 [5]

[1] For detailed composition of diets, see reference 11.

[2] Ethyl linoleate and fat-soluble vitamins were administered orally to the group fed the water-soluble diet at 200 mg/day per animal.

[3] Analysis of diets for Ca, P and Mg showed the following concentrations, in mg/100 g dry matter, steam-sterilized semipurified diet: Ca, 655; P, 642; Mg, 87.9 and Millipore-filtered water-soluble diet: Ca, 560; P, 708; Mg, 47.0.

[4] Mineral mixture contained: (in milligrams) ferrous gluconate, 35; KI, 3.0; Mn $(C_2H_3O_2)_2 \cdot 4H_2O$, 26; $ZnSO_4 \cdot H_2O$, 5.5; Cu $(C_2H_3O_2)_2 \cdot H_2O$, 2.5; $Co(C_2H_3O_2)_2 \cdot 4H_2O$, 0.9; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.6; Na_2SeO_3, 0.011; $Cr(C_2H_3O_2)_2 \cdot H_2O$, 0.48; calcium fructose 1,6-diphosphate, 5000; magnesium fructose 1,6-diphosphate, 500; NaCl, 380; and CH_3COOK, 1060.

[5] Mineral mixture contained: (in milligrams) NaCl, iodized, 515; $MgSO_4$, 400; Fe $(C_6H_5O_7)_2$, 60; $MnCO_3$, 20; CuO, 2.5; ZnO, 2.5 $CoCl_2 \cdot 6H_2O$, 0.05; NaF, 0.01; MoO_3, 0.005; KBr, 0.01; Na_2SeO_3, 0.01; $CaCO_3$, 1700; K_2HPO_4, 1000; and Na_2HPO_4, 1000.

weighed. The contents of the proximal, middle and distal third of the small intestine, representing duodenum, jejunum and ileum, respectively, and of cecum and colon were transferred quantitatively into preweighed vials and weighed. The feed, feces, urine, plasma, femurs, kidneys and the contents of various parts of the intestinal tract were frozen and stored at −20° until analyzed for calcium, phosphorus and magnesium.

Semipurified diet, feces, femurs, kidneys and intestinal contents were dried in a vacuum oven for 24 hours to determine dry weights. For the determination of calcium and magnesium, the above samples were dry-ashed in a muffle furnace at 550° overnight and dissolved in 3 ml of 6 N HCl; the liquid diet and urine were wet-ashed with a 2:1 mixture of HNO_3–$HClO_4$. Calcium and magnesium were determined with an atomic absorption spectrophotometer [2] in all above ashed samples diluted with 1% and 0.1% lanthanum oxide in 5% HCl, respectively. Serum calcium and magnesium were measured after diluting with 0.1% lanthanum chloride. Inorganic phosphorus in serum was analyzed by the Fisk-Subbarow method (12). Total phosphorus in all other materials was determined by a modified Fisk-Subbarow procedure, as described by Allen (13), after wet ashing the samples with $HClO_4$. The data were analyzed statistically by the *t* test.

RESULTS

The body weights of germfree (gf) and conventional (conv) rats fed liquid (L) diet were significantly ($P < 0.05$) lower than in corresponding groups fed semipurified (S) diet (mean weights ± SEM in grams: gf.S, 346 ± 8; conv.S, 345 ± 14; gf.L, 312 ± 9; conv.L, 298 ± 9). There was no significant difference in body weights between germfree and conventional status.

Tables 2, 3 and 4 summarize, for calcium, phosphorus and magnesium, respectively, data on absorption, excretion and retention in germfree and conventional rats fed semipurified or liquid diet. Calcium (table 2), phosphorus (table 3) and magnesium (table 4) concentrations in the plasma were not affected either by germfree status or by diet. The intakes of calcium and phosphorus by the different groups were approximately comparable. Due to lower magnesium content of the liquid diet, however, magnesium intake in rats fed liquid diet was lower than in semipurified diet-fed animals. Both germfree status and feeding of liquid diet increased apparent absorption (intake − fecal excretion expressed as % intake or mg per unit body weight) of calcium (table 2) and magnesium (table 4) as compared with their respective controls. In the conventional animals, however, the difference in absorption between the liquid and semipurified diet-fed groups did not reach statistical significance. There was little or no difference in phosphorus absorption between germfree and conventional animals, but feeding of liquid diet resulted in a

[2] Beckman Model 979, Beckman Instruments, Inc., Fullerton, California.

TABLE 2

Plasma calcium, and intake, absorption, excretion and retention of calcium in germfree and conventional rats fed water-soluble and semipurified diets

	Semipurified		Water-soluble	
	Germfree	Conventional	Germfree	Conventional
Plasma, mg/100 ml	11.1 ±0.34 [1]	11.0 ±0.24	11.3 ±0.34	11.1 ±0.20
Intake, mg/day/100 g body wt	32.2 ±0.83 [2]	33.4 ±0.95	29.3 ±0.96 [4]	33.4 ±0.72
Fecal, mg/day/100 g body wt	19.6 ±1.31 [2,3]	25.0 ±1.01	14.5 ±0.45 [4]	22.8 ±1.16
Absorption, mg/day/100 g body wt	12.7 ±0.70 [4]	8.4 ±0.50	14.9 ±0.77 [4]	10.6 ±0.93
Absorption, % intake	39.4 ±2.60 [2,4]	25.3 ±1.59	50.5 ±1.34 [4]	31.5 ±2.80
Urinary, mg/day/100 g body wt	1.25 ±0.08 [4]	0.68 ±0.14	1.21 ±0.05 [3]	0.83 ±0.16
Urinary, % intake	3.89 ±0.26 [4]	2.03 ±0.42	4.17 ±0.28 [3]	2.42 ±0.46
Retention, mg/day/100 g body wt	11.3 ±0.63 [2,4]	7.76 ±0.48	13.6 ±0.81 [4]	9.78 ±0.86
Retention, % intake	35.5 ±2.48 [2,4]	23.3 ±1.57	46.3 ±1.60 [4]	29.0 ±2.63

[1] Averages of 6 to 12 animals ± SEM.
[2] Difference from the germfree group fed water-soluble diet significant, $P < 0.05$.
[3,4] Difference from the conventional group fed similar diet significant, $P < 0.05$ and $P < 0.01$ respectively.

TABLE 3

Plasma phosphorus, and intake, absorption, excretion and retention of phosphorus in germfree and conventional rats fed water-soluble and semipurified diets

	Semipurified		Water-soluble	
	Germfree	Conventional	Germfree	Conventional
Plasma, mg/100 ml	7.56 ± 0.24 [1]	7.50 ± 0.30	7.40 ± 0.40	7.60 ± 0.44
Intake, mg/day/100 g body wt	31.5 ± 0.82 [3]	32.5 ± 1.03 [2]	37.8 ± 1.21 [4]	43.6 ± 0.93
Fecal, mg/day/100 g body wt	12.4 ± 0.80	14.6 ± 0.80 [3]	10.4 ± 0.49	11.3 ± 0.72
Absorption, mg/day/100 g body wt	19.0 ± 0.67 [3]	17.9 ± 0.62 [3]	27.3 ± 0.96 [4]	32.3 ± 0.39
Absorption, % intake	60.6 ± 1.98 [3]	55.2 ± 1.60 [3]	72.0 ± 0.96	74.1 ± 1.14
Urinary, mg/day/100 g body wt	3.73 ± 0.34 [3,4]	6.29 ± 0.34 [3]	14.9 ± 0.83 [4]	23.0 ± 0.43
Urinary, % intake	12.0 ± 1.16 [3,4]	19.5 ± 1.17 [3]	39.2 ± 1.18 [4]	52.9 ± 0.98
Retention, mg/day/100 g body wt	15.2 ± 0.64 [3,4]	11.6 ± 0.58 [3]	12.4 ± 0.34 [4]	9.2 ± 0.16
Retention, % intake	48.5 ± 1.67 [3,4]	35.7 ± 1.31 [3]	32.7 ± 1.11 [4]	21.3 ± 0.50

[1] Averages of 6 to 12 animals ± SEM.
[2,3] Difference between dietary groups within germfree or conventional status significant, $P < 0.05$ and $P < 0.01$, respectively.
[4] Difference from the conventional group fed similar diet significant, $P < 0.01$.

TABLE 4

Plasma magnesium, and intake, absorption, excretion and retention of magnesium in germfree and conventional rats fed water-soluble and semipurified diets

	Semipurified		Water-soluble	
	Germfree	Conventional	Germfree	Conventional
Plasma, mg/100 ml	1.89 ± 0.05 [1]	2.01 ± 0.06	2.00 ± 0.06	1.96 ± 0.06
Intake, mg/day/100 g body wt	4.32 ± 0.11 [3]	4.46 ± 0.14 [3]	2.50 ± 0.08 [5]	2.87 ± 0.06
Fecal, mg/day/100 g body wt	1.23 ± 0.12 [3,5]	2.66 ± 0.14 [3]	0.53 ± 0.04 [5]	1.55 ± 0.06
Fecal, % intake	28.0 ± 2.14 [2,5]	60.1 ± 3.38	21.0 ± 1.09 [5]	55.1 ± 3.02
Absorption, mg/day/100 g body wt	3.09 ± 0.06 [3,4]	1.80 ± 0.19 [3]	1.98 ± 0.05 [5]	1.32 ± 0.08
Absorption, % intake	72.0 ± 2.15 [2,5]	39.9 ± 3.38	79.0 ± 1.09 [5]	44.9 ± 3.02
Urinary, mg/day/100 g body wt	1.18 ± 0.08 [2,5]	0.59 ± 0.04	0.91 ± 0.04 [5]	0.61 ± 0.05
Urinary, % intake	27.7 ± 1.99 [2,5]	13.0 ± 1.20 [3]	36.1 ± 1.22 [5]	21.2 ± 1.62
Retention, mg/day/100 g body wt	1.91 ± 0.08 [3,5]	1.20 ± 0.21 [2]	1.06 ± 0.03 [5]	0.69 ± 0.09
Retention, % intake	44.2 ± 1.89 [5]	27.0 ± 3.68	42.7 ± 0.94 [5]	23.7 ± 2.86

[1] Averages of 6 to 12 animals ± SEM.
[2,3] Difference between dietary groups within germfree or conventional status significant, $P < 0.05$ and $P < 0.01$, respectively.
[4,5] Difference from conventional group fed similar diet significant, $P < 0.05$ and $P < 0.01$, respectively.

considerably higher absorption (table 3) in both groups of rats. Urinary excretion of calcium and magnesium were significantly increased in the germfree rats fed either diet compared with conventional controls. It is of interest that both germfree status and feeding of semipurified diet resulted in lower urinary phosphorus excretion than for the controls. In germfree rats fed either diet, the net retention of calcium, phosphorus and magnesium was significantly higher (varying from 131 to 159%) than in the conventional animals. In addition, the net retention of calcium was increased in all rats fed liquid diet, whereas phosphorus and magnesium retention values decreased in this group of animals (magnesium retention decreased only sightly).

Table 5 shows the distribution of calcium, phosphorus and magnesium in the contents of the intestinal tract of germfree and conventional rats fed semipurified diet. Comparisons were made in the various segments of intestinal tract between germfree and conventional rats by taking into consideration the concentrations and total amounts of each mineral present in the segments. Any differences in the concentration as well as the total amount of these minerals between germfree and conventional intestinal contents were taken as differences in absorption between germfree and conventional animals. Germfree status apparently resulted in a moderate decrease in the calcium and magnesium level of the various parts of the intestinal tract, suggesting an increased absorption

TABLE 5

Calcium, phosphorus and magnesium levels in intestinal contents of germfree and conventional rats fed semipurified diet

	Calcium		Phosphorus		Magnesium	
	Germfree	Conventional	Germfree	Conventional	Germfree	Conventional
Duodenum,						
mg/g dry matter	16.0 ±1.27 [1]	18.8 ±0.56	19.8 ±1.51 [2]	14.3 ±1.24	2.69 ±0.17	3.03 ±0.72
mg/total contents	0.33 ±0.033	0.44 ±0.032	0.41 ±0.05	0.33 ±0.038	0.054 ±0.004	0.069 ±0.014
Jejunum						
mg/g dry matter	14.8 ±0.89 [3]	21.9 ±1.02	11.7 ±2.29 [2]	19.2 ±1.32	2.96 ±0.16 [3]	4.01 ±0.16
mg/total contents	1.66 ±0.16 [4]	2.80 ±0.13	1.13 ±0.20 [3]	2.45 ±0.22	0.33 ±0.03 [3]	0.51 ±0.04
Ileum						
mg/g dry matter	19.2 ±0.48 [4]	29.2 ±0.50	24.2 ±1.61	26.4 ±1.33	3.02 ±0.21 [2]	4.66 ±0.50
mg/total contents	4.23 ±0.14 [4]	6.99 ±0.28	5.34 ±0.43	5.74 ±0.46	0.67 ±0.07 [2]	1.11 ±0.12
Cecum						
mg/g dry matter	38.9 ±1.45 [4]	64.7 ±1.58	22.2 ±1.05 [4]	41.8 ±1.79	3.76 ±0.28 [4]	7.61 ±0.47
mg/total contents	85.0 ±6.82 [4]	23.9 ±0.78	48.8 ±4.30 [4]	15.6 ±1.06	8.31 ±0.93 [4]	2.80 ±0.18
Colon						
mg/g dry matter	43.6 ±1.48 [4]	55.6 ±1.32	37.1 ±1.72	41.0 ±1.30	4.46 ±0.41 [2]	6.54 ±0.57
mg/total contents	12.5 ±0.42 [4]	17.3 ±0.60	10.7 ±0.61	12.0 ±0.69	1.27 ±0.11 [3]	2.03 ±0.18

[1] Averages ± SEM.
[2,3,4] Difference from conventional animal significant, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

of these minerals from the germfree intestine, and confirms the results summarized in tables 2 and 4. These differences reached statistical significance in all segments except the duodenum. The total amount of minerals present in the germfree cecum was higher than in the conventional cecum because of the characteristic increase of cecal contents in germfree rodents. Although the differences in phosphorus content of the various intestinal segments between germfree and conventional rats were irregular, the amounts of phosphorus present in the entire small intestine of germfree and conventional rats suggested no increase in absorption from the germfree small intestine, confirming the results presented in table 3.

Femur weights were about 16% higher in germfree rats compared with conventional animals (table 6). The levels of all three minerals in the femur expressed per unit of body weight were significantly higher in germfree rats (25 to 48% higher). The ratios of the minerals in germfree (Ca/P, 1.87; Ca/Mg, 49.1; P/Mg, 26.3) and conventional (Ca/P, 1.77; Ca/Mg, 50.2; P/Mg, 28.4) rats were similar. Kidney mineral levels were not affected by the germfree status.

Histopathological examination of kidneys from the germfree rats fed liquid and semipurified diets showed neither macroscopic nor microscopic lesions indicative of hydronephrosis and calcification. In earlier studies, germfree rats which showed hydronephrosis were raised on liquid diet containing glycerophosphate, calcium chloride and magnesium acetate in the salt mixture.[3] The presently used liquid diet, which contains calcium and magnesium salts of fructose 1, 6-diphosphate produced none of the kidney lesions, and thus appears to be nutritionally adequate for germfree rats.

DISCUSSION

The calcium and magnesium levels in the plasma of germfree and conventional rats were similar, suggesting that the increased absorption of these minerals under germfree conditions was not influenced by

[3] Pleasants, J. R. 1966 Development of chemically defined, water-soluble diets nutritionally adequate for germfree rats and mice. Ph.D. thesis, University of Notre Dame, Notre Dame, Indiana.

TABLE 6

Calcium, phosphorus and magnesium levels in femurs and kidneys of germfree and conventional rats fed semipurified diet

	Germfree	Conventional
Femur		
Weight	0.49 ±0.012 [1,2]	0.43 ±0.009
Per 100 g body wt	0.144±0.005 [3]	0.124±0.004
Ca, mg/g dry matter	224 ±8 [3]	198 ±4
Ca, mg/100 g body weight	32.9 ±1.48 [4]	24.6 ±1.01
P, mg/g dry matter	120 ±3	112 ±3
P, mg/100 g body weight	17.2 ±0.36 [4]	13.9 ±0.28
Mg, mg/g dry matter	4.56 ±0.17 [3]	3.94 ±0.12
Mg, mg/100 g body weight	0.65 ±0.03 [3]	0.44 ±0.05
Kidneys		
Ca, mg/g dry matter	0.77 ±0.04	0.82 ±0.04
Ca, mg/100 g body weight	0.115±0.006	0.129±0.005
P, mg/g dry matter	11.2 ±0.17	11.8 ±0.23
P, mg/100 g body weight	1.74 ±0.03 [4]	1.95 ±0.04
Mg, mg/g dry matter	0.54 ±0.06	0.58 ±0.04
Mg, mg/100 g body weight	0.090±0.014	0.091±0.008

[1] Averages ± SEM.
[2,3,4] Difference from conventional animal significant, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

their concentration in the plasma. Our data on calcium are in agreement with those of Bronner and Aubert (14), who showed that the serum calcium levels in the conventional rats failed to differ markedly even when the amount absorbed per day was increased to 100 times that circulating in the blood. Similarly, Tadayyon and Lutwak (15) showed that decreasing the absorption of calcium and magnesium by feeding certain fats in the diet had no effect on serum calcium and magnesium levels.

A number of factors have been shown to influence calcium and magnesium absorption from the intestinal tract. Absorption of calcium or magnesium, or both, was lowered by feeding poorly absorbable fats, and by high phosphorus and phytate intake (15–17), whereas absorption of these minerals was increased by coprophagy, low gastrointestinal pH, vitamin D, lactose, proteins, amino acids, antibiotics and hormones, to cite a few (16, 18–26). We have shown in this report that the absence of a microflora in the intestinal tract of rats caused a marked increase of calcium and magnesium absorption as evidenced by the data on absorption (tables 2 and 4) and distribution in various segments of intestinal contents (table 5). These data, however, do not exclude the possibility that differences in endogenous secretion of calcium and magnesium into the gut tract might contribute to the differences in apparent absorption observed between germfree and conventional animals. It has been shown that increase in age and dietary intake (26–29) can contribute markedly to increased endogenous excretion of these minerals. In our experiments, however, neither dietary intake nor age differed between germfree and conventional animals. Results of the present study also indicate the existence of a relationship between calcium and magnesium concentration in the various sections of the intestine. The ratios of the concentration of calcium to that of magnesium in the duodenum (D), jejunum (J), ileum (I), cecum (C) and colon (Co) in germfree (gf) and conventional (conv) rats calculated from table 5 are as follows: (gf.D, 5.9; conv.D, 6.2; gf.J, 5.0; conv.J, 5.5; gf.I, 6.4; conv.I, 6.3; gf.C, 10.3; conv.C, 8.5; gf.Co, 9.8; conv. Co. 8.5. The fact that the ratios in each segment of the small intestine were somewhat similar, and did not vary considerably between germfree and conventional animals, suggests some relationship between the absorption of calcium and that of magnesium in the small intestine. Also, increased calcium-magnesium ratio in the cecum suggests a major endogenous calcium secretion and little endogenous magnesium secretion into the cecum. This would render small intestine absorption data more valid and further reduces the

possibility that the endogenous secretions of these two minerals contributed to the apparent absorption data in these studies. The observation that urinary excretion of calcium was correlated with that of magnesium suggests a quantitative relationship between these two minerals even during excretion. Thus, the results offer evidence that the intestinal microflora plays an important role in the absorption of these two minerals from the intestinal tract. It is also justifiable to state that increased absorption of calcium and magnesium found in germfree animals was not a generalized phenomenon, because the germfree condition did not show any marked influence on the absorption of phosphorus (tables 3 and 5). Absorption studies using xylose, glucose and sodium (30), methionine [4] and fatty acids (31) also suggest that the effect of germfreeness on intestinal absorption is not a generalized phenomenon and varies depending on the compounds being studied.

Our results also indicate that feeding of liquid diet considerably increased phosphorus absorption and slightly enhanced calcium and magnesium absorption in both germfree and conventional rats. The calcium data confirmed the observations of Marcus and Lengemann (32). The increased absorption of these minerals from liquid diet may be ascribed to a lack of complexing materials in the liquid diet which would lower the absorption of these minerals by forming insoluble complexes.

Urinary excretion of calcium and magnesium was increased while the phosphorus excretion was decreased, under the germfree conditions. The increase in calcium and magnesium excretion presumably is part of the homeostatic mechanism combating the increased influx of these minerals. This appears in agreement with earlier studies (15, 29, 33). The reason for decrease in phosphorus excretion under germfree conditions could be related to a secondary increase in phosphorus retention to conserve the calcium-phosphorus ratio in the body fluids.

In the present study, germfree conditions thus increased the net retention of calcium, phosphorus and magnesium. This increase in retention was reflected in increased deposition of minerals in the bone. The fact that the content of the three minerals in the kidneys did not differ between germfree and conventional rats suggests that the increased absorption or net retention of these minerals observed under germfree conditions per se did not influence mineral content in soft tissues (kidneys). These results indicate that under the present experimental conditions no calcification of kidneys occurred as a result of the germfree status.

Since this investigation conclusively established that the germfree condition affected absorption, excretion, net retention and bone deposition of calcium, phosphorus and magnesium in rats, it is pertinent to elaborate on the possible mechanisms involved. Several speculative mechanisms are presented here.

(a) Morphological studies suggest that the absence of a viable microflora in the gut tract was associated with a reduction of mucosal surface area (34) in germfree animals. The functional capacity of the mucosal cell, however, is possibly increased in the germfree animal because the age of the cell is (somewhat) correlated with its absorptive function and because its average lifespan is increased under germfree conditions (35, 36). One might speculate that the germfree condition alters the morphological structure of the mucosa in a way that facilitates the passage of nutrients. This interpretation, however, would require that absorption of all nutrients transported passively or via low energy carrier systems be increased under germfree conditions. This is not the case. Some studies have indicated an increase in the absorption of xylose (30) and methionine,[5] and increased utilization of fat (37) whereas other studies have shown decreased availability of iron and copper (1) in germfree animals, and still other studies have shown no difference in fatty acid absorption (31, 38) under germfree conditions. Since the germfree state does not equally affect all the passive and low energy transport systems studied above, the increases

[4] Herskovic, T., J. Katz, M. H. Floch, R. P. Spencer and H. M. Spiro 1967 Small intestinal absorption and morphology in germfree, monocontaminated and conventional mice. Gastroenterology, 52: 1136 (abstract).

[5] See footnote 4.

in calcium and magnesium absorption observed in germfree rats do not necessarily result from changes in morphological structure of mucosa caused by germfree conditions.

(b) Another factor that has been shown to increase the absorption of calcium from the intestine is the presence of taurine-conjugated bile acids such as taurocholate and taurodeoxycholate (39, 40). Conjugated bile acids may enhance absorption directly by reducing the extent of formation of "insoluble" (difficult to absorb) complexes in the intestinal lumen, besides their possible influence on vitamin D uptake. Deconjugated bile acids have no such enhancing effect on calcium absorption (39, 40). Available data indicate that in the conventional animals (41) bile acids are present largely in the unconjugated form, especially in the lower parts of small intestine and in the cecum and large intestine, because of bacterial enzymatic hydrolysis, whereas in germfree rats, they are in a conjugated form such as taurocholate (42, 43). These conjugated bile acids, thus, could be responsible for an increased absorption of calcium and magnesium in the germfree rats.

(c) A third possibility is the influence of vitamin D on the absorption. The intestinal absorption of calcium, magnesium and other divalent ions appears to be enhanced by vitamin D (25, 44–48). Wasserman and Taylor (46, 49) showed that vitamin D induced a mucosal calcium-binding protein which is involved in calcium transport, and the degree of calcium absorption at any time was correlated with the amount of mucosal calcium-binding protein. Others have speculated that vitamin D alters the chemical or physical structure of the intestinal mucosa or both, so that passage of the cations is facilitated (47). In our case, an increased absorption of vitamin D from germfree small intestine could induce the synthesis of more carrier protein that would serve to transfer more calcium and magnesium across the intestine.

The deposition of more calcium in the bone of germfree rats than in conventional animals may be due either to increased accretion rates or to decreased resorption rates in the bones of germfree animals. Although our data do not distinguish between these two processes, it is possible that increases in bone size, calcium content of bone, and normal serum calcium levels may be mediated by thyrocalcitonin which acts to inhibit bone resorption (50), thereby increasing calcium content of bone and keeping serum calcium levels normal. Similar interpretations were made by Cohn et al. (27) who showed that the effect of high calcium intake (increased absorption, in our experiments) in decreasing bone resorption may be mediated by thyrocalcitonin. Germfree rats have shown reduced thyroid activity as measured by iodine-131 uptake (51), and there might be some relation between the two functions of thyroid.

In conclusion, this investigation indicated that the absence of viable intestinal microflora in the rat plays an important role in the absorption of calcium and magnesium, and in the net retention and bone concentrations of calcium, phosphorus and magnesium. In spite of the increased absorption of calcium and magnesium under germfree conditions, the kidneys of the germfree rats showed no calcification. Evidence presented here shows the existence of intestine-bone-kidney interrelationships for regulation of calcium, phosphorus and magnesium metabolism in the body through which the ratios among these minerals are maintained constant in the body fluids and in the bone.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Bernard F. Wostmann, Jr. for computer analysis of the data.

LITERATURE CITED

1. Reddy, B. S., J. R. Pleasants, D. R. Zimmerman and B. S. Wostmann 1965 Iron and copper utilization in rabbits as affected by diet and germfree status. J. Nutr., *87:* 189.
2. Edwards, H. A., Jr., and F. M. Boyd 1963 Effect of germfree enviroment on Ca^{47} metabolism. Poultry Sci., *42:* 1030.
3. Gustafsson, B. E., and A. Norman 1963 Urinary calculi in germfree rats. J. Exp. Med., *116:* 273.
4. Pleasants, J. R., B. S. Reddy and B. S. Wostmann 1969 Sudden death in germfree mice reared through successive generations on chemically defined liquid diet. In: Advances in Experimental Medicine and Biology, vol. 3, ed., E. A. Mirand. Plenum Publishing Corp., New York, p. 307.

5. National Research Council 1962 Nutrient Requirements of Laboratory Animals. Nat. Acad. Sci. Nat. Res. Counc., publ. 990, Washington, D. C.
6. Forbes, R. M. 1963 Mineral utilization in the rat. I. Effects of varying dietary ratios of calcium, magnesium and phosphorus. J. Nutr., *80:* 321.
7. Bunce, G. E., H. E. Sauberlich, P. G. Reeves and T. S. Oba 1965 Dietary phosphorus and magnesium deficiency in the rat. J. Nutr., *86:* 406.
8. Wostmann, B. S., J. R. Pleasants and B. S. Reddy 1967 Water-soluble and non-antigenic diets. In: Husbandry of Laboratory Animals, ed., M. L. Conalty. Academic Press, London, p. 187.
9. Reddy, B. S., and B. S. Wostmann 1966 Intestinal disaccharidase activities in the growing germfree and conventional rats. Arch. Biochem. Biophys., *113:* 609.
10. Wagner, M. 1959 Determination of germfree status. Ann. N. Y. Acad. Sci., *78:* 89.
11. Reddy, B. S., J. R. Pleasants and B. S. Wostmann 1969 Pancreatic enzymes in germfree and conventional rats fed chemically defined, water-soluble diet free from natural substrates. J. Nutr., *97:* 327.
12. Fisk, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.
13. Allen, R. J. L. 1940 The estimation of phosphorus. Biochem. J., *34:* 858.
14. Bronner, F., and J. P. Aubert 1965 Bone metabolism and regulation of the blood calcium level in rats. Amer. J. Physiol., *209:* 887.
15. Tadayyon, B., and L. Lutwak 1969 Interrelationship of triglycerides with calcium, magnesium and phosporus in the rat. J. Nutr., *97:* 246.
16. Meyer, D. L., and R. M. Forbes 1967 Effects of thyroid hormone and phosphorus loading on renal calcification and mineral metabolism of the rat. J. Nutr., *93:* 361.
17. Likuski, H. J. A., and R. M. Forbes 1965 Mineral utilization in the rat. IV. Effects of calcification and phytic acid on the utilization of dietary zinc. J. Nutr., *85:* 230.
18. Tadayyon, B., and L. Lutwak 1969 Role of coprophagy in utilization of triglycerides, calcium, magnesium and phosphorus in the rat. J. Nutr., *97:* 243.
19. Heggeness, F. W. 1959 Effect of antibiotics on the gastrointestinal absorption of calcium and magnesium in the rat. J. Nutr., *68:* 573.
20. Wasserman, R. H., C. L. Comar and M. M. Nold 1956 The influence of amino acids and other organic compounds on the gastrointestinal absorption of calcium^{45} and strontium^{89} in the rat. J. Nutr., *59:* 371.
21. Wasserman, R. H. 1964 Lactose-stimulated intestinal absorption of calcium: A theory. Nature, *201:* 997.
22. Scholz, R. W., and W. R. Featherston 1967 Influence of lactose and glucose on magnesium-28 retention in the chick. J. Nutr., *91:* 231.
23. Ali, R., and J. L. Evans 1967 Effect of dietary calcium, buffering capacity, lactose and EDTA on pH and calcium absorption from gastrointestinal segments in the growing rat. J. Nutr., *93:* 273.
24. Forbes, R. M. 1965 Mineral utilization in the rat. V. Effects of dietary thyroxine on mineral balance and tissue mineral composition with special reference to magnesium nutriture. J. Nutr., *86:* 193.
25. Engstrom, G. W., and H. F. DeLuca 1963 Effect of egg white diets on calcium metabolism in the rat. J. Nutr., *81:* 218.
26. Hansard, S. L., and H. M. Crowder 1957 The physiological behavior of calcium in the rat. J. Nutr., *62:* 325.
27. Cohn, S. T., T. Teree and E. Gusmono 1968 Effect of varying calcium intake on the parameters of calcium metabolism in the rat. Nutr., *94:* 261.
28. Benson, J. D., R. S. Emery and J. W. Thomas 1969 Effects of previous calcium intakes on adaptation to low and high calcium diets in rats. J. Nutr., *97:* 53.
29. Chutkow, J. G. 1964 Metabolism of magnesium in the normal rat. J. Lab. Clin. Med., *63:* 80.
30. Heneghan, J. B. 1963 Influence of microbial flora on xylose absorption in rats and mice. Amer. J. Physiol., *205:* 417.
31. Tennant, B., M. Reina-Guerra, D. Harrold and M. Goldman 1969 Influence of microorganisms on intestinal absorption: oleic acid ^{131}I and triolein ^{131}I absorption by germfree and conventional rats. J. Nutr., *97:* 65.
32. Marcus, C. S., and F. W. Lengemann 1962 Absorption of Ca^{45} and Sr^{85} from solid and liquid food at various levels of the alimentary tract of the rat. J. Nutr., *77:* 155.
33. Malm, O. J. 1963 Adaptation to alterations in calcium intake. In: The Transfer of Calcium and Stontium Across Biological Membranes, ed., R. H. Wasserman, Academic Press, New York, p. 143.
34. Gordon, H. A., and E. Bruckner-Kardoss 1961 Effect of normal microbial flora on intestinal surface area. Amer. J. Physiol., *201:* 175.
35. Abrams, G. D., H. Bauer and H. Sprinz 1963 Influence of the normal flora on mucosal morphology. Lab. Invest. *12:* 355.
36. Padykula, H. A., E. W. Strauss, A. J. Ladman and F. H. Gardner 1961 A morphologic and histochemical analysis of the human jejunal epithelium in nontropical sprue. Gastroenterology, *40:* 735.
37. Evrard, E., P. P. Hoet, H. Eyssen, H. Charlier and E. Sacquet 1964 Fecal lipids in germfree and conventional rats. Brit. J. Exp. Pathol., *45:* 409.
38. Wiech, N. L., J. G. Hamilton and O. N. Miller 1967 Absorption and metabolism of dietary triglycerides in germfree and conventional rats. J. Nutr., *93:* 324.

39. Webling, D. D'A., and E. S. Holdsworth 1965 Effect of bile, bile acids and detergents on calcium absorption in the chick. Biochem. J., 97: 408.
40. Webling, D. D'A., and E. S. Holdsworth 1966 Bile salts and calcium absorption. Biochem. J., *100:* 652.
41. Dietschy, J. M. 1968 Mechanism for the intestinal adaptation of bile acids. J. Lipid Res., 9: 297.
42. Kellogg, T. F., and B. S. Wostmann 1969 The quantitative fecal bile acid and neutral sterol excretion of germfree rats. J. Lipid Res., *10:* 495.
43. Gustafsson, B. E., S. Bergstrom, S. Lindstedt and A. Norman 1957 Turnover and nature of fecal bile acids in germfree and infected rats fed cholic acid-24C^{14}. Bile acids and steroids 41. Proc. Soc. Exp. Biol. Med., *94:* 467.
44. Worker, N. A., and B. B. Migicovsky 1961 Effect of vitamin D on the utilization of beryllium, magnesium, calcium, strontium and barium in the chick. J. Nutr., *74:* 490.
45. Meintzer, R. B., and H., Steenback 1955 Vitamin D and magnesium absorption. J. Nutr., 56: 285.
46. Wasserman, R. H., and A. N. Taylor 1968 Vitamin D-dependent calcium-binding protein. Response to some physiological and nutritional variables. J. Biol. Chem., 243: 2987.
47. Masuhara, T., and B. B. Migicovsky 1963 Vitamin D and the intestinal absorption of iron and cobalt. J. Nutr., *80:* 332.
48. Wasserman, R. H. 1962 Studies on vitamin D_3 and the intestinal absorption of calcium and other ions in the rachitic chick. J. Nutr., 77: 69.
49. Wasserman, R. H., and A. N. Taylor 1966 A vitamin D_3-induced Ca binding protein in chick intestinal homogenates. Science, *152:* 791.
50. MacIntyre, I. 1968 Calcitonin: a review of its discovery and an account of purification and action. Proc. Roy. Soc. B., *170:* 49.
51. Desplaces, A., D. Zagury and E. Sacquet 1963 Étude de la fonction thyroidienne du rat privé de bacteries (germfree). C. R. Acad. Sci., 257: 756.

Dependence of Chromium Transfer into the Rat Embryo on the Chemical Form [1]

WALTER MERTZ, E. E. ROGINSKI, F. J. FELDMAN AND D. E. THURMAN
Division of Biochemistry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

ABSTRACT The placental transfer of chromium was studied in rats using atomic absorption spectroscopy for determination of the stable isotope and scintillation counting for ^{51}Cr. Previous findings, that simple chromium compounds injected into pregnant rats do not label the litter, were confirmed. However, appreciable concentrations of ^{52}Cr were detected in all newborn animals. Feeding pregnant rats a diet in which a high chromium content was supplied by the natural ingredients resulted in increased chromium levels in the young, whereas giving 2 ppm chromium (as acetate) in the drinking water did not. Intragastric administration of ^{51}Cr, incorporated into brewer's yeast, to pregnant rats led to significant labeling of the newborn. These results indicate that chromium in the form of a natural complex, but not as a simple salt, is transported across the placenta.

Any trace element with an essential function must meet the criterion that it be present in the newborn organism. Most, if not all, essential elements are present in fetal or newborn tissues in considerably higher concentrations than during the following period of early life. Chromium, in particular, occurs at its highest levels in man immediately after birth and during the first few years of life (1). Schroeder et al. detected the element in most, but not in all newborn laboratory animals; they suggested that repeated pregnancies can deplete the mother animals of their liver chromium stores (1). Indirect evidence for a transfer of chromium from mother to fetus can be derived from Pribluda's analyses of the bones of 120 human embryos, in which the chromium content increased with increasing age (2). The same investigator demonstrated a steady decrease with time of gestation in the bones of pregnant rats (3). Another study noted continuously increasing tissue concentrations of the element in human embryos between the ages of 3.5 and 7 months, with a sharp decline at the time of birth (4).

On the other hand, Visek and collaborators were unable to detect any significant transfer into the young, following injection of pregnant rats with ^{51}Cr salts, regardless of chemical form or valence state of the element (5). Because absence of placental chromium transport, implicated in this latter study, is in apparent contrast to the analytically detectable presence of chromium in the fetus, and because it would present a strong challenge to the postulated essential role of chromium, the problem of transfer from mother into young was reinvestigated. To this end, female rats were given various treatments with different complexes of two isotopes of the element before or during gestation. The results reconcile both apparently contradictory sets of findings by demonstrating that placental transport depends on the complex in which chromium is administered.

EXPERIMENTAL

Male and female rats of the Sprague-Dawley strain (Walter Reed strain) were fed either a commercial laboratory ration [2] with a chromium content of approximately 500 ppb, or a 30% Torula yeast diet (6) of less than 100 ppb chromium. The diets and deionized drinking water, with or without 2 ppm chromium (as $Cr(CH_3COO)_3 \cdot H_2O$) were offered ad libitum. All experiments were performed with groups of at least five females. One adult male rat was

Received for publication June 9, 1969.

[1] A preliminary report of some of these findings has been made: Roginski, E. E., F. J. Feldman and W. Mertz 1968 Chromium in the newborn rat. Federation Proc., 27: 482 (abstract).

[2] G. and L. Baking Company, Frederick, Md.

kept in a cage with a female for 1 week, after which the mated female was kept separately and received, in addition to the experimental diet, fresh kale each day. Immediately after birth in most cases, but never later than 3 hours after delivery, the newborn litter on one hand, and the mother rat on the other, were counted for radioactivity in a small animal counter, with proper correction for background. Counting error at the 95% confidence level was calculated according to the formula (7): $E_{95} = 1.96\ (N_s/t_s + N_b/t_b)^{1/2}$, where N_s and N_b are the counting rates, and t_s and t_b the counting times for sample + background (s) and background (b), respectively. After completion of the counting, two or more of the young were killed with chloroform and analyzed for chromium by atomic absorption spectroscopy (8). Chromic chloride, $^{51}CrCl_3 \cdot 6H_2O$, specific activity ranging from 30 to 100 mCi/mg Cr, was injected intravenously into female rats, 5 μCi/rat, as indicated in the tables. In other experiments, it was administered by stomach tube daily, except for weekends, at 5 μCi/rat, or in one single dose of 250 μCi. Other rats received ^{51}Cr in form of glucose tolerance factor [3] from brewer's yeast. *Saccharomyces carlsbergensis* [4] was grown under aerobic conditions at 25° to a late log phase in Sabouraud's medium which had been adjusted to a concentration of 200 μg ^{52}Cr and 1.66 mCi ^{51}Cr per liter (as chromic chloride). The cells were collected by centrifugation and washed three times with deionized water. They were allowed to autolyze by incubation and shaking for 3 hours at 37° in 0.1 M $NaHCO_3$. Two milliliters of a 1:4 suspension (wet wt/v) of the autolysate were stomach-tubed into pregnant rats, five times during the second week of gestation.

In a second procedure (batch C, table 3) the amount of radioactivity in the yeast was increased by increasing the ^{51}Cr concentration to 2 mCi per liter and by stimulating chromium uptake through addition of glucose, according to Burkeholder and Mertz (9). The cells were treated as decribed above; in addition, the cell residue after autolysis was further broken up by boiling in 66% ethanol. A suspension was administered by stomach tube in different doses to three rats on days 15, 16 and 18 of gestation.[5]

RESULTS

The intravenous injection of one dose of chromic-51-chloride into female rats at the time of mating resulted in a typical whole body disappearance curve (fig. 1), similar to those previously observed in male rats (10). The slope of the curve remained unchanged when a litter of 11 young was born. The absence of a sharp break in the slope indicates that none of the mother's ^{51}Cr was lost to the young at birth. This is borne out by comparing the radioactivity in mother animals and litters (table 1). Neither intravenous injection nor stomach tubing of one dose of chromic-51-chloride into the females, before or at mating, resulted in appreciable transfer of ^{51}Cr into the young. Administration of the salt by stomach tube in repeated doses throughout the course of pregnancy led to a small degree of labeling of the litter, from 0.5 to 1.5% of the mother's total body activity.

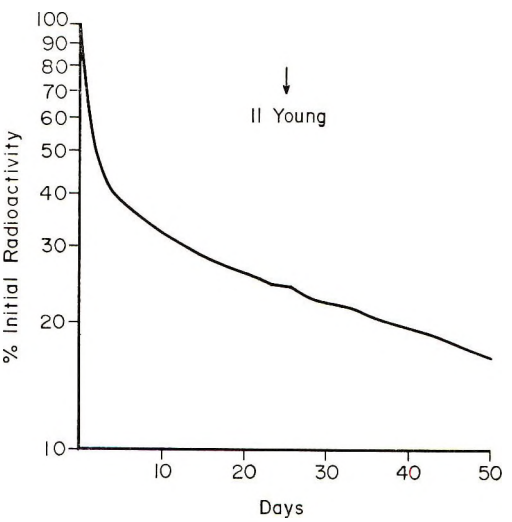

Fig. 1 Whole body disappearance of ^{51}Cr in a pregnant rat.

[3] Glucose tolerance factor (GTF) designates the yet unidentified chromium complex(es) extractable from brewer's yeast and other sources. Chromium in this form is of much greater biological activity than in the form of simple salts or aquo complexes.

[4] Carling Brewing Corporation, Baltimore, Md.

[5] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

TABLE 1

Placental transport of a chromium salt: concentration of ^{51}Cr in mothers and litters at birth [1]

Rat number	Number of young	Counts per minute		Ratio B/A × 100	Mode of administration
		In mother (A)	In young (B)		
2	11	24,500	0 [2]	0	iv injection at mating
3	12	20,390	(33)	0.16	iv injection at mating
8	13	14,090	(3)	0	iv injection 4½ weeks before mating
12	12	4,890	0	0	stomach-tubed at mating
13	11	6,840	0	0	stomach-tubed at mating
21	11	24,700	220 [3]	0.89	stomach-tubed repeatedly during gestation
22	11	2,289	36 [4]	1.57	stomach-tubed repeatedly during gestation
23	12	46,600	211 [3]	0.45	stomach-tubed repeatedly during gestation

[1] Chromic-51-chloride, without added carrier, administered to mother.
[2] Observed counts equal to or less than background. Figures in parentheses represent observations not significantly different from background.
[3] Counting error less than 8% of rate.
[4] Counting error 26.5% of rate.

TABLE 2

Influence of maternal diet on chromium-52 content of newborn rats

Maternal diet	Estimated daily chromium intake	Chromium in newborn	Average ± SE
	μg	*ppb*	*ppb*
Torula yeast diet	< 2	45, 42, 42, 50, 48, 33	43 ± 2.5
+ 2 ppm Cr in drinking water	42	39, 37, 57, 30	41 ± 5.8
Laboratory ration	10	300, 120, 95, 75, 63, 85, 65, 70, 75, 85, 79, 78, 82, 130, 116, 65, 64	97 ± 13.6

Analysis of chromium-52 levels in the newborn revealed an average concentration of 97 ppb in the young of mothers fed a commercial laboratory ration (approximately 500 ppb chromium) (table 2). Not included in these data are the values for two stillborn rats, with 1600 and 1160 ppb, respectively. When female rats were fed the low-chromium Torula yeast diet (less than 100 ppb chromium), their newborn contained only half of the amount of chromium found in the young of mothers fed the chromium-sufficient ration. These low concentrations could not be increased by supplementing the drinking water of the pregnant rats with 2 ppm chromium. Based on an estimated intake of 20 g food and 20 ml supplemented water per day, this latter regimen would furnish a total daily chromium intake of 42 μg, as compared with 10 μg for the same intake of stock diet and deionized water.

Chromium-51, synthesized into glucose tolerance factor by brewer's yeast was transported into the fetus. The average level incorporated into six litters was 20% that of the mother's radioactivity (table 3). Only one litter contained no measurable ^{51}Cr at birth; its mother had retained only one-third of the levels of the other five rats. Because of the small amount of radioactivity and the high counting error of approximately 20%, these experiments were repeated with a preparation of higher specific activity. The latter was again accumulated in the litter, to a higher degree than in the previous experiments. The accumulation in the litter of approximately 50% of the mother's radioactivity (counting error < 1%), when expressed on a

TABLE 3

Placental transport of a natural chromium complex: concentration of ^{51}Cr in mothers and litters at birth [1]

Rat number	Number of young	Net counts (10 min)		Ratio B/A × 100
		In mother (A)	In newborn (B)	
32 [2]	14	900	150	17
34 [2]	13	988	240	24
35 [2]	12	1096	183	17
44 [3]	10	1006	350	35
45 [3]	13	1195	294	25
42 [3]	8	283	0	0
Average (N = 6) [4]		911 ± 132	203 ± 50	20 ± 5
71 [5]	15	321,600	172,400	54
72 [5]	11	209,400	132,400	63
73 [5]	15	34,700	14,200	41

[1] After 3 to 5 stomach-tubed doses of ^{51}Cr as yeast extract to mothers.
[2] Batch A.
[3] Batch B.
[4] Mean ± SE.
[5] Batch C, high specific activity. Rat 73 received approximately one-fifth of the dose given to rats 71 and 72.

body-weight basis (60 to 70 g for litter *vs.* 300 g for mother), strongly suggests active transport of chromium against a gradient.

DISCUSSION

The first part of the data confirms the findings of Visek and collaborators (5), that simple chromium compounds do not penetrate into the fetus when given to the pregnant mother in one dose. This fact would be incompatible with the postulated essential role of chromium, if it were taken by itself. But one must take into account that chromium-52 was detected in all newborn rats, even in those which had been used in the isotope experiment and had not accumulated any of the ^{51}Cr. This apparent discrepancy can be reconciled in one of three ways. One could assume that the organism discriminates between the two isotopes of chromium. While some discrimination has been proven for isotopes of lighter elements (11), all known evidence is against discrimination between isotopes of the mass of chromium. Secondly, one could assume that the maternal pool from which the fetal chromium is derived has a very slow turnover, therefore requiring a certain period of time for an appreciable incorporation of label. This possibility is supported by the observation that administration of $^{51}CrCl_3$ throughout the pregnancy results in a slightly better transport of the element into the fetus, but it is not compatible with the failure of one dose of the salt to cross the placenta, even when injected into the female 7.5 weeks before birth of the litter.

The third alternative is to assume that the mechanism of placental chromium transport discriminates against the chemical form in which the chromium ion is offered. This alternative is proven by the observation that ^{51}Cr in the biological form, isolated from autolyzed brewer's yeast (GTF), easily labels the fetus when administered to the mother. Since even high concentrations of chromic acetate in the drinking water do not cross into the fetus, it must be concluded that the fetal chromium is derived from one or more specific chromium complex(es) in the diet. The identity of these complexes in various rations with glucose tolerance factor remains to be proved; however, the data obtained with GTF from brewer's yeast lend strong support to the possibility.

The rat is not entirely dependent on the dietary chromium complex(es). It has a limited capability to build small, daily doses of "inorganic" chromium into a complex which can cross the placental barrier. The very small degree of this synthesis is evident from the data in the second part of table 1, and from the failure of chromic acetate in the drinking water to lead to an analytically recognizable elevation of chromium levels in the young. If

it could be unequivocally proven that chromium is essential for the fetus, one would have to assume that the element is present as a part of a new vitamin, i.e., of an essential compound which the animal cannot synthesize itself. However, the essentiality of chromium for the fetus has not yet been established.

The nearly absolute requirement for a specific chromium complex sets the placental transport system apart from the various chromium-responsive systems of glucose metabolism. Even though glucose-tolerance-factor chromium is at least ten times more active than simple chromium salts,[6] the latter do produce significant effects with isolated fat tissue in vitro (12), upon oral or intravenous administration in the rat (10, 13), in sheep [7] and squirrel monkeys (14) and in man (15–17). This difference in the requirement for a specific complex of chromium may suggest two different sites of action, one of which can, the other of which cannot utilize simple salts of the element. But it is also possible that the organic complex (GTF) is the only biologically active form and that the limited ability of the rat to synthesize this form can meet the requirement of carbohydrate metabolism in the adult animal, but not that of the growing fetus. In any case, the results of this study suggest that great caution must be exercised in the interpretation of experiments with chromium-51-chloride in the rat.

LITERATURE CITED

1. Schroeder, H. A., J. J. Balassa and I. H. Tipton 1962 Abnormal trace metals in man: Chromium. J. Chron. Dis., *15:* 941.
2. Pribluda, L. A. 1963 The chromium content of hollow bones of the human fetus. Dokl. Akad. Nauk. Belorussk. SSR, *7:* 135. (Chem. Abstr., *59:* 3142.).
3. Pribluda, L. A. 1963 Chromium content of the long bones of rats at different stages of pregnancy. Dokl. Akad. Nauk. Belorussk. SSR, *7:* 206. (Chem. Abstr., *59:* 4331.)
4. Mikosha, A. 1959 Trace elements in human embryos. Nauk. Zap. Stanislav's Med. Inst., *1959* (3): 85. (Chem. Abstr., *59:* 7969.)
5. Visek, W. J., I. B. Whitney, U. S. G. Kuhn III and C. L. Comar 1953 Metabolism of Cr^{51} by animals as influenced by chemical state. Proc. Soc. Exp. Biol. Med., *84:* 610.
6. Mertz, W., and K. Schwarz 1959 Relation of glucose tolerance factor to impaired glucose tolerance in rats on stock diets. Amer. J. Physiol., *196:* 614.
7. Kinsman, S., ed. 1957 Radiological Health Handbook. U. S. Dept. Health, Educ. Welf., Cincinnati, Ohio, p. 129.
8. Feldman, F. J., E. C. Knoblock and W. C. Purdy 1967 The determination of chromium in biological materials by atomic absorption spectroscopy. Anal. Chim. Acta, *38:* 489.
9. Burkeholder, J. N., and W. Mertz 1967 Properties and effects of chromium III fractions obtained from brewer's yeast. Proceedings, 7th Intern. Congr. Nutr., vol. *5:* 701.
10. Mertz, W., E. E. Roginski and R. C. Reba 1965 Biological activity and fate of intravenous chromium (III) in the rat. Amer. J. Physiol., *209:* 489.
11. Bowen, H. J. M. 1966 Trace Elements in Biochemistry. Academic Press, New York, p. 147–149.
12. Mertz, W., E. E. Roginski and K. Schwarz 1961 Effect of trivalent chromium complexes on glucose uptake by epididymal fat tissue of rats. J. Biol. Chem., *236:* 318.
13. Schwarz, K., and W. Mertz 1959 Chromium (III) and the glucose tolerance factor. Arch. Biochem. Biophys., *85:* 292.
14. Davidson, I. W. F., and W. L. Blackwell 1968 Changes in carbohydrate metabolism of squirrel monkeys with chromium dietary supplementation. Proc. Soc. Exp. Biol. Med., *127:* 66.
15. Glinsmann, W. H., and W. Mertz 1966 Effect of trivalent chromium on glucose tolerance. Metab. Clin. Exp., *15:* 510.
16. Hopkins, L. L., Jr., O. Ransome-Kuti and A. S. Majaj 1968 Improvement of impaired carbohydrate metabolism by chromium (III) in malnourished infants. Amer. J. Clin. Nutr., *21:* 203.
17. Levine, R. A., D. H. P. Streeten and R. J. Doisy 1968 Effect of oral chromium supplementation on the glucose tolerance of elderly human subjects. Metab. Clin. Exp., *17:* 114.

[6] Burkeholder, J. N., and W. Mertz 1967 Effect of yeast fractions on glucose utilization by yeast mammalian systems. Federation Proc., *26:* 307 (abstract).

[7] Britton, R. A., G. A. McLaren and D. A. Jett 1968 Influence of cane molasses on NPN utilization and fiber digestibility. J. Animal Sci., in press (abstract).

The Meal Eating Response of the Chicken — Species Differences, and the Role of Partial Starvation [1]

P. GRIMINGER, V. VILLAMIL AND H. FISHER
Department of Nutrition, Rutgers University, New Brunswick, New Jersey

ABSTRACT Chickens with access to two 1-hour meals daily from 1 week to 17 weeks of age consumed starvation levels of food and exhibited hypercholesterolemia in comparison with ad libitum-fed controls. Five daily 1-hour meals allowed 5-week-old chickens to consume as much feed as, or at least not significantly less than, their ad libitum-fed controls. Nevertheless, the meal eaters utilized their food less efficiently than the nibblers and gained less weight. In another test, with 2-week-old chicks, the effects of two or five daily 1-hour meals on food consumption, growth, body composition, blood cholesterol and glucose tolerance were measured over a 30-day period. Both meal eating regimens caused depressed food consumption and growth and gave rise to hypercholesterolemia, decreased body fat, increased body water, and an elevated fasting plasma glucose level. Five daily 1-hour meals were insufficient to permit maximum weight gains of 2-week-old chicks during their period of rapid growth. An experiment with young female rats (5 weeks after weaning) makes it appear doubtful that a basic difference exists between rats and chickens in their response to meal eating.

Interest in the metabolic consequences of feeding frequency goes back to the early days of nutritional investigations (1). Most of the methodology now used in such experimentation, however, can be traced to the force-feeding experiment of Levin (2) or to the "training" study of Tepperman et al. (3), published in 1944 and 1943, respectively. Levin observed that force-feeding rats twice daily resulted in higher carcass fat levels than ad libitum feeding, while Tepperman and co-workers noted increased lipogenesis for rats trained to eat during a restricted period, once daily. As quoted in his review paper (4), Cohn and associates found that "pair gaining" or "pair feeding" force-fed adult rats with ad libitum-fed controls also resulted in characteristic changes in body composition. While in this sense "meal eating" referred to the force feeding of the day's ration in one or two meals, the term has also been used for the "trained" voluntary consumption of a day's food intake during a few restricted time periods (5–10).

Adult rats of some strains are able to consume their daily food needs in one or two limited time periods per day (1); by contrast, it has been demonstrated that growing rats trained to meal eat cannot consume as much as their nibbling controls (9). Cohn et al. (5) have reported that 8- to 10-week-old chickens will consume less food in two 1-hour meals daily than their ad libitum-fed controls. Younger birds were even less able to consume in two 1-hour periods that quantity of food which they would ingest during 24-hour access to food (6, 8); moreover, the meal-eating regimen caused depressed growth and decreased body fat. This observation appears to be in direct contrast to some of the observations made with meal-fed rats.

The variable feeding techniques used, as well as large age differences of experimental animals, may have contributed to the inference that there is, indeed, a difference in feeding-frequency response between chickens and rats.

The experimentation reported here was carried out to determine the extent to which the meal-eating response of growing chickens discussed earlier was a reflection of the reduced food intake of this feeding regimen when compared with nibbling. The number of daily meals given to the chickens was aimed at allowing them to

Received for publication May 26, 1969.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey. The source of the experimental data other than the rat study was the thesis research performed in partial fulfillment of the requirements for the M.S. degree by one of the authors (V. V.).

consume as much food as their ad libitum-fed controls "nibbled." The effects of such meal eating regimens were compared with the other regimens in terms of food consumption, weight gain, blood cholesterol, body composition and glucose tolerance.

EXPERIMENTAL

Experiment 1. Crossbred cockerels (New Hampshire males × Columbian females) were randomly distributed into two lots of 80 meal eaters and 40 nibblers. A conventional starter ration containing, by calculation, 21.2% protein, and 270 metabolizable calories/100 g was fed in this and subsequent experiments. The nibblers had continuous access to food while the meal eaters, during the first week, were given access to food twice daily for 2 hours from 8 to 10 AM and again from 3 to 5 PM. When they were 1 week old, 20 nibblers and all the meal eaters were cropectomized according to the procedure of Fisher and Weiss (11) to insure "true" meal-eating in the absence of the crop, which acts as a storage organ. During as well as after the recovery period, the meal eaters were allowed to eat twice daily for 1 hour from 8 to 9 AM and from 3 to 4 PM. The experiment was initiated when the birds were 2 weeks old, the normal and cropectomized nibblers being designated as lots 1 and 2, respectively; all other birds received two 1-hour meals. After a 3-week period (period 1) the birds were fasted overnight, and blood was taken from five birds from each treatment for determination of plasma cholesterol levels. For period 2, the meal eaters were subdivided into three new groupings of 20 birds each. Birds in lot 3 were permitted to eat ad libitum, and those in lot 4 had access to food twice daily for 1 hour; the birds in lot 5 were nibblers pair-fed with lot 4, receiving a day's allowance once every 24 hours. Lots 1 and 2 were composed of 20 normal and 14 cropectomized nibblers, respectively. Food consumption was recorded daily for birds in lots 4 and 5, while weekly records were kept for all other lots.

Birds were continued on experiment until they had reached an average body weight of 1500 g. Lots 1 and 2 were discontinued after 8 weeks and 2 days on the experiment (periods 1 and 2). Birds in lot 3 reached the stated body weight after 10 weeks but were, for technical reasons, kept until 12 weeks of age. The average weight of lot 4 exceeded 1500 g after 17 weeks. The pair-fed birds of lot 5 were discontinued at the same time, even though they had not quite reached the target weight. As each lot was discontinued, plasma cholesterol (12) was determined for 10 birds.

Experiments 2 and 3. Hyline cockerels were cropectomized at 1 week of age and allowed to recover for another week, under conditions of ad libitum food intake. In experiment 2, three lots of 15 birds were fed ad libitum, or either four or three times daily, for 0.5 hour periods each for 1 week. Since weight gains for the time-restricted lots were appreciably lower than for the controls, the former were again permitted free access to food for 1 week, and then redistributed, by weight, into two lots (4 and 5). The lot originally fed ad libitum was discarded, and for 1 week lot 4 was fed ad libitum, and lot 5 four times daily for 1 hour. Since this treatment was also insufficient to obtain comparable food intake, each lot was further subdivided for 1 week, one sub-lot receiving food ad libitum, and the other, five 1-hour meals per day.

Chicks for experiment 3 were randomly distributed into two lots of 30 birds each. Lot 1 was permitted to eat ad libitum, while lot 2 was allowed to eat five times daily for 1 hour, at 7:30 and 11 AM, and at 3:30, 7:00, and 12:00 PM.

The two lots were maintained on their respective regimen for 10 days. After this training period, they were subdivided according to body weight into six lots of 10 chickens each, so that all three lots in either A or B would have similar average starting weights. The design was as follows:

— A — Trained as nibblers		
1A Nibblers	2A 5 meals	3A 2 meals
— B — Trained as meal eaters		
1B Nibblers	2B 5 meals	3B 2 meals

The chickens were maintained on these feeding regimens for an additional 20 days. Those in lots 3A and 3B were allowed to eat their second meal from 3:30 to 4:30 PM. Daily consumption records were kept for the birds in the meal eating lots, and weekly records for those in the two nibbling lots. All birds were weighed weekly. At the end of the 10-day training period as well as at the conclusion of the experiment, plasma cholesterol levels were determined following on overnight fast.

At the end of the experiment, a glucose tolerance test was carried out on all birds from lots 1A, 2B and 3A. For this test the birds were fasted overnight (4:30 PM to 9:00 AM) and weighed in the morning. Initial blood samples were drawn from a wing vein of each bird. Approximately 2 g glucose per kilogram body weight was then given by syringe into the esophagus of each bird as a 50% solution. Additional blood samples were drawn from the wing vein at 10 minutes, and by heart puncture at 20, 40, and 80 mintues after glucose infusion. All blood samples were immediately placed in 3% trichloroacetic acid solution, and were analyzed for glucose by the *O*-toluidine method of Hyvärinen and Nikkilä (13). At the end of the experiment all birds were killed with chloroform and dried to constant weight at 85°. Body composition was determined on pooled samples, each sample consisting of two birds of similar dry weights from a given lot. Fat was determined by chloroform–methanol (2:1 v/v) extraction. Carcass nitrogen was determined by the Kjeldahl method and converted to body protein (N × 6.25).

An experiment that was not part of this series, but is mentioned for the purpose of discussing interspecies comparisons, was conducted with Carworth (CNF strain) female rats. They were fed a casein-purified (16% protein) pelleted rat diet that had previously been shown to support good growth. Upon reaching 150 g body weight, they were trained for 1 week to eat their daily ration in one 2-hour meal per day. They were then distributed, by weight, into lots of 24 rats each. One of these lots was fed ad libitum and the other one for 2 hours a day. Body weight and food consumption records were kept on a weekly basis; at the end of the experiment, blood was drawn for cholesterol determination, the animals were killed and body composition was determined on an individual basis.

RESULTS

Experiment 1. Throughout this experiment the meal eaters consumed considerably less feed and gained at correspondingly slower rates than the nibblers (table 1). Chickens in the two nibbling lots responded similarly, the absence of the crop exerting no apparent effect on growth or feed consumption. Birds in lot 3 initially responded like the other meal eaters with

TABLE 1

Effect of continuous access to food, or of two daily meals, on growth, food consumption, and plasma cholesterol of growing chickens (exp. 1)

Lot [1]	Feeding pattern [2]		Period 1 [3]		Period 2 [3]			Final body [4] weight	Final plasma [5] cholesterol
	Period		Gain	Food cons.	No. of weeks	Avg gains	Food cons.		
	1	2							
			g	*g*		*g*	*g*	*g*	*mg/100 ml*
1	Nib (20) [6]	Nib (20)	125	291	5.3	198	569	1575 ± 28	87 ± 8 a
2	Nib (14)	Nib (13)	133	287	5.3	194	549	1549 ± 34	93 ± 9 a
3	Meal (21)	Nib (20)	29	88	9 [7]	184	532	1798 ± 30 [7]	92 ± 4 a
4	Meal (20)	Meal (15)	29	71	14.1	108	385	1523 ± 67	135 ± 5 b
5	Meal (20)	Pair-fed (16)	29	74	14.1	96	381	1351 ± 91	151 ± 7 c

[1] Lot 1, intact crops; all other lots cropectomized.
[2] Nib: ad libitum feeding. Meal: two 1-hour feedings daily. Pair-fed: pair-fed with lot 4, but food made available all at one time, and left until finished. Period 1, 3 weeks; period 2, variable.
[3] Gain and food consumption figures are averages per bird per week.
[4] Arithmetic means ± SE.
[5] Arithmetic means ± SE; lots with a different letter differ significantly ($P < 0.001$).
[6] Number of birds at end of experimental period.
[7] Average weight comparable to lots 1 and 2 was reached after 7 weeks.

greatly decreased consumption and weight gain. During period 2, however, these birds grew rapidly and required only 2 additional weeks, in comparison with lots 1 and 2, to exceed 1500 g of body weight. The meal eaters (lot 4) and the pair-fed nibblers (lot 5) responded with greatly depressed growth rates. Birds in lot 5 consumed their daily ration in a 3-hour period after it was given to them each morning, even though they were allowed access to it throughout the day. In lot 4, five birds died, and four were lost in lot 5.

Plasma cholesterol levels for nibblers and meal eaters did not differ significantly at the end of period 1. Final cholesterol values, however, were significantly different ($P < 0.001$) when meal eaters and pair-fed nibblers were compared with each other or with the ad libitum-fed nibblers (table 1). On the basis of the lack of difference between lots 2 and 3, and the significant difference between lots 4 and 5, as well as the observations of others (14) we believe that the increase in cholesterol values in lots 4 and 5 is not due to the greater age of these chickens. It could also be argued that these chickens, while of greater actual age, were probably of similar physiological age as those in the full-fed lots.

Experiment 2. On the three or four 1-hour feedings per day, birds still could not consume food quantities equivalent to those of their nibbling controls. With five meals a day, however, the birds, after a week on their respective eating schedules, did consume quantities of food similar to those consumed by the controls (table 2). Even then, however, the nibblers grew at a faster rate than the meal eaters: 57% versus 49% gain over the 1-week experimental period for lot 4 (prior treatment: nibbling) and 87% versus 70% for lot 5 (prior treatment: meal eaters).

Experiment 3. During the 10-day training period (period 1, table 3), meal eaters consumed appreciably less feed than nibblers, even with five meals daily. Similar differences were also observed during period 2. However, the ability of the meal eaters to increase their consumption improved with time, and by the end of the experiment, birds in lots 2A and 2B ate, on a daily basis, nearly as much as their ad libitum-fed controls. While the regimens during the initial training period may not have had a lasting effect on feed intake of the birds during period 2, birds trained to meal eat five times daily (lot 2B) came closer to consuming the quantity eaten by the controls (lot 1B) than did birds not initially trained to meal eat (lot 2A vs. 1A). Both groups of meal eaters, first exposed to this regimen during period 2, required only 1 day before they were able to consume as much as the trained meal eaters continued on this regimen.

No significant differences were found between plasma cholesterol levels of meal eaters and nibblers at the end of the 10-day training period (the birds were then approximately 4 weeks old). Such differences ($P < 0.001$) were, however, noted at the conclusion of the experimental period (at approximately 7 weeks of age). Birds with access to food twice daily had significantly higher cholesterol levels than did birds with ad libitum access to food (untrained birds, $P < 0.01$; previously trained birds, $P < 0.05$). In addition, birds in the untrained group on two meals daily had

TABLE 2

Effect of food access time on food consumption and body weights of growing chickens (exp. 2)

Lot [1]	Feeding pattern	Daily food consumption	Body weights [2]		Grams gain/gram food consumed
			Initial	Final	
		g/bird	*g*	*g*	
4A	Nibbling	85	696±23	1095±37	0.67
4B	5 meals	81	612±25	914±24	0.53
5A	Nibbling	90	548±29	1026±41	0.76
5B	5 meals	90	506±29	859±40	0.56

[1] Lots 4A and 5B, seven birds; lots 4B and 5A, eight birds each. Prior treatment: lots 4, nibblers; lots 5, meal eaters. Experimental period: 1 week.
[2] Arithmetic means of weights at beginning and end of test period ± SE.

TABLE 3

Effect of two or five daily meals on food consumption, body weight, plasma cholesterol, and body composition (exp. 3) [1]

Lot	Experimental lots [2]		Period 1 [3]			Period 2 [3]					
	Period		Avg food cons./ bird/ day	Body weight		Avg food cons./ bird/ day	Body weight, final	Plasma cholest., final	Body composition		
	1	2		Initial	Final				Fat	Water	Protein (N × 6.25)
			g	*g*	*g*	*g*	*g*	*mg/100 ml*	%	%	%
1A	Nib					54	609 ± 15	106 ± 5	6.7	69.0 ± 6	22.0
2A	Nib	5 meals	31	143 ± 3	276 ± 5	38	489 ± 17	124 ± 6	4.5	70.6 ± 3	22.2
3A		2 meals				23	370 ± 17	156 ± 11	4.0	71.2 ± 1	22.2
1B		Nib				51	586 ± 12	111 ± 5	6.4	69.6 ± 2	21.8
2B	5 meals	5 meals	22	145 ± 4	219 ± 5	42	516 ± 20	123 ± 8	5.2	70.1 ± 3	21.9
3B		2 meals				22	332 ± 18	139 ± 9	3.7	71.6 ± 3	22.0

[1] Arithmetic means; standard errors were calculated for all parameters which are based on data from individual chicks.
[2] Nib: fed ad libitum; 5 or 2 meals: food made available for five or two 1-hour periods daily.
[3] Period 1, 10 days; period 2, 20 days.

significantly ($P < 0.01$) higher blood cholesterol levels than did those on five meals.

The carcasses of the nibblers contained higher levels of fat, balanced by a lower water content (table 3). Average carcass fat content of the six lots was highly correlated with average body weight ($r = 0.966$).

The initial (fasting) blood glucose levels of the meal eating birds were appreciably higher than those of the ad libitum eating controls (table 4). Although differences between treatments decreased in later samplings, the pattern remained essentially the same during the 80-minute glucose tolerance test. All groups showed a glucose peak at the 40-minute sampling time. Analysis of variance gave highly significant F- values for treatments as well as for time of sampling ($P < 0.001$) but no significant interaction between these two sources of variation.

DISCUSSION

Two daily 1-hour meals markedly depressed food consumption and growth of young chickens. Since birds pair-fed with the meal eaters, *without* a time limitation, did not grow better than the meal eaters, it would appear that the results obtained are due to the restricted food intake rather than to the frequency of eating per se. The partial starvation effect of two daily meals manifested itself in considerable mortality (5 of 20 and 4 of 20, respectively, for lots 4 and 5) and in elevated plasma cholesterol levels for the meal eaters and the birds pair-fed with them. Elevated plasma cholesterol levels for birds fed one 2-hour meal daily are also apparent from the data of Leveille and Hanson (8); Cohn et al. (15), who fed a cholesterol-containing diet, also found a much higher blood cholesterol level in birds fed two 1-hour meals daily.

Fewer than five daily 1-hour meals restricted feed consumption significantly below that of control nibblers; we thought, however, that more than five meals daily would begin to simulate the chicken's normal feeding habits (nibbling), and no longer reflect a distinct meal eating pattern. Our results suggest that adaptation to a 5-meal pattern depends upon the age

of the chicken. Five-week-old chickens on five daily meals for 1 week consumed as much as, or at least not significantly less than, their ad libitum-fed controls. The same regimen given to 2-week-old birds did not, however, allow for food consumption and growth equal to that of the nibbling controls. The initial depression in food intake was apparently sufficient to prevent the meal eaters from catching up to the controls after 4 weeks on this regimen. The inability of the younger chickens to adapt in the same way the 5-week-old birds did in experiment 2 may be a reflection of greater intestinal volume capacity, in relation to growth needs, for the latter. It is, nevertheless, probable that the results of experiment 3, at least as far as the birds receiving five meals are concerned, reflect primarily meal eating rather than the effects of partial starvation and slow growth.

The effect on food consumption, growth, and body composition of Leghorn chickens given two 1-hour meals daily was similar to the effect of such a regimen on chickens from a heavier breed (6). The decrease in body fat for meal-fed, growing chickens appears to be in direct contrast with the increased body fat reported for rats on restricted feeding regimens (16). It must be remembered, however, that the rats were force-fed in the latter study, whereas in the present experiments with voluntary food intake, equal body weights and food intake could not be maintained. Even when equal food intake was achieved (experiment 3, table 2) the meal eaters gained more slowly than the nibblers, corroborating the earlier findings of Feigenbaum et al. (6).

There seems little doubt that results of studies on feeding frequency greatly depend on the manner in which they are conducted. In a rat experiment recently carried out in our laboratory, 24 rats on a 2-hour/day feeding regimen gained only half as much, in a 4-week period, as ad libitum-fed rats with initially equal weights, although both groups had been trained for meal eating. There was little overlapping in the final weight distribution of the two groups, and none in the distribution of gains, and the ad libitum-fed animals had 54% more body fat than the controls (table 5). This was not at all in agreement with results obtained by Braun et al. (17). Personal communication with one of the authors revealed, however, that their rats were fed in groups and not, as is our case, in individual cages. Furthermore, a large number of rats was used to permit selection of rats with overlapping weights from

TABLE 4

Effect of two or five daily meals on glucose tolerance (exp. 3)

Experimental lots [1]			Blood glucose (min after dosing)				
Lot	Period 1	Period 2	0	10	20	40	80
			mg/100 ml				
1A	Nib	Nib	108 ± 5 [2]	201 ± 16	307 ± 22	348 ± 23	268 ± 12
2B	5 meals	5 meals	162 ± 8	251 ± 18	326 ± 18	335 ± 16	274 ± 17
3A	Nib	2 meals	164 ± 6	292 ± 17	356 ± 13	367 ± 8	288 ± 18

[1] Nib: fed ad libitum; 5 or 2 meals: food made available for five or two 1-hour feeding periods per day.
[2] Arithmetic means ± SE.

TABLE 5

Effect of feeding frequency on weight and composition of rats [1]

	Feeding method	
	Ad libitum	Restricted [2]
Avg weight at end, g	239 (216–274) [3]	193 (168–234)
Avg gain, g	92 (74–120)	46 (21– 72)
Avg carcass fat, %	4.0 ± 0.14 [4]	2.6 ± 0.14

[1] 24 rats per lot; all were fed one 2-hour meal per day during week preceding experiment.
[2] One 2-hour meal each day during 4-week experimental period.
[3] Arithmetic means and ranges.
[4] Arithmetic means ± SE.

the nibbling and from the meal eating groups.

At this time it is not clear whether there really exists a significant species difference between rats and chickens since varying results have been obtained, even in the rat, depending on the environmental conditions mentioned above as well as on additional factors such as temperature, breed, and even composition of diet.

The significant elevation of plasma cholesterol levels for chickens given only two meals daily was probably partly due to starvation,[2] whereas the elevated levels for birds on five daily meals were, more likely, a direct response to feeding frequency.

Meal eating has been implicated in the susceptibility of alloxanized rats to diabetes (18, 19). The glucose tolerance test has been used in only a few studies related to meal eating (20, 21). The results reported here suggest a possible involvement of feeding frequency with glucose tolerance. Further research in this area will be required to understand fully the importance of the initial elevation of glucose levels in meal-fed growing chickens.

LITERATURE CITED

1. Fabry, P. 1967 Metabolic consequences of the pattern of food intake. In: Handbook of Physiology, section 6, *1:* 31, ed., C. F. Code. Williams & Wilkins Co., Baltimore, Md.
2. Levin, L. 1944 Some effects of increased food consumption on the composition of carcass and liver of hypophysectomized rats. Amer. J. Physiol., *141:* 143.
3. Tepperman, J., J. R. Brobeck and C. N. H. Long 1943 The effects of hypothalamic hyperphagia and of alterations in feeding habits on the metabolism of the albino rat. Yale J. Biol. Med., *15:* 855.
4. Cohn, C. 1963 Feeding frequency and body composition. Ann. N. Y. Acad. Sci., *110:* 395.
5. Cohn, C., R. Pick and L. Katz 1961 Effect of meal eating compared to nibbling upon atherosclerosis in chickens. Circulation Res., *9:* 139.
6. Feigenbaum, A. S., H. Fisher and H. S. Weiss 1962 Effect of "meal eating" versus "nibbling" on body composition and digestive organ weight of normal and cropectomized chickens. Amer. J. Clin. Nutr., *11:* 312.
7. Lepkovsky, S., A. Chari-Bitron, R. M. Lemon, R. C. Ostwald and M. K. Dimick 1960 Metabolic and anatomic adaptations in chickens "trained" to eat their daily food in two hours. Poultry Sci., *39:* 385.
8. Leveille, G. A., and R. W. Hanson 1965 Influence of periodicity of eating in the chicken. Amer. J. Physiol., *209:* 153.
9. Okey, R., G. Scheier and M. Reed 1960 Food restriction and cholesterol metabolism. J. Amer. Diet. Ass., *36:* 441.
10. Leveille, G. A., and E. K. O'Hea 1967 Influence of periodicity of eating on energy metabolism in the rat. J. Nutr., *93:* 541.
11. Fisher, H., and H. S. Weiss 1956 Feed consumption in relation to dietary bulk and energy level: The effect of surgical removal of the crop. Poultry Sci., *35:* 418.
12. Zlatkis, F., B. Zak and A. J. Boyle 1953 A new method for the direct determination of serum cholesterol. J. Lab. Clin. Med., *41:* 486.
13. Hyvärinen, A., and E. A. Nikkilä 1962 Specific determination of blood glucose with O-toluidine. Clin. Chim. Acta, *7:* 140.
14. Feigenbaum, A. S., H. Fisher, G. A. Leveille, H. S. Weiss and P. Griminger 1961 The polyunsaturated fatty acids and cholesterol concentrations of plasma and aorta and their relationship to avian atherosclerosis. J. Amer. Oil Chem. Soc., 38: 93.
15. Cohn, C., R. Pick and L. N. Katz 1961 Effect of meal eating compared to nibbling upon atherosclerosis in chickens. Circulation Res., *9:* 139.
16. Cohn, C., and D. Joseph 1959 Changes in body composition attendant on force feeding. Amer. J. Physiol., *196:* 965.
17. Braun, T., M. Kohout and P. Fabry 1967 The effect of feeding frequency on fatty acid composition of rat adipose tissue. Can. J. Biochem., *45:* 1470.
18. Cohn, C., and D. Joseph 1960 Comparison of "meal eating" and "nibbling" on severity of alloxan diabetes. Proc. Soc. Exp. Biol. Med., *105:* 575.
19. Houssay, B. A., and C. Martinez 1947 Experimental diabetes and diet. Science, *105:* 548.
20. Gwinup, G., R. C. Byron, W. Roush, F. Kruger and G. J. Hamwi 1963 Effect of nibbling versus gorging on glucose tolerance. Lancet, 2: 165.
21. Fabry, P., J. Fodor, Z. Hejl, T. Braun and K. Zvolankova 1964 The frequency of meals — its relation to overweight, hypercholesterolaemia, and decreased glucose tolerance. Lancet, 2: 614.

[2] Yacowitz, H., S. G. Kahn, S. Wind and B. Amrein 1957 Hypercholesterolemia of starvation as related to changes in plasma volume. Circulation *16:* 485 (abstract). These authors observed this phenomenon in the chicken; similar observations for man, rabbit, and rat have also been published.

Effect of Coprophagy on Digestion and Mineral Excretion in the Guinea Pig

H. F. HINTZ
Equine Research Program, Cornell University, Ithaca, New York

ABSTRACT The prevention of coprophagy decreased the apparent digestibility of dry matter, organic matter, crude protein, ether extract and acid detergent fiber when guinea pigs were fed alfalfa meal or a semipurified diet. The feces of guinea pigs fed the semipurified diet were analyzed for eight minerals by mass spectroscopy, and the prevention of coprophagy increased the amount of minerals appearing in the feces. The addition of 250 ppm molybdenum to the semipurified diet did not increase the amount of copper in the feces. It was concluded that coprophagy is important in the utilization of nutrients by the guinea pig and that coprophagy must be considered in digestion trials with any rodents.

The importance of coprophagy in the nutrition of the rat and rabbit has received considerable study but little work has been conducted with guinea pigs. For example, coprophagy is not mentioned by Reid (1) in her review of the nutrition of the guinea pig, nor is the guinea pig discussed in a recent review dealing with coprophagy (2). Therefore, the following trials were initiated to study the effect of coprophagy on digestion and mineral excretion in the guinea pig.

EXPERIMENTAL

Trial 1. The digestibility of pelleted alfalfa meal was determined with five male guinea pigs, 3 months old and weighing 320 to 400 g, first without and then with the prevention of coprophagy. Coprophagy was prevented by taping four wooden tongue depressors lengthwise to the body to prevent bending of the spine. The animals were housed in metabolism cages which permitted separation of urine and feces. Preliminary periods of 10 days of constant diet intake and fecal collection periods of 7 days were used. The animals were fed 30 g of alfalfa meal daily and distilled water was given ad libitum. Approximately 300 mg of ascorbic acid were added daily to the drinking water. The feeds and feces were analyzed for dry matter, crude protein, ether extract and ash according to methods described by AOAC (3). Acid detergent fiber (ADF) was determined according to the method described by Van Soest (4). ADF, which estimates the ligno-cellulose content was used rather than the more conventional crude fiber because it gives a more accurate representation of the total fibrous matter (5). The data were statistically evaluated by means of the *t* test for paired observations (6).

Trial 2. Sixteen male guinea pigs weighing 200 to 300 g were used to study the effect of coprophagy on digestion and mineral excretion when fed a semipurified diet [1] with or without 250 ppm molybdenum as sodium molybdate. Molybdenum was added as a variable because it is known to interfere with the utilization of copper in the guinea pig (7) and it was thought there might be a molybdenum-coprophagy interaction. Food intake was restricted to 5 g/100 g of body weight per day. Approximately 250 mg of ascorbic acid were added daily to the drinking water. The experimental design was a 4 × 4 latin square replicated 4 times; however, period 4 was omitted. The prevention of coprophagy and collection of feces and urine were accomplished in the manner described for trial 1. Preliminary periods of 10 days and collection periods of 7 days were used. Distilled water was given ad libitum. Pooled fecal samples

Received for publication May 21, 1969.

[1] The composition of the diet was: (in percent) dried skim milk, 30; cornstarch, 30; Solka Floc, 15; casein, 10; glucose, 7; Torula yeast, 5; corn oil, 3; and (in ppm) Fe (as $FeSO_4$), 100; Mg (as $MgSO_4$), 800; Zn (as $ZnCO_3$), 20; Mn (as $MnSO_4 \cdot H_2O$), 40; Cu (as Cu acetate), 10; and (in IU/kg diet) vitamin A (as vitamin A palmitate), 2000; vitamin D_3, 200.

were analyzed for minerals by mass spectroscopy.

RESULTS AND DISCUSSION

Trial 1. The guinea pigs consumed 29.5 g/day and 29.0 g/day when coprophagy was allowed and prevented, respectively. The prevention of coprophagy significantly decreased digestibility of dry matter, organic matter, protein, ether extract and ADF of alfalfa meal (table 1). The amount of ash recovered in the feces of guinea pigs that practiced coprophagy was 33.8% of that in the feed compared to 42.7% when coprophagy was prevented.

Trial 2. One animal died in each of the three periods and two animals removed the tape during the collection period. Period 4 was omitted because the differences between treatments observed in the first 3 periods were so large that a further period seemed unnecessary. Therefore, there was a total of 39 observations. As in trial 1, the prevention of coprophagy significantly decreased digestibility of dry matter, protein and ADF (table 2). ADF is primarily cellulose and lignin. The diet in trial 2 contained 0.5% lignin but the alfalfa meal contained 6.9% lignin and, therefore, the lignin fraction of ADF in the diet in trial 1 probably caused most of the large difference in digestibility of ADF between trials 1 and 2. When coprophagy was prevented the guinea pigs lost 3.2 g/day, but when coprophagy was allowed they gained 0.7 g/day. Molybdenum did not affect gain.

The prevention of coprophagy also significantly increased the amount of minerals excreted in the feces (table 3). Prevention of coprophagy appears to be a method of inducing mineral deficiency, particularly of zinc and copper because the amount of these minerals recovered in the feces was almost twice the amount in the feed. Thus the guinea pigs were apparently less efficient in the recovery of endogenous minerals when coprophagy was prevented. The mineral content of the feces may also have been increased, however, as a consequence of weight loss associated with the prevention of coprophagy rather than entirely due to the prevention of coprophagy per se. The addition of molybdenum appeared to decrease the amount of minerals, except zinc, present

TABLE 1

Effect of coprophagy on apparent digestion of dehydrated alfalfa meal by guinea pigs

Component	Composition of alfalfa	Apparent digestibility		SD of mean difference	P
		Coprophagy allowed	Coprophagy prevented		
	%	%	%	%	
Dry matter	90.8	53.5	45.5	1.97	< 0.02
Organic matter	81.2	52.6	45.8	1.40	< 0.01
Crude protein	15.2	59.1	44.9	1.77	< 0.01
Ether extract	3.7	55.3	36.2	1.51	< 0.001
ADF	34.3	32.6	22.3	1.30	< 0.01

TABLE 2

Effect of coprophagy and molybdenum on apparent digestion of a semipurified diet by guinea pigs

Component	Composition of diet	Apparent digestibility			
		Coprophagy		No coprophagy	
		Basal (11) [1]	250 ppm Mo (11)	Basal (10)	250 ppm Mo (7)
	%	%	%	%	%
Dry matter	90.5	94.4 ± 1.08 [2]	94.8 ± 0.86	85.4 ± 1.90	86.8 ± 1.07
Crude protein	21.3	91.1 ± 1.29	92.3 ± 0.97	78.4 ± 2.63	80.4 ± 1.87
Ether extract	3.5	98.2 ± 1.05	97.0 ± 0.80	91.0 ± 1.45	90.0 ± 1.05
ADF	15.5	88.2 ± 1.80	89.1 ± 2.42	63.2 ± 4.86	66.8 ± 3.16

[1] Number in parentheses is number of observations.
[2] Mean ± SE.

TABLE 3

Effect of coprophagy on fecal mineral excretion [1]

Mineral	Coprophagy allowed		Coprophagy prevented	
	Basal	250 ppm Mo	Basal	250 ppm Mo
	%	%	%	%
P	13.8	13.6	35.6	27.5
Ca	4.9	4.8	13.3	8.8
K	9.0	8.0	22.7	16.5
Mg	6.4	6.8	18.4	14.1
Zn	80.7	67.2	174.3	189.6
Mn	44.7	42.7	106.0	92.2
Cu	82.3	75.3	176.2	164.9
Fe	24.0	27.7	71.1	40.2

[1] Values obtained with pooled samples and expressed in % excretion (amount in feces/amount in feed, × 100).

in the feces when coprophagy was prevented but not when coprophagy was allowed. The addition of molybdenum did not increase the amount of copper appearing in the feces which suggests that the level of molybdenum used in these trials does not interfere with copper absorption in the guinea pig. Mills (8) suggested that molybdenum impaired copper absorption in ruminants but not in rats.

The effect of prevention of coprophagy on apparent digestion of dry matter, protein and fiber was similar to results obtained with rabbits (9). Tadayyon and Lutwak (10) also reported that the prevention of coprophagy decreased the apparent digestibility of calcium, magnesium and phosphorus in rats.

The results demonstrate that coprophagy is important in the absorption of dietary nutrients and probably enables the guinea pig to utilize some B vitamins and amino acid synthesized by the microflora of the lower gut (11). Howard (12) stated that, as far as is known, coprophagy occurs only in rats and rabbits and possibly in poultry; it has, however, been observed in hamsters[2] and mountain beavers (13), and probably all rodents practice it (14). Coprophagy has also been observed in young foals (15), dogs (16) and some primates (17). Thus, coprophagy may be more important than previously recognized and must be considered in digestion trials.

LITERATURE CITED

1. Reid, M. E. 1962 Nutrient requirements of the guinea pig. In: Nutrient Requirements of Laboratory Animals. NAS-NRC, pub. 990.
2. Hotzel, D., and R. H. Barnes 1966 Contributions of the intestinal microflora to the nutrition of the host. Vitamins Hormones, *24:* 115.
3. A.O.A.C. 1960 Official Methods of Analysis, 9th ed., Assoc. Offic. Agr. Chem., Washington, D. C.
4. Van Soest, P. J. 1963 Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. J. Assoc. Offic. Agr. Chem., *46:* 829.
5. Fonnesbeck, P. V. 1969 Partitioning the nutrients of forage for horses. J. Anim. Sci., *28:* 624.
6. Steel, R.G.D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.
7. Arthur, D. 1965 Interrelationships of molybdenum and copper in the diet of the guinea pig. J. Nutr., *87:* 69.
8. Mills, C. F. 1964 Metabolic interrelationships in the utilization of trace elements. Proc. Nutr. Soc., *23:* 38.
9. Thacker, E. J., and C. Brandt 1955 Coprophagy in the rabbit. J. Nutr., *55:* 375.
10. Tadayyon, B., and L. Lutwak 1969 Role of coprophagy in utilization of triglycerides, calcium, magnesium and phosphorus in the rat. J. Nutr., *97:* 243.
11. Kellner, W. 1956 Untersuchungen zur symbiontischen Bedeutung der Blinddarmflora der Nagetiere. Z. Morphol. Oekol. Tiere, *44:* 518.
12. Howard, B. H. 1967 Intestinal microorganisms of ruminants and other vertebrates. In: Symbiosis, vol. 2, ed., S. M. Henry. Academic Press, New York.
13. Ingles, L. G. 1961 Reingestion in the mountain beaver. J. Mammalogy, *42:* 411.
14. Frank, I., Hadeler, U. und W. Harder 1951 Zur Ernaehrungsphysiologie der Nagetiere. Über die Bedeutung der Coecotropie und die

[2] H. F. Hintz, personal observation.

Zusammensetzung der Coecotrophe. Pfluegers Arch., 253: 173.
15. Russell, A. 1948 The development of helminthiasis in Thoroughbred foals. J. Comp. Pathol., 58: 107.
16. Fox, M. W. 1965 Canine Behavior. Charles C Thomas, Springfield, Illinois.
17. Oxnard, C. E. 1964 Some variations in the amount of vitamin B_{12} in the serum of the rhesus monkey. Nature, 201: 1188.

Effect of Iron on Growth, Cytochromes, Glycogen and Fatty Acids of *Tetrahymena pyriformis* [1,2]

AUSTIN L. SHUG, CHARLES ELSON AND EARL SHRAGO
Veterans Administration Hospital, and Departments of Nutritional Sciences and Medicine, University of Wisconsin, Madison, Wisconsin

ABSTRACT Although the iron concentration in 2% proteose-peptone was found to be about the same as in chemically defined media, the addition of inorganic iron salts produced a dramatic acceleration of growth and marked alterations in the metabolism of the ciliated protozoan, *Tetrahymena pyriformis*. In well aerated cultures, iron supplementation resulted in marked increases in pyridine hemochromogen concentration, conversion of heme to cytochrome, glycogen concentration, degree of unsaturation of fatty acids and cell population.

The ciliated protozoan *Tetrahymena pyriformis* bears a striking resemblance to mammals in certain aspects of its metabolism and nutritional requirements. The recent discovery that this unicellular organism contains an adrenergic system (1), and the isolation of epinephrine (2) and serotonin (3) from the organism, indicate that it would make a promising model system for the study of metabolic controls in higher mammals. It has been used to study protein quality (4) and the literature is replete with references (5) indicating the validity of using *Tetrahymena* as a model for the study of nutritional interrelationships in mammals.

Under conditions of carbohydrate deprivation, the organism is highly glyconeogenic and can synthesize up to 23% of its dry weight in glycogen (6). During this process there is a net conversion of lipid to carbohydrate by way of an active glyoxalate cycle (7). By varying the constituents of the growth medium one can simulate in *Tetrahymena* a condition representative of diabetes in higher animals. An important observation was the finding that glyconeogenesis was facilitated by poor aeration or relative anaerobiosis of the growth medium (8), and studies were therefore initiated to determine the relationship of the electron transport system to glyconeogenesis (9, 10). As part of this investigation, inorganic iron salts were added to a basic 2% proteose-peptone medium in attempts to increase the synthesis of cytochromes. An unsupplemented proteose-peptone medium supports good growth and is widely used for experimental purposes with *Tetrahymena*. It was therefore surprising to observe certain qualitative changes in the metabolism as well as a general enhancement of growth of the organism. These metabolic changes do not appear to be simply a reflection of accelerated growth. The present communication is a detailed report of the effects of iron on the overall metabolism of *Tetrahymena*.

MATERIALS AND METHODS

T. pyriformis, strain E, was maintained on 2% sterile proteose-peptone (Difco) medium prepared with distilled deionized water.[3] In most studies, 400 ml of medium in 2-liter Erlenmeyer flasks or 100 ml of medium in 500-ml Erlenmeyer flasks were used. Additional constituents were added aseptically. Experimental cultures were started with a 1% inoculum of log-phase cells and grown at 25° under good aeration by shaking on a New Brunswick gyrorotary shaker at 150 rpm. Unless otherwise stated, well-aerated cultures were used. Poorly aerated standing cultures at 25° were grown in 500-ml Erlenmeyer flasks containing 250 ml of medium. The cells were harvested by sedimentation at 600 × *g* in a

Received for publication June 30, 1969.
[1] Supported in part by Public Health Service Grant no. GM-14033.
[2] This manuscript is approved for publication by the Director of the Research Division of the College of Agricultural and Life Sciences, University of Wisconsin.
[3] Continental Water Company, Chicago, Ill.

refrigerated International PR-2 centrifuge. The packed cells were washed twice and taken up in 0.15 M NaCl.

Assay procedures. Growth was measured turbidimetrically with a Bausch and Lomb spectrophotometer at 660 mμ. Aliquots were taken for cell counting and sizing with a Model B Coulter Counter calibrated with ragweed pollen. Dry weight determinations were made on aliquots of washed cell suspension dried to constant weight. A protein fraction was prepared from an aliquot of cells which had been precipitated with an equal volume of 0.6 M perchloric acid. The precipitate was washed consecutively with hot 95% ethanol, a 3:1 ethanol–ether mixture, hot 0.6 M perchloric acid, 95% ethanol, and finally taken up in 0.1 N NaOH for protein determination by the biuret method (11). For glycogen determinations an aliquot of cells was made to 30% with respect to KOH, saponified for 15 minutes in a boiling water bath, and centrifuged to remove insoluble material. The precipitate was washed once with 30% KOH and an equal volume of 95% EtOH was added to the pooled supernatant fractions. The precipitate was washed twice with 60% EtOH, dried, and taken up in a known volume of H_2O. Glycogen was analyzed by the anthrone procedure (12).

The incorporation of 2-^{14}C-acetate into lipids and glycogen was determined after 24 and 48 hours of growth. Fifty-milliliter aliquots of the cultures were incubated an additional 2 hours with 50 mg Na acetate and 1.0 μCi 2-^{14}C-acetate. The cells were cooled, centrifuged, and washed three times with distilled water. The washed cells were homogenized and diluted with 20 volumes chloroform–methanol (2:1). After centrifugation, the supernate was thoroughly mixed with 20% of its volume of a pure solvent upper phase salt solution (13) and allowed to separate by standing overnight. The upper phase was removed and the lower phase was again washed. The chloroform layer was collected and evaporated to dryness. The residue was taken up in a small volume of chloroform for counting. The precipitate from the homogenate was washed twice with 10% trichloroacetic acid. The pooled washes were diluted with 95% ethanol to a final concentration of 66%. The precipitated glycogen was washed three times with 60% ethanol, air dried, and dissolved in water for counting.

In other experiments, the lipid residues were either separated by thin-layer chromatographic techniques into a phospholipid fraction, a glyceride fraction and a free acid fraction, or converted to methyl esters by the methanol–sulfuric acid procedure of Bowles et al. (14). Following thin-layer chromatography on Eastman Chromagram silica gel sheets with petroleum ether–diethyl ether–acetic acid system (85:15:1), the fractions were made visible using iodine vapors and the fractions corresponding to standards were cut from the plate and placed in scintillation counting vials containing Packard "Permablend" scintillation mixture. All radioactive samples were counted using a Packard Tri-carb Spectrometer equipped with an Automatic External Standardization device. Counts were corrected for background and quenching.

The fatty acid methyl esters were chromatographed using an F and M Model 400 gas chromatograph equipped with ⅛ inch by 6 ft. glass U column packed with 17% diethylene glycol succinate polyester on 60-80 mesh Chromosorb W. The instrument was operated isothermally at 165°. The peaks on the chromatogram were identified by comparing their retention times with the retention times of fatty acid methyl esters in NIH mixtures B and D. Semilog plots of retention time and carbon number were used to determine equivalent chain lengths (15) of acids that were not identified by comparison with standards. The acids identified were the same as those identified by Lees and Korn (16). The linearity of response of the flame ionization detector was ascertained by injection of a series of dilutions of NIH Mixtures B and D. Quantitative results obtained by determining the peak areas (height × width at half height) agreed to within 1% with the stated values.

Inorganic iron was determined by the method of Henry et al. (17), and iron-binding capacity by that of Ramsay (18). Hemin was isolated from an aliquot of cells with 12 volumes of an extraction solvent containing one part 2% strontium chloride hexahydrate in concentrated glacial acetic

acid and three parts acetone, according to Labbe and Nishida (19). For isolation of radioactive hemin from 2-^{14}C-acetate, 20 mg of carrier hemin was added to the filtered extract. Precipitation was induced by evaporation of the acetone and the precipitate was washed twice with 50% acetic acid and once with ethanol and diethyl ether. The material was then plated and counted with a Nuclear-Chicago gas-flow counter. Mitochondria were prepared according to Kobayashi (20), and hemoproteins determined quantitatively as pyridine hemochromogens (21). Spectra were plotted with a Carey model 11 recording spectrophotometer.

RESULTS AND DISCUSSION

An unsupplemented 2% proteose-peptone broth supports good growth and is the most common medium used in studies of *Tetrahymena*. Figure 1 illustrates the stimulation of the growth pattern by addition of 5 μg ferric chloride ($FeCl_3 \cdot 6H_2O$) per milliliter of proteose-peptone broth. There is little gross effect of iron during the initial 24 hours of growth. The generation time has been shortened by 10 minutes ($G.T._{Fe}$, 230 minutes; $G.T._c$, 240 minutes). By 48 hours there is a considerable increase in cell population in the iron-fortified medium which reaches a peak by 60 hours and then decreases with cell death. The median size of the iron-grown cells is 14% greater than the control cells. It thus appears that proteose-peptone does not contain enough iron to ensure optimum growth of *Tetrahymena*. Analysis of the 2% proteose-peptone broth indicated the iron concentration was 0.42 μg/ml, a value very close to that specified in chemically defined media for this organism. The optimum iron concentration in defined media is 0.5 μg/ml (22). The unsaturated iron binding capacity of proteose-peptone was also determined and found to be 0.21 μg/ml. It appears that the total iron binding capacity (0.6 μg/ml) of proteose-pep-

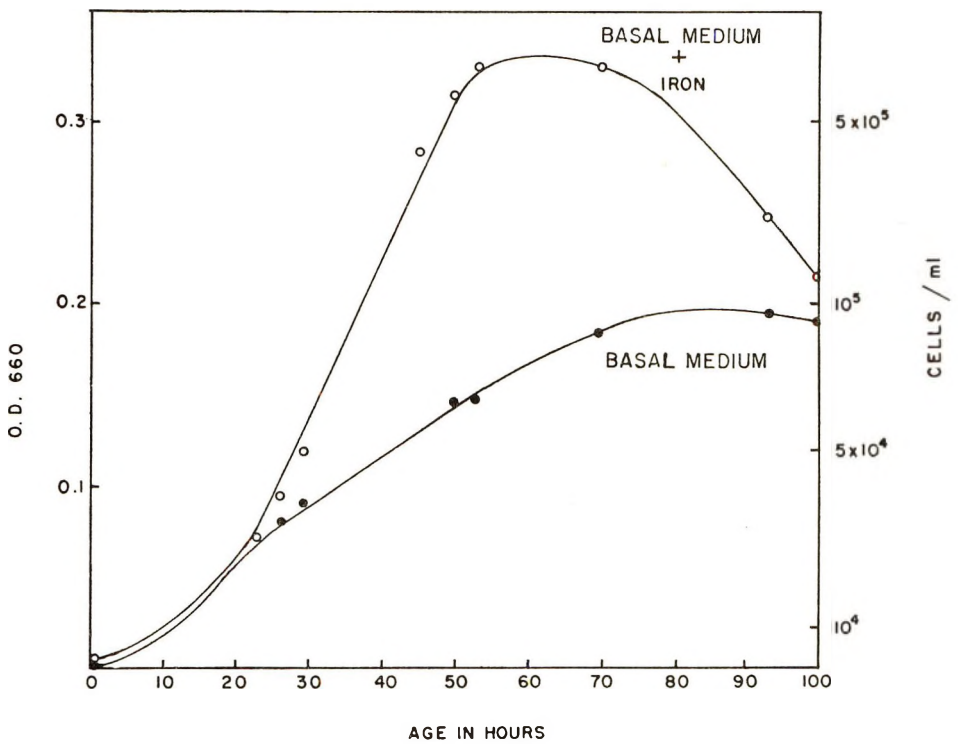

Fig. 1 Comparison of growth in iron and non-iron-grown cultures. $FeCl_3 \cdot 6H_2O$ added at 5 μg/ml medium — a concentration previously noted to give optimum growth.

tone medium is sufficient to bind all the potentially free iron and thereby make it less available to the cell.

The uptake of iron by the cell culture at 48 hours is linear up to at least 5 μg $FeCl_3 \cdot 6H_2O$/ml (fig. 2). Growth, as measured by dry weight, and synthesis of glycogen appear to be near maximum at a concentration of 2 μg $FeCl_3 \cdot 6H_2O$/ml. Considering that iron makes up 20% of $FeCl_3 \cdot 6H_2O$, 2 μg ferric chloride contains 0.4 μg iron of which 0.2 μg could be expected to be protein bound. The remainder would be more freely available to the cell. As the proteose-peptone broth is utilized, more iron also becomes available to the organisms in the supplemented medium.

The effect of iron on the growth pattern characteristics was examined in more detail (fig. 3). The maximum effect on dry weight, protein and glycogen formation occurs by the third day and subsequent decreases indicate that the culture has reached the stationary phase with the onset of accelerated cell death. By contrast, the culture without additional iron grows much slower and synthesizes considerably less protein and glycogen.

The large accumulation of glycogen in *Tetrahymena* is characteristic of the organism in the stationary phase of growth and reflects an active glyconeogenic capacity. On a basal medium the glycogen level can reach 23% of the dry weight of the culture in the late stationary phase of growth (6). In figure 4 the amount is approximately 17% at 4 days of growth under aerated conditions. The striking increase in the level of glycogen in the iron-grown cultures (37%) is somewhat unexpected because an amount of this magnitude has not been observed unless the medium is supplemented with glucose. The calculations give percentages of dry weight and, therefore, the increased glycogen is not merely a reflection of accelerated growth but must represent some qualitative metabolic change in the organism. This change is shown in table 1 by the high rate of incorporation of 2-^{14}C-acetate into glycogen of iron-supplemented cells after 48 hours of growth. This rate is not

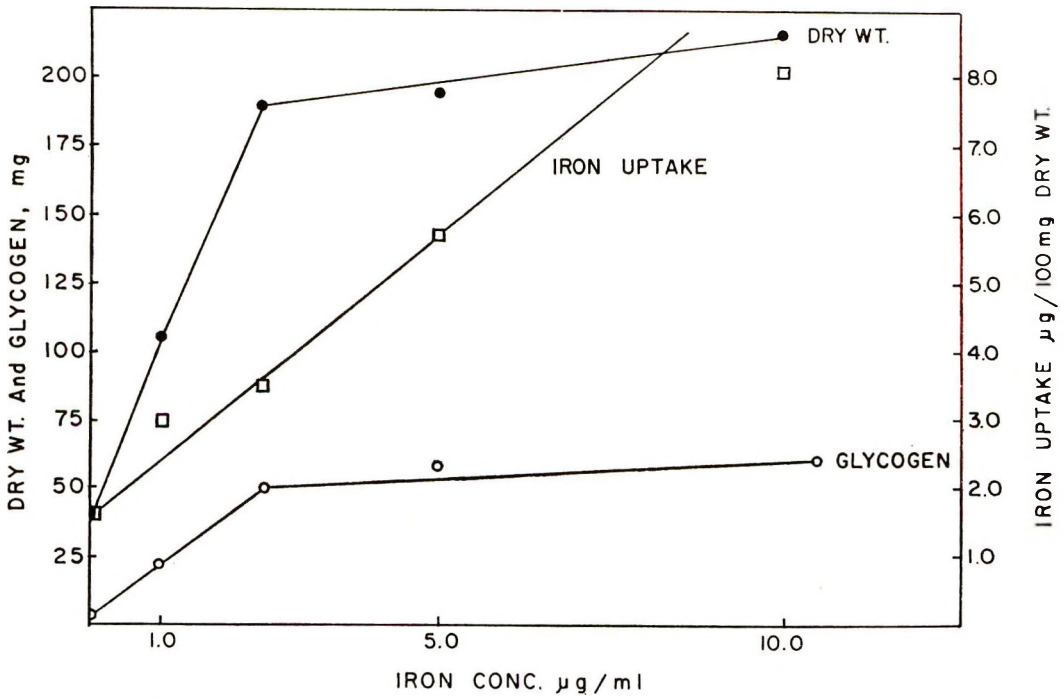

Fig. 2 Relationship of dry weight, glycogen formation, and Fe accumulation to $FeCl_3 \cdot 6H_2O$ concentration in the medium.

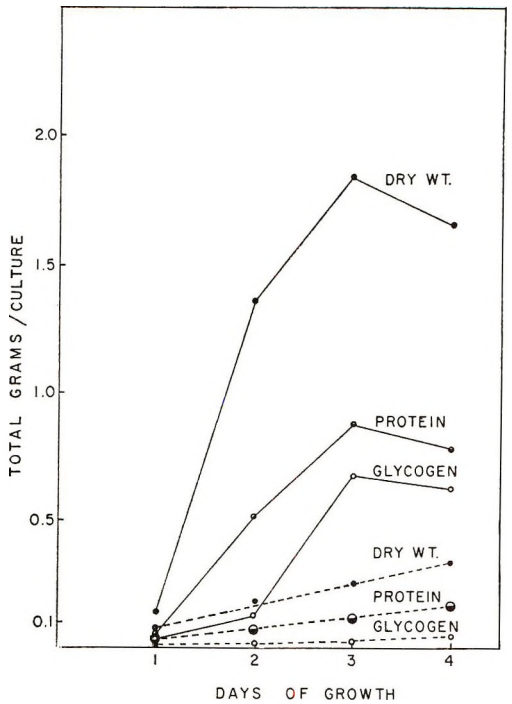

Fig. 3 Changes in various metabolic parameters during the growth cycle with and without addition of 1 μg Fe/ml. With Fe, ●——●——●; without Fe. ●- - - -●- - - -●.

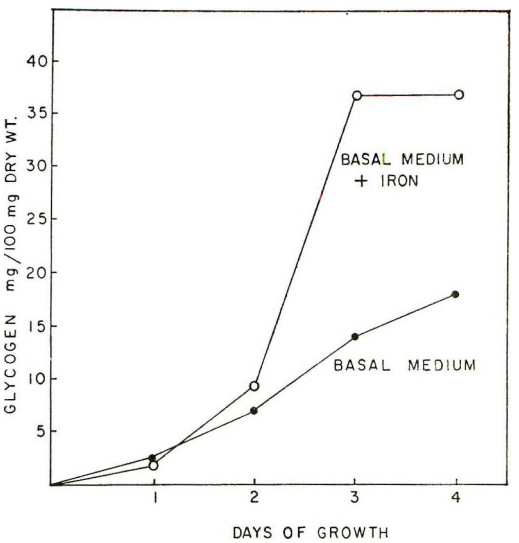

Fig. 4 Effect of 1 μg Fe/ml on glycogen synthesis.

reached by the control cells at any point of the growth cycle.

A marked effect of iron on the desaturation of fatty acids was also observed in *Tetrahymena*. As illustrated in table 2 the relative content of γ-linolenic acid is increased. The change appears to be due to a conversion of linoleic to γ-linolenic acid which apparently proceeds at a more rapid rate in iron grown cells. If chain length is ignored, little change is noted in the content of the monounsaturated fatty acids. Chain elongation also proceeds at a greater rate in iron-grown cells. Table 3 shows that cells grown in iron-fortified cultures maintain a greater relative quantity of γ-linolenic acid well into the stationary phase.

The increased ratio of polyunsaturated fatty acids in iron-grown cells suggests a general increase in the formation of phospholipids. After 44 hours of growth, ^{14}C-palmitic and ^{14}C-linoleic acid were added to control and iron-supplemented cultures for 4 hours. Table 4 shows that proportionately more of the labeled fatty acids are incorporated into the phospholipid fraction and less into the triglyceride fraction in the iron grown cells, compared with the controls. It has been noted that there is a decrease in lipid content of aging *Tetrahymena* cultures with a net conversion of lipids to carbohydrates via an active glyoxylate cycle (23). At cell populations

TABLE 1

Incorporation of 2-^{14}C-acetate after 24 and 48 hours into lipids and glycogen by Tetrahymena pyriformis cells [1]

	24 hr		48 hr	
	Lipid	Glycogen	Lipid	Glycogen
	cpm	*cpm*	*cpm*	*cpm*
Control	3580	30	10730	140
Fe [2]	2930	80	29030	24900

[1] Experimental procedures given in text.
[2] 1 μg Fe/ml.

TABLE 2

Effect of iron on the fatty acid patterns of Tetrahymena pyriformis lipids at 48 hours

Acid	Total lipids control (8) [1]	Iron [2] (7)
$C_{14:0}$	10.1 ± 0.83 [3]	7.4 ± 0.53
$C_{14:1}$	3.7 ± 0.44	2.3 ± 0.19
$C_{16:0}$	12.8 ± 1.01	7.8 ± 0.24
$C_{16:1}$	15.6 ± 0.98	12.2 ± 0.45
$C_{18:0}$	4.4 ± 0.98	4.0 ± 0.28
$C_{18:1}$	8.9 ± 0.40	14.4 ± 1.00
$C_{19:0}$	5.3 ± 0.44	9.9 ± 0.52
$C_{18:2}$	18.4 ± 1.04	14.1 ± 0.49
$C_{18:3}$	20.7 ± 1.21	28.0 ± 0.77

[1] Number of determinations in parentheses.
[2] 1 μg Fe/ml.
[3] SEM.

TABLE 3

Ratio of linoleic to linolenic acid in Tetrahymena pyriformis lipids

Time	Control	Iron [1]	
hr			
24	0.94(4) [2]	0.70(2)	
48	1.06 ± 0.09 [3](16)	0.55 ± 0.03(11)	$P < 0.005$
72	1.41(4)	0.52(2)	
96	1.11(2)	0.66(2)	

[1] 1 μg Fe/ml.
[2] Number of determinations in parentheses.
[3] SEM.

TABLE 4

Incorporation of ^{14}C fatty acids into lipid fractions of Tetrahymena pyriformis at 48 hours

	% Distribution		
	PL	FFA	TG
Control + palmitic acid	34.8	5.1	60.1
Control + linoleic acid	38.1	8.7	53.2
Fe [1] + palmitic acid	55.5	19.1	25.2
Fe + linoleic acid	48.5	26.4	25.2

[1] 1 μg Fe/ml.

between 1×10^5 and 5×10^5 cells/ml, the organisms enter a circadian pattern of cell division (24). At 48 hours, the iron-grown cultures have reached the population while the control cells do not achieve this population level until 80 hours. Although there is a greater total incorporation of ^{14}C-acetate into heme by the iron-grown cells, the incorporation per milligram protein proceeds at nearly the same rate. Of interest is the rapid decrease in the ^{14}C from 2-^{14}C-acetate in heme after 48 hours of growth in the iron cells (fig. 5). This precedes the rapid increase in glycogen concentration in these

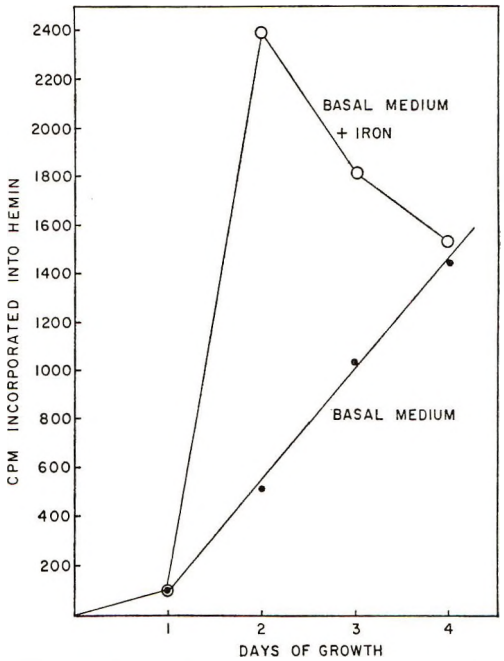

Fig. 5 Effect of 1 μg Fe/ml on incorporation of 2-^{14}C-acetate into hemin.

cells (fig. 3). The heme ^{14}C continued to increase in the control cells throughout a 120-hour period.

Figure 6 shows the concentration of pyridine hemochromogens per milligram protein in iron-grown cells at 48 hours when the cells have completed the log-phase of growth, and in control cells at 72 hours when they have reached the same point in the growth cycle. The iron-grown cells contain a threefold greater concentration of pyridine hemochromogens. There is a marked increase in the components of iron-grown cells corresponding to cytochromes *b* and c (556 mμ) and *a* (587 mμ). The presence of a pyridine hemochromogen corresponding to cytochrome *a* confirms an earlier report (1) that *Tetrahymena pyriformis* contains an a-type cytochrome oxidase. It becomes apparent that the heme synthesized by the iron-grown cells is more efficiently converted to pyridine hemochromogens. A primary effect of iron supplementation could be an increased concentration or activity of certain heme enzymes, particularly in the electron transport chain. The subsequent formation of more ATP

would in turn stimulate the growth and glycogen synthesis of the organism.

An effect of iron supplementation on the growth of standing cultures of *Tetrahymena* has been reported (25). Because standing cultures grow more slowly due to poor aeration, a comparison of effects of iron was made in standing (table 5) and shaking well-aerated cultures (fig. 2). The effect of iron on the dry weight, protein and glycogen content of standing cells is considerably less than its effect on shaking well-aerated cells. This is consistent with the results that the predominant iron effect is related to the mitochondrial enzymes which in turn are dependent upon oxygen availability.

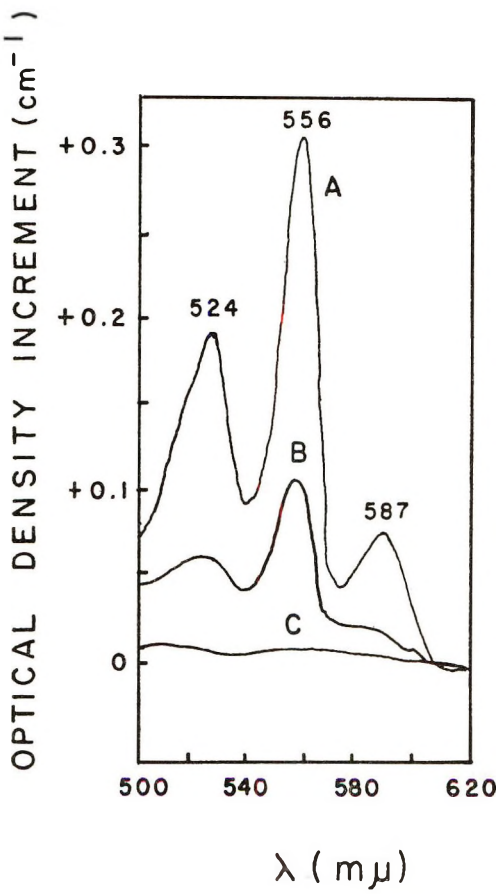

Fig. 6 Comparison of pyridine hemachromogen spectra in Fe (1 μg Fe/ml) and non-Fe-grown cells. A), Fe-grown cells (reduced-oxidized); B), Non-Fe-grown cells (reduced-oxidized); C), Baseline (oxidized-oxidized).

TABLE 5

Effect of iron on Tetrahymena grown under standing conditions for 48 hours

$FeCl_3 \cdot 6H_2O$	Dry wt	Protein	Glycogen	
μg/ml	*mg*	*mg*	*mg*	*% dry wt*
None	67	51	11	16
4.0	116	96	26	22
10.0	101	81	16	16

A similar type of growth response could not be elicited by the addition of other divalent cations such as cobalt and copper. Enrichment of the proteose-peptone medium with yeast extract, glucose or acetate, or a combination of these, is known to increase growth, but the quantitative effects of additional iron appear to be unique. The unusually high glycogen content (37% dry weight) is not apparent during any phase of growth without additional iron. The highest value reported for cells not grown on glucose was approximately 23% of the dry weight (6).

The complete explanation of the iron effect is not known at this time. Presumably, iron permits maximum synthesis of the cytochrome chain in the electron transport system as well as nonheme iron components of other enzymes. An important nonheme iron-containing enzyme acts in the desaturation of fatty acids. The increased proportion of linolenic acid in the iron-grown cells indicates that this enzyme may be involved.

LITERATURE CITED

1. Blum, J. J. 1967 An adrenergic control system in *Tetrahymena*. Proc. Nat. Acad. Sci., *58:* 81.
2. Janakidevi, K., V. C. Dewey and G. W. Kidder 1966 The biosynthesis of catecholamines in two genera of protozoa. J. Biol. Chem., *241:* 2576.
3. Janakidevi, K., V. C. Dewey and G. W. Kidder 1966 Serotonin in protozoa. Arch. Biochem. Biophys., *113:* 758.
4. Pilcher, H. L., and H. H. Williams 1954 Microbiological evaluation of protein quality. II. Studies of the responses of *Tetrahymena pyriformis* W to intact proteins. J. Nutr., *53:* 589.
5. Hutner, S. H., and L. Provasoli 1965 Comparative physiology: Nutrition. Annu. Rev. Physiol., *27:* 19.
6. Manners, D. J., and J. F. Ryley 1952 Studies on the metabolism of the protozoan: II. The glycogen of the ciliate *Tetrahymena pyriformis*. Biochem. J., *52:* 480.

7. Hogg, J. F., and H. L. Kornberg 1963 The metabolism of C_2-compounds in micro-organisms. 9. Role of the glyoxylate cycle in protozoal glyconeogenesis. Biochem. J., 86: 462.
8. Levy, M. R., and O. H. Scherbaum 1965 Glyconeogenesis in growing and non-growing cultures of *Tetrahymena*. J. Gen. Microbiol., 38: 221.
9. Shrago, E., W. Brech and K. Templeton 1967 Glyconeogenesis in *Tetrahymena pyriformis*. Relationship of enzyme adaption to the carbon pathway. J. Biol. Chem., 242: 4060.
10. Shug, A. L., S. Ferguson and E. Shrago 1968 Stimulation of growth and metabolism in *Tetrahymena pyriformis* by antimycin. Biochem. Biophys. Res. Commun., 32: 81.
11. Gornall, A. G., C. S. Bardawell and M. M. David 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177*: 751.
12. Morris, D. L. 1948 Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science, 48: 254.
13. Folch, J., M. Lees and G. H. Sloane Stanley 1957 A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., 226: 497.
14. Bowles, C. Y., J. G. Hamilton, J. E. Muldrey, W. T. Miyamasu, G. A. Reynolds and N. Schalley 1966 The simultaneous determination of both the quantity and the fatty acid composition of the triglycerides in three to ten microliters of plasma. J. Amer. Oil Chem. Soc., 43: 2.
15. Hofstetter, H. H., N. Sen and R. T. Holman 1965 Characteristics of unsaturated fatty acids by gas-liquid chromatography. J. Amer. Oil Chem. Soc., 42: 537.
16. Lees, A. M., and E. D. Korn 1966 Metabolism of unsaturated fatty acids in protozoa. Biochemistry, 5: 1475.
17. Henry, R. J., C. Sobel and N. Chiamori 1958 The determination of serum iron and iron-binding capacity. Clin. Chim. Acta, 3: 523.
18. Ramsay, W. N. M. 1957 Determination of total iron-binding capacity of serum. Clin. Chim. Acta, 2: 221.
19. Labbe, R. F., and G. Nishida 1957 A new method of hemin isolation. Biochim. Biophys. Acta, 26: 437.
20. Kobayashi, S. 1965 Preparation and properties of mitochondria from the ciliated protozoan, *Tetrahymena*. J. Biochem., 58: 444.
21. Rieske, J. S. 1967 The quantitative determination of mitochondrial hemoproteins. In: Methods in Enzymology, vol. 10, eds., R. W. Estabrook and M. E. Pullman, Academic Press, New York, p. 488.
22. Johnson, W. H. 1956 Nutrition of protozoa. Annu. Rev. Microbiol., *10*: 193.
23. Levy, M. R., and O. H. Scherbaum 1965 Induction of the glyoxylate cycle in *Tetrahymena*. Arch. Biochem. Biophys., *109*: 116.
24. Wille, J. J., Jr., and C. F. Ehret 1968 Light synchronization of an endogenous circadian rhythm of cell division in *Tetrahymena*. J. Protozool., *15*: 785.
25. Connor, R. C., and S. G. Cline 1964 Iron deficiency and the metabolism of *Tetrahymena pyriformis*. J. Protozool., *11*: 486.

Effect of Vitamin E and Selenium Deficiencies on Lysosomal and Cytoplasmic Enzymes in Sheep Tissues [1]

J. G. BUCHANAN-SMITH,[2,4] E. C. NELSON [3] AND A. D. TILLMAN [2]
Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma [5]

ABSTRACT Forty-eight ewe and twelve ram lambs, about 4 months of age, were fed a purified diet during growth and reproduction. The experimental design was a 2 × 2 factorial arrangement in which the factors were subcutaneous injections of vitamin E (700 IU as *d*-α-tocopheryl acetate) and selenium (5 mg as sodium selenate), given separately and in combination at weekly intervals. Ewes and rams on the basal treatment died of muscular dystrophy before day 230 of the experiment and the activities of blood plasma creatine phosphokinase, lactate dehydrogenase, α-hydroxy butyrate dehydrogenase and glutamate-oxaloacetate transaminase were elevated. Elevations of these enzymes in blood plasma were transient in the basal plus selenium-treated sheep. Total activity of acid protease and total free activities of β-glucuronidase were elevated in skeletal muscle of selenium-treated sheep. Free activity of acid protease in skeletal muscle was not affected by treatment. Total activity of acid protease was greater in livers of the vitamin E-treated sheep but vitamin E deficiency affected neither the activity of acid protease in liver nor the stability of liver lysosomes. Treatment had no effect on lysosomal enzymes in uterus and placenta.

Elevated activities of both the total and free forms of several lysosomal enzymes in skeletal muscle and kidneys have been observed in vitamin E-deficient rabbits and rats (1, 2). The increase in free forms of the enzymes is believed to reflect a decreased stability of the lysosome due to lipid peroxidation (1). Elevated total activities of lysosomal enzymes have been found in skeletal muscle of young lambs (3); there is question whether this was due to a deficiency of vitamin E or selenium. Free activities of the lysosomal enzymes were not determined. Vitamin E deficiency in the rabbit also resulted in elevated activities of two lysosomal enzymes in liver, although the elevations were not as significant as those found in muscle (1). There are no reports on the effects of vitamin E and selenium deficiencies on lysosomes of sheep tissues other than muscle.

An experiment was designed to study the effects of vitamin E and selenium deficiencies during growth and reproduction in sheep (4). The purpose of this report is to present data on lysosomal enzyme activities in the tissues of these sheep. In addition, the activities of several cytoplasmic enzymes, which are indicative of the course of vitamin E deficiency in the young lamb (5), are presented for the post-weaned sheep used in this experiment.

EXPERIMENTAL

Animals and diets. Two trials involving both ewe and ram lambs were conducted. Vitamin E or selenium, or both, were injected into sheep fed a basal diet (table 1) in a 2 × 2 factorial design. The basal diet was analyzed for selenium (6), and contained less than 0.005 ppm.[6] Molecular-distilled corn oil [7] was used in the basal and vitamin E-deficient diets, whereas regular corn oil, containing about 0.28 IU/g of vitamin E (8), was used in the vitamin E-supplemented treatments. Seven hundred IU of vitamin E [8] (*d*-α-tocopheryl acetate) were injected subcutaneously into each sheep once weekly. One milligram of selenium as sodium selenate was injected weekly to the ewes for the first 155 days

Received for publication March 3, 1969.
[1] Supported in part by Public Health Service Research Grant AM-09191, National Institute of Arthritis and Metabolic Diseases.
[2] Department of Animal Sciences and Industry.
[3] Department of Biochemistry.
[4] Present address: Department of Animal Science, University of Guelph, Guelph, Ontario, Canada.
[5] Approved by the Director.
[6] Courtesy Dr. W. H. Allaway, U. S. Soil, Plant and Nutrition Laboratory, Ithaca, N. Y.
[7] Provided gratis by Dr. Stanley R. Ames, Distillation Products Industries, Rochester, N. Y.
[8] See footnote 7.

TABLE 1

Composition of the basal diet

	%
Starch	34.37
Dextrose	24.37
Cellulose	31.00
Urea [1]	4.20
Corn oil [2]	1.00
Choline chloride	0.10
Minerals [3]	4.95
Vitamins [4]	+

[1] Crystalline urea. Courtesy Nipak Chemical Co., Pryor, Oklahoma.
[2] Sheep on the basal and basal plus selenium treatments were fed molecular-distilled corn oil.
[3] Described by Clifford et al. (7).
[4] Added so as to contain 4,400 IU vitamin A and 550 IU vitamin D_3 per kg.

of the trial, after which the amount was increased to 5 mg/week. The rams received 5 mg/week throughout the trial.

Forty-eight ewes, about 4 months of age, were assigned at random, 12 each, to the four treatments. They were kept indoors in wooden pens on slatted floors and fed the basal diet ad libitum for a 140-day growth phase; afterwards the level of feed to each animal was restricted to 1.3 kg/day. To determine the effect of vitamin E and selenium on reproduction, the ewes were bred between days 140 and 200 of the trial. Six ewes from each treatment were killed at different stages of gestation, between days 239 and 302 of the trial. Samples of skeletal muscle (quadriceps), uterus and placenta, and liver were immediately placed in ice-cold 0.25 M sucrose. Blood was collected and plasma harvested by centrifugation at 5,000 × *g* for 20 minutes at 5°. Lysosomal enzymes were assayed immediately and cytoplasmic enzymes were assayed within 72 hours.

Twelve ram lambs, about 4 months of age, were randomly assigned, three each, to the same four treatments described for the ewes. They were fed the basal diet ad libitum for 140 days. Blood samples were collected from all rams at biweekly intervals for 140 days. Plasma was collected as previously described and cytoplasmic enzymes assayed.

Methods of analysis. Acid protease was assayed using the method of Dingle et al. (9), 1 unit of activity being expressed as the amount of enzyme required to release 1 μg of tyrosine in 1 hour with hemoglobin as substrate. The activity of β-glucuronidase, expressed in international units, was assayed using phenolphthalein glucuronide as substrate (10) and measuring the released phenolphthalein by the method of Tappel et al. (11).

The total and free activity and subcellular distribution of acid protease were determined in liver which was pretreated by expulsion through a 1-mm stainless steel screen using a screw press.[9] Ten percent homogenates of liver were prepared in ice-cold 0.25 M sucrose using three up and down strokes on a Potter-Elvehjem tissue grinder with a motor-driven grooved Teflon piston. Liver homogenates were centrifuged at 600 × *g* for 10 minutes and the pellet formed was suspended in 0.25 M sucrose and recentrifuged; the supernates were combined and centrifuged at 18,000 × *g* for 30 minutes. Free activity of acid protease was determined in the 18,000 × *g* supernate. Lysosomal bound activity of acid protease was determined by suspending the pellet formed at 18,000 × *g* in 0.25 M sucrose containing Triton X-100 [10] (final concentration of 0.5%), then recentrifuging and assaying the released enzyme in the supernate. Activity of acid protease was determined in the 600 × *g* pellet, and total activity for liver was found by adding this value to the free and lysosomal bound activities.

Stability of liver lysosomes was determined by incubating a 10% suspension of the lysosome-rich precipitate, obtained by centrifuging liver homogenates at 18,000 × *g*, in 0.25 M sucrose at 37°. After incubation, activity of acid protease was determined in the supernates obtained by centrifuging the suspensions at 18,000 × *g* for 30 minutes. The values obtained were divided by total lysosomal bound activity and expressed as a percentage.

The total and free activity of acid protease and β-glucuronidase were determined in skeletal muscle and uterus and placenta. Either 20 or 40% homogenates were prepared by blending the tissues for 30 seconds in ice-cold 0.25 M sucrose using an omni-mixer. Total activities of the enzymes in skeletal muscle and in uterus and placenta were determined by adding detergent [11] to the homogenate to give a final concentration of 0.5%. The homogenate

[9] Harvard Apparatus Co., Inc., Dover, Massachusetts.
[10] Rohm and Haas, Philadelphia, Pennsylvania.
[11] See footnote 10.

was centrifuged at 18,000 × *g* for 30 minutes and the assays performed on suitable aliquots of the supernate. Free activities of the enzymes were determined in the same way but without detergent.

Creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and α-hydroxy butyrate dehydrogenase (HBD) activities were determined on suitable aliquots of the skeletal muscle and uterus and placenta supernates, obtained for assay of lysosomal enzymes, and on blood plasma (12–14). In addition, glutamate-oxaloacetate transaminate (GOT), glutamate dehydrogenase (GDH) and ornithine carbamyl transferase (OCT) determinations were made on plasma (15–17).

Data were subjected to analysis of variance, and differences between means were tested for significance using a multiple range test (18).

RESULTS AND DISCUSSION

Ewes and rams on the basal treatment died from muscular dystrophy between days 80 and 230 of the experiment (4). Selenium treatment delayed but did not prevent the deaths of these animals. Elevated GOT activity was found in blood plasma of these sheep (4). Activities for GOT, LDH, HBD and CPK in blood plasma of the rams during the first 140 days of the experiment are shown in figure 1. Cyclic variation was observed in these values, all enzymes being elevated ($P < 0.05$) at 42 and 84 days on trial. In most of the ewes and rams fed on the basal diet, plasma enzyme values remained elevated until death. On the other hand, in basal plus selenium-treated rams, enzyme values returned to normal before the end of the 140-day period. These data, when considered along with animal survival, indicate a role for selenium in temporarily alleviating the dystrophy.

GDH and OCT activities were not elevated in the blood plasma of vitamin E-deficient sheep. Since these enzymes are concentrated in liver (16, 17), it appears unlikely that there was any hepatic necrosis in the vitamin E-deficient sheep. Histopathological findings confirmed this observation. This is contrary to findings in young lambs fed vitamin E-deficient diets (5).

Because of deaths, ewes were killed only from those treatments in which vitamin E or selenium were given. Activities for CPK, LDH and HBD were elevated in the blood plasma of the killed ewes which were treated with selenium (table 2). Thus it was apparent that the basal plus selenium-treated sheep were suffering from muscular dystrophy at the time of killing. The pathology of the skeletal muscle of the vitamin E-deficient animals was similar to that described previously (19, 20).

Activities of CPK, LDH and HBD in skeletal muscle were not affected ($P > 0.05$) by treatment (table 3), but their activities were depressed to less than 50% in

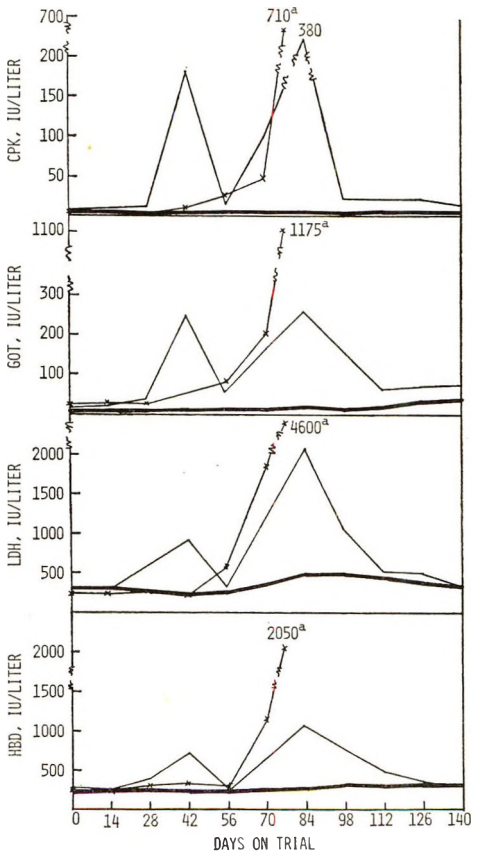

Fig. 1 Activities of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), α-hydroxy butyrate dehydrogenase (HBD) and glutamate-oxaloacetate transaminase (GOT) in blood plasma of rams during the first 140 days of the experiment. Basal, x——x; basal + Se, ———; basal + E, and basal + E + Se, _____. (Three rams per treatment.) [a]Rams fed the basal died.

TABLE 2

Effect of vitamin E and selenium on the activities of creatine phosphokinase, lactate dehydrogenase and α-hydroxy butyrate dehydrogenase in blood plasma [1]

Treatment	Creatine phosphokinase		Lactate dehydrogenase		α-Hydroxy butyrate dehydrogenase	
	Number	Activity	Number	Activity	Number	Activity
		IU/liter		*IU/liter*		*IU/liter*
Basal + selenium	4	197.4 ± 43.6 [2,a]	4	937.5 ± 150.0 [a]	4	606.9 ± 80.1 [a]
Basal + vitamin E	6	22.4 ± 39.8 [b]	5	371.5 ± 134.1 [b]	5	257.7 ± 71.6 [b]
Basal + vitamin E + selenium	6	29.8 ± 39.8 [b]	4	369.6 ± 150.0 [b]	4	250.8 ± 80.1 [b]

[1] Sheep on the basal treatment died.
[2] Mean ± S.E.
[a,b] Values in the same column bearing different superscripts differ significantly ($P < 0.05$).

the skeletal muscle of vitamin E-deficient sheep killed at the terminal stages. Plasma activities of the same enzymes in the vitamin E-deficient sheep were elevated 10-fold or more, supporting the idea (5) that muscle may be the primary source of elevated enzyme activities in blood plasma of vitamin E-deficient sheep.

Total activities of acid protease and β-glucuronidase were greatest ($P < 0.01$) in the skeletal muscle of the basal plus selenium-treated sheep (table 3). Similar results have been found in rabbits (1) but the results of the present experiment indicate that the enzymes may be affected more in older sheep than in young lambs (3). The total activities of acid protease and β-glucuronidase in skeletal muscle were elevated 82- and 19-fold, respectively, in sheep in the terminal stages of vitamin E deficiency. Thus the stage of deficiency probably affects this phenomenon. Selenium omission did not affect ($P > 0.05$) the activities, free or total, of either acid protease or β-glucuronidase in skeletal muscle.

The free activity of β-glucuronidase was also elevated ($P < 0.05$) in skeletal muscle of vitamin E-deficient sheep (basal + Se, table 3). However, free activity of acid protease in skeletal muscle was not affected by treatment ($P > 0.05$) and was detected in only one vitamin E-deficient ewe. This finding is contrary to that in skeletal muscle of the vitamin E-deficient rabbit, in which free acid protease is increased (1). However, variations in the ratios of free to total activities of lysosomal enzymes were found in skeletal muscle of the vitamin E-deficient rabbit (1). A difference in the ratio of free to total activity between β-glucuronidase and acid protease indicates that the two enzymes might not be distributed in the same proportions in different subcellular particles in the skeletal muscles.

Histopathology of vitamin E-deficient sheep in the present experiment indicated that there was a proliferation of macrophages in skeletal muscle. The complement of lysosomal enzymes in macrophages could differ from that in a skeletal muscle cell. Results of Weinstock and Lukacs indicated that the pathological muscle cell itself is the source of increased lysosomal enzyme activity in nutritional muscular dystrophy of rabbits (21). It appears that those species exhibiting muscle dystrophy in vitamin E deficiency, e.g., rabbit, sheep, chicken, rat and hamster, simultaneously contain elevated activities of lysosomal enzymes in their dystrophic tissue (1, 3, 22, 23). Results of the present experiment indicate that a significant fraction of the elevated activity probably remains bound within the cell.

When expressed on the basis of total activity in liver, the activity of acid protease was greater ($P < 0.05$) in livers of vitamin E-treated sheep than in those from animals receiving the combination (table 4). The differences were not significant, however, when expressed as concentration per unit of liver. Differences in total activity of liver acid protease between selenium-treated sheep and vitamin E plus selenium-treated sheep were not significant ($P > 0.05$). Even though the differences were not significant ($P > 0.05$), the results on subcellular distribution (table 4) and lysosomal membrane stability (fig. 2) when considered together, might indicate

TABLE 3

Effect of vitamin E and selenium on the total and free activities of acid protease and β-glucuronidase and the total activities of creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and α-hydroxy butyrate dehydrogenase (HBD) in skeletal muscle [1]

Treatment	Acid protease			β-Glucuronidase			Cytoplasmic enzymes			
	No. sheep	Activity (free) [2]	Activity (total) [2]	No. sheep	Activity (free) [2]	Activity (total) [2]	No. sheep	CPK activity	LDH activity	HBD activity
		Units/mg[2]			*m IU/g*				*IU/g*	
Basal + Se	6	0.022 [3] (±0.013)	0.433 [a] (±0.091)	5	1.87 [a] (±0.334)	4.27 [a] (±0.282)	4	348.1 (±25.9)	471.4 (±69.8)	119.6 (±13.0)
Basal + vit E	6	0	0.046 [b] (±0.091)	4	0.18 [b] (±0.373)	1.06 [b] (±0.315)	4	379.9 (25.9)	547.7 (±69.8)	133.3 (±13.0)
Basal + vit E + Se	6	0	0.042 [b] (±0.091)	5	0.15 [b] (±0.334)	0.51 [b] (±0.282)	4	362.0 (±25.9)	378.0 (±69.8)	105.5 (±13.0)

[1] Sheep on the basal treatment died.
[2] For definition of acid protease units and determination of total and free activity, see Experimental.
[3] Mean (± SE).
[a,b] Values in the same column bearing different superscripts differ significantly ($P < 0.01$).

TABLE 4

The effect of vitamin E and selenium on the total activity and subcellular distribution of acid protease in liver [1]

Treatment	No. sheep	Live wt	Liver wt	Activity (total)		Subcellular distribution		
						Activity (bound)	Activity (free)	Activity (free) / Activity (bound) + activity (free)
		kg	*g*	*units/mg*	*units/liver/g live wt*	*units/mg*		%
Basal + Se	6	55.2 [2]	664 [a]	17.70	215.5 [a,b]	6.52	3.34	31.7
Basal + vit E	6	48.8	546 [b]	24.64	273.8 [a]	9.28	5.28	35.2
Basal + vit E + Se	6	53.0	516 [b]	17.70	166.9 [b]	6.60	3.10	26.4
		(±3.21) [3]	(±44.7)	(±2.87)	(±30.2)	(±1.08)	(±1.11)	(±6.3)

[1] Sheep on the basal treatment died.
[2] Mean.
[3] SE of treatment means.
[a,b] Values in the same column bearing different superscripts differ significantly ($P < 0.05$).

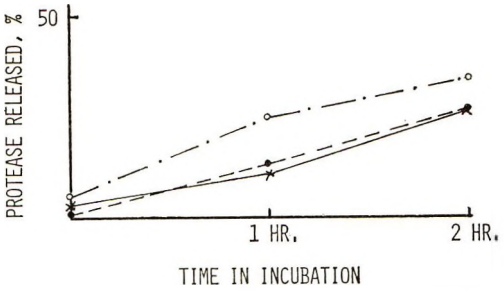

Fig. 2 Effect of treatments on stability of liver lysosomes as measured by release of acid protease. Basal + Se, ●— — —●; basal + E, ○—— • ——○; basal + E + Se, x——x. Each point is the mean of six observations, and the standard error of the combined means of 18 observations per treatment is 2.6%.

that lysosomes from livers of vitamin E-treated sheep were less stable than those taken from livers of sheep on other treatments.

Stability of liver lysosomes is decreased by excess vitamin A (9). Concentrations of vitamin A (μg/g) in livers of sheep in the present experiment were 155.2(± 19.2),[12] 183.2(± 19.2) and 207.2(± 19.2) for basal plus selenium, basal plus vitamin E and basal plus vitamin E plus selenium treatments, respectively. These values are 10-fold greater than others reported (24) but treatment differences were not significant ($P > 0.05$). Thus it appears improbable that vitamin A per se affected the results obtained. Results of the present experiment indicate that vitamin E deficiency did not affect the subcellular distribution or total activities of lysosomal enzymes in liver or stability of the lysosome.

It has been observed that total activities of rabbit lysosomal enzymes in liver were increased by vitamin E deficiency (1). It is believed that this result is related to lipid peroxidation in the same tissue (25) but it is not known whether lipid peroxidation took place in the liver of these vitamin E-deficient sheep. Results of the present experiment indicate that selenium deficiency may increase total activities of lysosomal enzymes and decrease lysosome stability in liver, but a metabolic role for selenium at the subcellular level independent of vitamin E has not been demonstrated (26). Liver necrosis was not found in livers of these selenium-deficient sheep and other ewes on the same selenium-deficient treatment have survived 12 months after the present animals were killed.

Treatments did not affect ($P > 0.05$) the total and free activities of acid protease and β-glucuronidase in the uterus and placenta (table 5). The results show that a greater proportion of the total activities of acid protease and β-glucuronidase was free in uterus and placenta than in skeletal muscle. No pathology was observed in the uteri and placentae of any sheep in this experiment. In the rat, vitamin E deficiency did not affect lysosomal enzyme activities of uterus and placenta until after fetal resorption had taken place, at which time activities increased (27). In the present experiment, activities of lysosomal en-

[12] Mean (± SE).

TABLE 5

The effect of vitamin E and selenium on the total and free activities of acid protease and β-glucuronidase in uterus and placenta [1]

Treatment	Acid protease			β-Glucuronidase		
	No. sheep	Activity (free) [2]	Activity (total)	No. sheep	Activity (free)	Activity (total)
		units/mg [2]			*mIU/g*	
Basal + Se	6	5.7 [3] (± 1.65)	8.6 (± 3.30)	5	15.4 (± 3.07)	22.6 (± 5.30)
Basal + vit E	6	5.9 (± 1.65)	10.7 (± 3.30)	4	11.6 (± 3.44)	17.8 (± 5.90)
Basal + vit E + Se	6	2.1 (± 1.65)	5.2 (± 3.30)	5	7.3 (± 3.07)	15.1 (± 5.30)

[1] Sheep on the basal treatment died.
[2] For definition of acid protease units and determination of total and free activity, see Experimental.
[3] Mean (± SE).

zymes in the uterus and placenta increased with duration of pregnancy.

Skeletal muscle was the only tissue taken from the vitamin E-deficient sheep of this experiment that contained elevated total activities of lysosomal enzymes, and the only tissue that was dystrophic. This result is similar to that found in the vitamin E-deficient rabbit (1); in the sheep, however, the total and free activities of acid protease in liver were not affected by vitamin E deficiency. Furthermore, the free activity of acid protease was not elevated in the sheep skeletal muscle; thus vitamin E deficiency in sheep may involve some dysfunction in skeletal muscle in addition to lipid peroxidation (1). Because the activity of acid protease was elevated in liver of vitamin E-treated sheep (table 4), there is a need to determine if selenium has a function in sheep liver independent of vitamin E. Selenium affected the pattern of enzyme activities in blood plasma of vitamin E-deficient sheep (fig. 1) and thus may have a specific function in the vitamin E-deficient animal.

ACKNOWLEDGMENTS

The authors wish to acknowledge technical assistance given by Mrs. Marcia Mayberry, Miss Sue Ward and Mr. John Milner.

LITERATURE CITED

1. Zalkin, H., A. L. Tappel, K. A. Caldwell, S. Shibko, I. D. Desai and T. A. Holliday 1962 Increased lysosomal enzymes in muscular dystrophy of vitamin E-deficient rabbits. J. Biol. Chem., 237: 2678.
2. Moore, T., I. M. Sharman, M. G. Stanton and J. T. Dingle 1967 Nutrition and lysosomal activity. The influence of vitamin E deficiency and its duration on the stability of lysosomes in the kidneys of rats. Biochem. J., *103:* 923.
3. Desai, I. D. 1966 Activity of lysosomal enzymes in white muscle disease. Nature, *209:* 1349.
4. Buchanan-Smith, J. G., E. C. Nelson, B. I. Osburn, M. E. Wells and A. D. Tillman 1969 Effects of vitamin E and selenium deficiencies in sheep fed a purified diet during growth and reproduction. J. Anim. Sci. (In press).
5. Boyd, J. W. 1964 Serum enzyme changes in lambs with experimentally-induced acute muscular dystrophy. Res. Vet. Sci., *5:* 419.
6. Watkinson, J. H. 1966 Fluorometric determination of traces of selenium. Anal. Chem., *32:* 981.
7. Clifford, A. J., R. D. Goodrich and A. D. Tillman 1967 Effects of supplementing ruminant all-concentrate and purified diets with vitamins of the B complex. J. Anim. Sci., *26:* 400.
8. Herting, David C., and Emma-Jane E. Drury 1963 Vitamin E content of vegetable oils and fats. J. Nutr., *81:* 335.
9. Dingle, J. T., I. M. Sharman and T. Moore 1966 Nutrition and lysosomal activity. The influence of vitamin A status on proteolytic activity of extracts from the livers and kidneys of rats. Biochem. J., *98:* 476.
10. Gianetto, R., and C. de Duve 1955 Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, beta-glucuronidase and cathepsin by rat-liver particles. Biochem. J., 59: 433.
11. Tappel, A. L., H. Zalkin, K. A. Caldwell, I. D. Desai and S. Shibko 1962 Increased lysosomal enzymes in genetic muscular dystrophy. Arch. Biochem. Biophys., *96:* 340.
12. Wiesmann, U., J. P. Colombo, A. Adam and R. Richterich 1966 Determination of cysteine activated creatine kinase in serum. Enzymol. Biol. Clin., *7:* 266.
13. Henry, R. J. 1964 Clinical Chemistry. Principles and Technics. Harper and Row, New York, N. Y., p. 605–611.
14. Elliot, B. A., and J. H. Wilkinson 1961 Serum "alpha-hydroxy butyric dehydrogenase" in myocardial infarction and in liver disease. Lancet, *1:* 698.
15. Henry, R. J. 1964 Clinical Chemistry. Principles and Technics. Harper and Row, New York, N. Y., p. 512–518.
16. Schmidt, Ellen 1965 Glutamic dehydrogenase. In: Methods of Enzymatic Analysis, ed., Hans-Ulrich Bergmeyer. Academic Press, New York, N. Y., p. 752.
17. Carper, H. A., and A. R. Roesler 1968 A sensitive method for the determination of serum ornithine carbamyl transferase (OCT) in domestic animals. Amer. J. Vet. Clin. Path., 2: 15.
18. Duncan, D. B. 1955 Multiple range and multiple *F* tests. Biometrics, *11:* 1.
19. Blaxter, Kenneth L., and Fred Brown 1952 Vitamin E in the nutrition of farm animals. Nutr. Abstr. Rev., *22:* 1.
20. Muth, O. H. 1955 White muscle disease (myopathy) in lambs and calves. I. Occurrence and nature of the disease under Oregon conditions. J. Amer. Vet. Med. Ass., *126:* 355.
21. Weinstock, I. M., and M. Lukacs 1965 Enzyme studies in muscular dystrophy. V. Vitamin E-deficiency and denervation atrophy. Enzymol. Biol. Clin., *5:* 89.
22. Bunyan, J., J. Green, A. T. Diplock and D. Robinson 1967 Lysosomal enzymes and vitamin E deficiency. 1. Muscular dystrophy, encephalomalacia and exudative diathesis in the chick. Brit. J. Nutr., *21:* 127.
23. Koszalka, Thomas B., Karl E. Mason and George Krol 1961 Relation of vitamin E to proteolytic and autolytic activity of skeletal muscle. J. Nutr., *73:* 78.
24. Rousseau, J. E., Jr., Martha W. Dicks, R. Teichman, C. F. Helmboldt, E. L. Bacon, R. M. Prouty, K. L. Dolge, H. D. Eaton, E. L.

Jungherr and Geoffrey Beall 1957 Relationship between plasma, liver and dietary tocopherol in calves, lambs and pigs. J. Anim. Sci., *16:* 612.
25. Zalkin, H., and A. L. Tappel 1960 Studies of the mechanism of vitamin E action. IV. Lipid peroxidation in the vitamin E-deficient rabbit. Arch. Biochem. Biophys., *88:* 113.
26. Bull, R. C., and J. E. Oldfield 1967 Selenium involvement in the oxidation by rat liver tissue of certain tricarboxylic acid cycle intermediates. J. Nutr., *91:* 237.
27. Bunyan, J., J. Green, A. T. Diplock and D. Robinson 1967 Lysosomal enzymes and vitamin E deficiency. 2. Gestation-resorption in the rat. Brit. J. Nutr., *21:* 137.