THE JOURNAL OF NUTRITION

OFFICIAL ORGAN OF THE
AMERICAN INSTITUTE OF NUTRITION

RICHARD H. BARNES, *Editor*
Graduate School of Nutrition
Cornell University, Savage Hall
Ithaca, New York

HAROLD H. WILLIAMS
Associate Editor

E. NEIGE TODHUNTER
Biographical Editor

EDITORIAL BOARD

GERALD F. COMBS
RUTH M. LEVERTON
GEORGE V. MANN
MAX O. SCHULTZE
A. E. AXELROD
GEORGE K. DAVIS
RICHARD H. FOLLIS, JR.
E. L. R. STOKSTAD
JOHN G. BIERI
HARRY P. BROQUIST
R. GAURTH HANSEN
RALPH T. HOLMAN
GEORGE M. BRIGGS
R. M. FORBES
JULES HIRSCH
E. E. HOWE

VOLUME 82

JANUARY – APRIL 1964

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PENNSYLVANIA

THE JOURNAL OF NUTRITION ®
® *Trade mark registered, U. S. Patent Office*

Copyright © 1964 by
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

All rights reserved

Contents

No. 1 JANUARY 1964

Sir Jack Cecil Drummond, F.R.S. — A Biographical Sketch. *Alice M. Copping* 1

Effect of Dietary Restriction of Pregnant Rats on Body Weight Gain of the Offspring. *Bacon F. Chow and Chi-Jen Lee* 10

Effect of Degree of Fatty Acid Unsaturation in Tocopherol Deficiency-induced Creatinuria. *L. A. Witting and M. K. Horwitt* 19

Phosphorus Requirement of the Baby Pig. *E. R. Miller, D. E. Ullrey, C. L. Zutaut, Betty V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke* 34

Zinc Deficiency Syndrome in the Young Lamb. *E. A. Ott, W. H. Smith, Martin Stob and W. M. Beeson* 41

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. N. Williams, Jr.* 51

Supplementation of Wheat Gluten Protein. *W. L. Banks, Jr., J. B. Allison and R. W. Wannemacher, Jr.* 61

A Determination of the Essential Amino Acid Proportions Needed to Allow Rapid Growth in Chicks. *D. C. Dobson, J. O. Anderson and R. E. Warnick* 67

Relative Potency of Diets Containing Amino Acids and Proteins for Promoting Growth of Rats. *W. D. Salmon* 76

Fatty Livers Produced in Albino Rats by Excess Niacin in High Fat Diets. I. Alterations in Enzyme and Coenzyme Systems Induced by Supplementing 40% Fat Diets with 0.1% of Niacin. *Lora Long Rikans, Dorothy Arata and Dena C. Cederquist* 83

The Amino Acid Composition and Nutritive Value of Proteins. V. Amino Acid Requirements as a Pattern for Protein Evaluation. *P. B. Rama Rao, H. W. Norton and B. Connor Johnson* 88

Effect of Barbituric Acid, Chlortetracycline and Carbohydrates upon Growth and Gastrointestinal Urease Activity of Chicks. *A. P. Alvares, L. H. Harbers and W. J. Visek* 93

Absorption of Preformed Vitamin A from Ligatured Poultry Intestinal Sections. *T. E. Shellenberger, D. B. Parrish and P. E. Sanford* 99

Vanadium Toxicity and Distribution in Chicks and Rats. *John N. Hathcock, Charles H. Hill and Gennard Matrone* 106

Mineral Balance Studies with the Baby Pig: Effects of Dietary Phosphorus Level upon Calcium and Phosphorus Balance. *E. R. Miller, D. E. Ullrey, C. L. Zutaut, J. A. Hoefer and R. W. Luecke* 111

Adaptation of the Chick to Dietary Energy Source. *W. E. Donaldson* .. 115

Effect of Calcium, Phosphorus and Manganese on the Intestinal Synthesis of Thiamine in Rats. *S. K. Meghal and M. C. Nath* . . . 121

Effect of the Quality of Dietary Protein on Nitrogen Compounds in the Urine of Rats. *Shuhachi Kiriyama and Kiyoshi Ashida* . . . 127

Pantothenic Acid Deficiency in Cats. *S. N. Gershoff and L. S. Gottlieb* . . . 135

Influence of High Protein Diets on Vitamin A Metabolism and Adrenal Hypertrophy in the Chick. *G. S. Stoewsand and M. L. Scott* . . . 139

Heat of Combustion Values of the Protein and Fat in the Body and Wool of Sheep. *O. L. Paladines, J. T. Reid, A. Bensadoun and B. D. H. van Niekerk* . . . 145

Some Biochemical Lesions Associated with Liver Fat Accumulation in Threonine-deficient Rats. *Dorothy Arata, Catherine Carroll and Dena C. Cederquist* . . . 150

Influence of Nicotinic, Picolinic and Pyridine-3-sulfonic Acids on Cholesterol Metabolism in the Rat. *David Kritchevsky and Shirley A. Tepper* . . . 157

No. 2 FEBRUARY 1964

Influences of Dietary Carbohydrate-Fat Combinations on Various Functions Associated with Glycolysis and Lipogenesis in Rats. II. Glucose vs. Sucrose with Corn Oil and Two Hydrogenated Oils. *Catherine Carroll* . . . 163

Hypertension and Cardiovascular Abnormalities in Starved-Refed Swine. *G. S. Smith, Jack L. Smith, M. S. Mameesh, J. Simon and B. Connor Johnson* . . . 173

Energy Intake and Fertility of Male Chickens. *J. E. Parker and G. H. Arscott* . . . 183

Effect of Stress from High Protein Diets on Vitamin A Metabolism in Chicks. *G. S. Stoewsand and M. L. Scott* . . . 188

Studies on the Digestion of Soybeans. *H. J. H. de Muelenaere* . . . 197

Free Amino Acids in Plasma and Tissues of Rats Fed a Vitamin B_6-deficient Diet. *Marian E. Swendseid, Juanita Villalobos and Barbara Friedrich* . . . 206

Effects of Dietary Supplements on Young Rats Fed High Levels of Zinc. *Aden C. Magee and Sandra Spahr* . . . 209

Protein and Lysine as Factors in the Cariogenicity of a Cereal Diet. *Mary L. Dodds* . . . 217

Protein-Energy Relationship in Adult Rats. I. Effects of Varying Amounts of Dietary Protein and Food Energy on Nitrogen Utilization. *Pilar A. Garcia and Charlotte E. Roderuck* . . . 224

Protein-Energy Relationship in Adult Rats. II. Qualitative and Quantitative Variations in Dietary Nitrogen and Effects on Deposition of Hepatic Fat and on Nitrogen Utilization. *Pilar A. Garcia and Charlotte E. Roderuck* . . . 231

Antagonism of Poorly Invertible D-Amino Acids Toward Growth Promotion by Readily Invertible D-Amino Acids. *Shrinivas H. Kamath and Clarence P. Berg* 237

Antagonism of the D-Forms of the Essential Amino Acids Toward the Promotion of Growth by D-Histidine. *Shrinivas H. Kamath and Clarence P. Berg* 243

Influence of Various Chelating Agents on the Availability of Zinc. *Pran Vohra and F. H. Kratzer* 249

Reproductive Performance of Rats Receiving Various Levels of Dietary Protein and Fat. *L. R. Richardson, Jackie Godwin, Stella Wilkes and Martha Cannon* 257

Effect of Vitamin A and Vitamin A Acid on Cerebrospinal Fluid Pressure and Blood and Liver Vitamin A Concentrations in the Pig. *E. C. Nelson, B. A. Dehority, H. S. Teague, A. P. Grifo, Jr. and V. L. Sanger* 263

Chemical Pathology of Acute Amino Acid Deficiencies. VI. Influence of Fat Intake on the Morphologic and Biochemical Changes in Young Rats Force-Fed a Threonine-Devoid Diet. *Herschel Sidransky and Ethel Verney* 269

Exclusion of the Exocrine Pancreatic Secretion: Effect on Digestibility of Soybean and Milk Protein by Baby Pigs at Various Ages. *Jerome C. Pekas, Virgil W. Hays and Alan M. Thompson* 277

Effect of Zinc Toxicity on Calcium, Phosphorus and Magnesium Metabolism of Young Rats. *Alta K. Stewart and Aden C. Magee* 287

No. 3 MARCH 1964

Lactose and Calcium Transport in Gut Sacs. *Yet-oy Chang and D. M. Hegsted* 297

Effects of Dietary Creatine and Related Compounds on Tissue Glycogen Deposition in Rats. *W. R. Todd, Marilouise Harris and Lester Laastuen* 301

A Mechanism of Interaction between Dietary Protein Levels and Coccidiosis in Chicks. *W. M. Britton, C. H. Hill and C. W. Barber* 306

Effects of Egg Oil, Cholesterol, Cholic Acid and Choline upon Plasma and Liver Lipids. *Jane B. Walker and Gladys A. Emerson* 311

Importance of Dietary Copper in the Formation of Aortic Elastin. *Barry Starcher, Charles H. Hill and Gennard Matrone* 318

Dietary Fat and the Structure and Properties of Rat Erythrocytes. II. Stability of the Erythrocyte. *Brian L. Walker and F. A. Kummerow* 323

Dietary Fat and the Structure and Properties of Rat Erythrocytes III. Response of Erythrocyte Fatty Acids to Various Dietary Fats. *Brian L. Walker and F. A. Kummerow* 329

Metabolic Studies of an Amino Acid Imbalance in Cold-exposed Rats. *George J. Klain and Robert L. Winders* 333

Serum Lipid Levels, Fat, Nitrogen, and Mineral Metabolism of Young Men Associated with Kind of Dietary Carbohydrate. *M. Isabel Irwin, Doris D. Taylor and Ruth M. Feeley* 338

Vitamin A in Serum and Liver of Weanling Pigs Fed a Protein-deficient Diet and Fixed Intakes per Unit of Live Weight of Carotene in Oil. *H. D. Eaton, W. B. Boucher and P. C. Shah* 343

Nutrient-to-Calorie Ratios in Applied Nutrition. *E. W. Crampton* 353

Anti-rachitic Effects of Soybean Preparations for Turkey Poults. *C. W. Carlson, J. McGinnis and L. S. Jensen* 366

Carbohydrate and Fat Metabolism and Response to Insulin in Vitamin B_6-deficient Rats. *Agnes M. Huber, Stanley N. Gershoff and D. Mark Hegsted* 371

Tissue Distribution of α-Aminoisobutyric Acid and Nitrogen Metabolism in the Rat. II. Effects of Environmental Temperature and Dietary Imbalance. *L. A. Bavetta and M. E. Nimni* 379

Amino Acid Composition of Rye Flour and the Influence of Amino Acid Supplementation of Rye Flour and Bread on Growth, Nitrogen Efficiency Ratio and Liver Fat in the Growing Rat. *R. Kihlberg and L.-E. Ericson* 385

Relationship between Tryptophan — Niacin Metabolism and Changes in Nitrogen Balance. *Virginia M. Vivian* 395

No. 4 APRIL 1964

Nutritional Studies with the Guinea Pig. X. Determination of the Linoleic Acid Requirement. *M. E. Reid, J. G. Bieri, P. A. Plack and E. L. Andrews* 401

Response of Digestive Enzymes to Dietary Protein. *Jean Twombly Snook and J. H. Meyer* 409

Nonspecific Role of Arginine in the Promotion of Muscular Dystrophy in the Chick. *K. J. Jenkins, D. C. Hill and Lilian M. Hutcheson* 415

Nutrition and Biochemistry of Survival During Newcastle Disease Virus Infection. II. Relation of Clinical Symptoms and Starvation to Nucleic and Free Amino Acids of Avian Liver. *Robert L. Squibb* 422

Nutrition and Biochemistry of Survival During Newcastle Disease Virus Infection. III. Relation of Dietary Protein to Nucleic and Free Amino Acids of Avian Liver. *Robert L. Squibb* 427

Liver Lipid Accumulation in Isoleucine-deficient Rats. *R. L. Lyman, C. R. Cook and Mary Ann Williams* 432

Effect of Potassium on Juxtaglomerular Cells and the Adrenal Zona Glomerulosa of Rats. *Phyllis M. Hartroft and Elizabeth Sowa* 439

Adrenal Lipids of Cholesterol-fed Guinea Pigs and Rats. *Rosemarie Ostwald, Angela Shannon, P. Miljanich and R. L. Lyman* 443

Studies on the Utilization of Tempeh Protein by Weanling Rats. *L. R. Hackler, K. H. Steinkraus, J. P. Van Buren and D. B. Hand* 452

Effect of Thyroxine, Thiouracil and Ambient Temperature on the Utilization of Vitamin A by Vitamin A-deficient Rats. *Thomas A. Anderson, Farris Hubbert, Jr. and C. B. Roubicek* ... 457

Influence of Protein and Energy on Growth and Protein Utilization in the Growing Chicken. *J. D. Summers, S. J. Slinger, I. R. Sibbald and W. F. Pepper* ... 463

Effect of Water Intake on Nitrogen Metabolism in Dogs. *Ricardo Bressani and J. Edgar Braham* ... 469

Nutrition of Salmonoid Fishes. XI. Iodide Requirements of Chinook Salmon. *A. N. Woodall and Gilles LaRoche* ... 475

Effect of Pyridoxine Deficiency upon Valine Incorporation into Tissue Proteins of the Rat. *Anthony C. Trakatellis and A. E. Axelrod* ... 483

Effect of Zinc Nutrition upon Uptake and Retention of Zinc-65 in the Chick. *T. R. Zeigler, R. M. Leach, Jr., M. L. Scott, F. Huegin, R. K. McEvoy and W. H. Strain* ... 489

Effect of Selenium, Sulfur and Sulfur Amino Acids on Nutritional Muscular Dystrophy in the Lamb. *H. F. Hintz and D. E. Hogue* ... 495

The Role of Dispensable Amino Acids in the Nutrition of the Rat. *L. H. Breuer, Jr., W. G. Pond, R. G. Warner and J. K. Loosli* ... 499

Rachitogenic Activity of Soybean Fractions. *C. W. Carlson, H. C. Saxena, L. S. Jensen and James McGinnis* ... 507

Index to Volume 82 ... 513

SIR JACK CECIL DRUMMOND, F.R.S.

(1891 — 1952)

Published with the permission of the Controller of Her Britannic Majesty's Stationary Office

© British Crown

Sir Jack Cecil Drummond, F.R.S.

Sir Jack Cecil Drummond, F.R.S. — A Biographical Sketch

(January 12, 1891 — August 4, 1952)

The advance of biochemistry and the growth of scientific nutrition from biochemical bases owe much to the work and personality of Sir Jack Drummond. His interests in biochemistry and nutrition were extraordinarily diverse and he had a great gift for imparting enthusiasm to others. From this talent, which Drummond well knew how to develop, the world at large has derived great benefit in the teachers and research workers who were trained in the Department of Biochemistry at University College, London. Drummond had abounding mental and physical energy and was accustomed to work hard and play hard. His vitality was infectious and he was most successful in training others for senior posts. Moreover he frequently infused some of his own entertaining ideas into those around him. His lectures were never dull and his suggestions on research problems were always illuminating and sometimes even startling. They certainly aided many a struggling young research worker to arrive at a satisfactory development of a difficult problem.

Early years. Jack Cecil Drummond was born on January 12, 1891, at Leicester, the only son of Major John Drummond of the Royal Artillery and of Mrs. Drummond. His parents died in India when he was very young and he was brought up by an uncle and aunt, Captain and Mrs. Spinks, in a somewhat rigid nonconformist atmosphere. His early education was at the Roan School, Greenwich, and at the Strand School of King's College. From there he went in 1909 to East London College (now Queen Mary College) and three years later he was graduated with a Bachelor of Science degree from the University of London with first class honours in Chemistry. During his student days at East London he met Mabel Straw as a fellow student and later married her. Many biochemists in Great Britain and other countries remember her kindly grace as hostess in the Drummonds' home at Chorleywood in Hertfordshire and at functions associated with Biochemical Society meetings. In the early days of the Society, Biochemical dinners were exclusively for men and Mrs. Drummond started a delightful custom of entertaining women scientists and wives of the biochemists at rival parties. In the course of the years men and women came to dine together and Mrs. Drummond was always a happy member of the parties of the early nineteen-thirties. Overseas students and visitors were happily received by the Drummonds in their home. Both took country pursuits of gardening, golfing, walking, watching birds with great pleasure and introduced others to these delights through their own knowledge. A week-end at Chorleywood was a memorable occasion for many a young student from abroad.

Drummond's first post after graduation was as a research assistant at King's College, University of London, where he came into contact with Professor W. D. Halliburton and Dr. Otto Rosenheim, so that his initial chemical training was diverted into biochemical channels. Though he moved from King's College to the Research Institute of the Cancer Hospital in 1914, Drummond's career was profoundly influenced by both Halliburton and Rosenheim. At the Research Institute he worked under Dr. Casimir Funk who was in charge of biochemical research. In 1912 Funk, then at the Lister Institute, had published a paper on "The aetiology of the deficiency diseases" (J. State Med., 1912, *20*, 341) in which he introduced the term "Vitamine" for the first time. The origin of the term was in Funk's attempts to isolate from rice polishings an antipolyneuritic principle. The product was a basic nitrogenous substance, possibly an amine, and on this

basis Funk suggested that vitamines might be nitrogenous substances essential in the diet of birds, man and animals. Drummond himself some years later, in 1920, suggested the change to "vitamins," with the omission of the final "e," as the term to cover these essential substances since some were not amines. Thus Drummond was introduced to the new ideas fertilising the field of nutrition. His second scientific publication was written jointly with Funk on the chemical investigation of the phosphotungstic precipitate from rice polishings. Studies of amino-acid composition of dietary proteins were in progress at the same time as the vitamin studies and Drummond investigated the possible effects of deficiency of protein and of vitamins on the growth of tumours in experimental animals. Although by dietary means no inhibition of tumour growth could be demonstrated without serious general nutrition failure in the host animal, such negative information was invaluable for planning future studies of the relation between nutrition and tumour growth.

With the outbreak of war in August 1914, Drummond found to his regret that his health did not permit him to serve in the Armed Forces. He turned his energies to research on food problems carried out at the Cancer Research Institute under Dr. Alexander Paine who taught him the essentials of methods of feeding diets of controlled composition to rats and other experimental animals. War-time studies included comparative tests on butter and margarine and other butter substitutes leading to an interest in fat-soluble as well as water-soluble vitamins. Moreover the researches on animals and the chemical analyses were never purely laboratory exercises but led to practical applications as illustrated by a paper in 1918 on "Some aspects of infant feeding." In this publication he pointed out the demand for accessory food factors during the period of growth and the depletion of the tissues of the mother if the maternal diet was inadequate. He used his experimental observations to illustrate the point that many substitutes for mother's milk might be seriously deficient in both water-soluble and fat-soluble vitamins.

University College, 1919–1929. By 1918 Drummond was already recognised as an expert on nutrition and he received the degree of Doctor of Science. In this year also he was appointed to succeed Funk as Physiological Chemist to the Cancer Hospital. A year later he moved, on the invitation of Professor E. H. Starling, to University College, London. Here he was made Reader in Physiological Chemistry in 1920 and in 1922 he was appointed to the new Chair of Biochemistry at the College. At this time Katharine Coward, who was later appointed Head of the Nutrition Department of the Pharmaceutical Society of Great Britain, came to work under Drummond on the fat-soluble accessory food substance, vitamin A. Their joint publications over the years 1920–25 form the framework of much of the fundamental study of vitamin A in foodstuffs and in pharmaceutical preparations such as cod liver oil. Drummond's interest in the practical side of the problem took him into the Norwegian fishing industry to investigate the processing of cod liver oil and other fish liver oils. His interest in vitamin A did not preclude attention to vitamin D. In collaboration with S. S. Zilva and J. Golding, research was carried out on the relation of fat-soluble vitamins to rickets and growth in pigs, and on feeding cod liver oils to cows in winter. The origin of fat-soluble vitamins in fish liver oils was investigated with the aid of marine biologists, and studies were made on the chemical nature of vitamin A. An important observation made in 1920 with the help of O. Rosenheim on the relation of lipochrome pigments to vitamin A was the first indication of the relation between carotene and vitamin A. Unfortunately the animal tests in this early study gave negative results. Some years later it was shown that the preparation of the doses of carotene in ethyloleate had permitted oxidation of the pigment so that its activity was not demonstrated. Moreover it was not then appreciated that carotene in solution was highly susceptible to oxidative destruction by exposure to light. Drummond himself, in a communication at a meeting of the Biochemical Society, took full responsibility for the initial error of observation. This was a gesture characteristic of the

scientific stature and human generosity of the man which endeared him to those who were privileged to work in his laboratories and under his guidance.

Although in the years 1920–30 much of Drummond's attention was given to vitamin A and other matters relating to fats and fat-soluble vitamins D and E, his interest in proteins and in water-soluble vitamins was maintained. The physiological role of vitamin B was under investigation and some of the early work on the complex nature of vitamin B came from his department. At this time the Department of Biochemistry at University College was small and was part of the Department of Physiology and Biochemistry in the Medical Sciences Faculty, but the impetus of the research under Drummond soon made the department an important centre which exerted an enormous influence on the growing subject of biochemistry. At the time of his death in 1952 no fewer than nine of his pupils were Professors of Biochemistry in Great Britain or in other countries. By 1925 scientists from many countries were anxious to join Drummond's research group. They were made welcome and the international exchange of ideas was one of the most valuable aspects of research in the Department of Biochemistry.

With all the research activity there was no neglect of the teaching of medical students and of undergraduates who studied biochemistry as part of a degree in physiology. Drummond's own lectures were inevitably stimulating and he naturally attracted a strong team of other teachers. He preferred to lecture on the aspects of biochemistry which belong to the field of nutrition, but his field of vision was wide and allowed a full participation in the teaching programme. If all medical students could be inspired by as stimulating a teacher as Drummond, their subsequent attention to problems of nutrition might be more extensive. Much of the distinguished medical research of the past 30 years at University College Hospital and other centres derives from men and women who had premedical biochemistry training from Drummond.

Expanding interests, 1929–1939. In his second decade at University College nutrition became the paramount interest in Drummond's life. His biochemistry department was running smoothly in the hands of well-tried colleagues and he was able to develop practical ideas on the urgent problems of nutrition arising from the economic crisis of 1931. He had also had some field experience of nutritional deficiencies beginning with a visit to the fishermen of Newfoundland in the course of a study of the cod liver oil industry. There he observed nutritional deficiencies among the people caused by the lack of the very substance present in the products they prepared. He surveyed this and related problems in the Lane Medical Lectures which he was invited to give at Stanford University, California, in 1933. He gave a Harvey Lecture in New York in the same year and also the Harben Lectures of the Royal Society of Arts. In these public lectures he gave a panoramic view of the modern ideas on the functions of the vitamins. His able exposition of such subjects in a way which was intelligible to a lay audience and at the same time of interest to more scientific minds led to extensive demands on his time for lectures in many countries. This was freely given for he enjoyed travel and communication with others of like mind. One of his most extensive journeys was in 1936 under a short-term Fellowship from the Rockefeller Foundation when he visited Holland, Germany, Czechoslovakia, Austria, Hungary, Switzerland, Poland, Russia, Finland, Sweden, Norway and Denmark in order to study nutritional conditions. He had for many years been interested in the "Peoples' League of Health," an organization founded in 1917 by Miss Olga Nethersole, C.B.E., in Great Britain. This organization endeavoured to help and to educate people in the use of food especially in the stringency of unemployment and other situations leading to poverty and poor nutrition and Drummond was its constant advisor. He became involved in an advisory capacity with many other bodies concerned with nutrition including the Ministry of Health, the British Medical Association. and the Health Section of the League of Nations.

During this time he was beginning to look into the historical aspects of diet and nutrition. His foreword to "The Schoolboy,

His Nutrition and Development," by G. E. Friend (1935) is an admirable example of his historical knowledge of his subject. After a look into the further past he makes some excellent comments on the nutrition lessons of the 1914–18 war and goes on to say:

"It is a disheartening task to search the wreckage of our post-war world in the hopes of finding some benefit, however small, which the upheaval brought directly or indirectly to mankind. One there is, although it is seldom recognized. It is the vast progress and powerful stimulus to further work in the field of nutritional research for which the food problems of the war years were to a large extent responsible. Few people adequately realise how extensive have been the advances made in nutrition and in the field of practical dietetics during the past 20 years. Already as a result of these labours the standard of national physique is noticeably improving in this country and the florid forms of nutritional disorders, once so common, are fast becoming rarities.

"But in spite of this progress there is yet a long way to go and in no direction is greater guidance needed than where the feeding of school children is concerned. How deplorably the subject has been neglected we are only just beginning to appreciate. Almost entirely ignored by the medical profession until recent years, its treatment by those responsible for the provision and preparation of school diets was, with relatively few notable exceptions, deplorable for a long time. Nor are the parents free from blame. Many a well-to-do father has given far greater attention to the feeding of his own horses, dogs or farm stock than to the diet on which his son might be subsisting at a famous public school."

This introduction to a most useful treatise on adolescent nutrition was written about the same time as a chapter on "The Vitamins" contributed to a "Textbook of Biochemistry," edited by B. Harrow and C. P. Sherwin. In his department, research was in progress on fat-soluble vitamins A, D and E, on various B vitamins, on carbohydrate and protein problems. And in every moment he could find his historical researches were proceeding apace. Here he was happily aided by his secretary, Anne Wilbraham, who in 1940 became his second wife. Their joint labours were given first in a delightful series of lectures at the Royal Society of Arts (J. Roy. Soc. Arts, 1938, 76, 191; 214; 246). The greatly expanded text was published in 1939 as "The Englishman's Food. Five Centuries of English Diet." This enthralling history of diet and dietary practices was the culmination of Drummond's preparatory work for the tremendous part which he was to play as nutritional adviser in the war years and the years of recovery.

The war years. On the outbreak of war in 1939 Drummond was given leave from the University to be Chief Adviser on Food Contamination and as a result of his initial activities a manual on "Food and its Protection from Poison Gas" was published from the Ministry of Food in 1940. With his broad vision of nutrition he was by no means fully occupied with the food contamination work. His own University department had temporarily been moved to Wales, but he kept some academic research going at the Courtauld Institute of Biochemistry at the Middlesex Hospital, London, by courtesy of the Director, Professor E. C. (later Sir Charles) Dodds. On February 1, 1940, Drummond was officially appointed Scientific Adviser to the Ministry of Food. Immediately prior to this official appointment he had submitted to the Ministry of Food a memorandum, "On certain nutritional aspects of the food position," in which he reviewed the pre-war nutritional position of Great Britain and the probable effects on it of wartime conditions, particularly in relation to the poorer sections of the population. He stressed the need for providing bread of high nutritive value, for increasing the consumption of potatoes, oatmeal, cheese and green vegetables, for supplying not less than a pint of milk a day to expectant and nursing mothers and to all children up to the age of 15, and for fortifying margarine with vitamins A and D. He drew attention to the value of the lean meat of the pig as a source of vitamin B, and at the same time made it clear that there was no special need for large supplies of meat provided adequate supplies of cheese and fish were available.

Within a very short time after his appointment, his knowledge and influence were clearly reflected in the programme of food inports for the second year of the war, to which was appended "A Survey of War-time Nutrition." This set out in detail Drummond's views on nutritional strategy. In the light of the history of nutrition in the 1914–18 war he made strong recommendations against measures to reduce the country's total consumption of food. He was uniquely equipped to see the problems of food supplies and nutritional demands as a whole and his personal success in collaborating with statisticians and economists produced a food plan for which Great Britain must always be profoundly grateful. He reviewed the food situation of the first world war and endeavoured to plan to avoid the nutritional failures arising in those years. A comprehensive rationing system spread supplies evenly over the population and reviews of home production showed the quota of essential nutrients that could be provided from full use of resources, and the amounts which must be imported in order to balance the dietary budget. Increased imports were advised of cheese, dried and condensed milk, canned fatty fish and dried pulses. Concentrates of vitamins A and D for adding to margarine were considered an import priority. He recommended that imports of fruit, other than oranges, nuts and eggs in shell should be reduced as they were wasteful of shipping space.

Recommendations were made also for satisfying the vitamin C requirement by increased consumption of potatoes and for increasing the vitamin B_1 supply by raising the extraction rate of flour or by fortifying white flour with synthetic vitamin B_1. The fortification of flour with vitamin B_1 was favoured by many as it left a larger share of milling products for feeding pigs and poultry. However the Medical Research Council Memorandum of August, 1940 (Lancet, 1940, 2, 1943) strongly recommended bread based on a flour of 80 to 85% extraction. Dr. T. Moran, Director of the British Flour Millers' Research Association, was closely involved in all the discussions and studies of the bread problem. Experimental work on flours of 72, 80, 82½, 85% and higher extractions was carried out under the guidance of the Accessory Food Factors Committee of the Lister Institute and the Medical Research Council. As a result of the studies the Ministry of Food in 1941 raised the extraction rate of flour to 85% and fortified the flour with calcium to ensure that the daily intake of this essential nutrient should be satisfactory for all groups of the population at a time when milk, eggs and meat were in short supply. The flour and bread orders were only one expression of Drummond's determination that the nutritional value of the British diet should not merely be maintained but should be improved under war-time conditions.

He was fully aware of modern advances in food technology and encouraged the importing of dehydrated foods to improve protein supplies. Large quantities of dried eggs and dried milk were thus obtained and his appreciation of the difficulties of the housewife in handling unusual foods led to the information talks and leaflets for which the Ministry of Food became famous. Every opportunity was taken to teach simple nutrition in all sections of the community. For this purpose the Food Advice Service of the Ministry was established and staffed with trained dietitians able to help interpret the ideas to the people.

Another valuable contribution to good nutrition and good health with which Drummond was closely associated was the provision of special foods for mothers and children. He was acutely aware of the special needs of vulnerable groups and as a result of his efforts schemes were evolved for cheap supplies and priority rationing of fresh milk, for provision of blackcurrant syrup, rose-hip syrup, concentrated orange juice and cod liver oil as sources of vitamins. There were also generous allowances of rationed foods allocated for school meals. It is a measure of Drummond's sense of history that the provision of welfare foods for mothers and children has been carried on beyond the end of the war and beyond the end of rationing as a permanent requirement for the health of the population.

Another great enterprise begun by the Ministry of Food under its strenuous Scientific Advisor was the Household Dietary Survey. This began in 1941 and is still

carried on as a check on the diet of representative samples of the population.

The work of the Ministry of Food and the Ministry of Health in the distribution of food supplies was recognized in 1947 by the Lasker Group Award of the American Public Health Association. The citation gives at length the details of the work of the two ministries in maintaining and even improving health under war time stress and recommends awards to the Ministries and to the four leaders concerned: Lord Woolton, Sir Jack Drummond, Sir Wilson Jameson and Sir John Boyd Orr.

With all his commitments in the Ministry of Food, Drummond still found time to keep in touch with his University Department which from 1941 was at Leatherhead in Surrey. From 1941–1944 he was Fullerian Professor of Physiology in the Royal Institution and during his tenure of this Chair he gave four courses of lectures on nutritional subjects and two Friday evening discourses. He was concerned also in international discussions on nutrition. When the nutritional situation in Malta became serious in 1942 he flew out to review it himself and to advise on relief measures. In 1943 he was delegate to the Hot Springs Conference in Virginia so that he was present at the planning sessions which led to the formation of the Food and Agriculture Organization of the United Nations.

Post-war period. In 1943 Drummond became Chairman of the Hospital Catering and Diet Committee of the King Edward's Hospital Fund for London and continued to advise on hopital food services until 1949. He was the first President of the Hospital Caterers' Association.

Towards the end of the war he became adviser on nutrition to S.H.A.E.F. and to the Allied Post-War Requirements Bureau. One of his first tasks was the relief of the starving Dutch people before hostilities had finally ceased. A characteristic side line of this terrific effort was his visit to his old friends, Professor and Mrs. Jansen in Amsterdam, to assure them of the safety of their son from whom they had not heard for some years since his escape from occupied Holland.

Many well deserved honours came to Drummond for his work in the Ministry of Food. He was knighted in 1944 and in the same year he became a Fellow of the Royal Society. In 1946 he received the U. S. Medal of Freedom with Silver Palms and was elected an Honorary Member of the New York Academy of Sciences. He was made a Commander (Civil Division) of the Order of Orange Nassau for his services to Holland. The University of Paris conferred on him the Degree of Doctor *honoris causa.*

In 1945 he accepted appointment as Director of Research to Boots Pure Drug Company at Nottingham and resigned his chair of Biochemistry at University College. He did not go to Nottingham until 1946 because he was still involved as nutritional adviser to the Control Commissioners in Germany and Austria.

Very soon after he had taken up his research post with Boots Pure Drug Company he was elected to the Board of Directors of the Company. Here again his wide experience and his ability to understand and work with all sorts of people gave him a full outlet for his energies. He still continued to travel and to exert his valuable personal influence for the solution of diverse problems of industry and research. He was a powerful catalyst in the process of lowering barriers of reaction between scientists in industry and in academic situations.

The bibliography relating to Drummond's publications runs to nearly 200 titles yet he found time in life for so very many other things than work. He was no mean artist and could quickly illustrate a point of design on paper. He appreciated art in pictures, in the theater and in music. Moreover he showed those who worked with him the way towards appreciation of the finer things of life. He was good company and enjoyed good food and wine. He liked dancing and often organized parties from his Department to dances and entertainments of the societies to which he belonged. He was gay and debonair and seemed enternally young. He made friends quickly but displayed a tonic critical faculty that had a profound effect on all who had contact with him in his many spheres of influence.

He took life with both hands and made great use of his opportunities, so that the news of the murder on August 4, 1952, of Sir Jack and Lady Drummond and their ten-year-old daughter while they were on camping holiday in France caused infinite dismay to all who had known them. Although he was 61 at the time of his death he was described as a man of 40 by a French newspaper reporter who came to the scene of the crime before the identity of the victims was established. This is perhaps a last tribute to his youthful vitality.

Those of us who were his pupils at University College or his colleagues at the Ministry of Food will for years to come continue to be inspired by his achievements and to try to carry on the progress of nutrition.

Alice M. Copping
Queen Elizabeth College,
University of London,
London, W.8., England

Effect of Dietary Restriction of Pregnant Rats on Body Weight Gain of the Offspring[1]

BACON F. CHOW AND CHI-JEN LEE
Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

ABSTRACT The effect of dietary restriction on various parameters of nutriture has been reported by a number of investigators. In all these studies, the restriction was imposed at various ages after birth. Our present study utilizes the method of dietary restriction but differs from others in that the restriction was imposed on the mother during either the gestation or lactation periods, or both. Its effect on the body weight gain and other characteristics of the offspring are reported. Restriction during gestation and lactation of the dietary intake of the rats by as little as 25% of that consumed by the unrestricted group, resulted in growth stunting of the progenies, anemia and a decreased resistance to hypothermia. Similar effects were observed when the dietary restriction was imposed during the period of gestation only. These adverse effects could be corrected by pituitary extract or growth hormone when the hormone was administered shortly after weaning, but not 3 months after birth.

The effect of dietary restriction on the various parameters of nutriture has been the subject of many studies (1–6). In all these reports the restriction was imposed after birth and at various ages after birth. Our present study utilizes the method of dietary restriction, but differs from others in that this stress was imposed on the *mother* during the gestation or lactation periods, or both. Its effects on body weight gain and other characteristics of the offspring are reported here. This communication contains data obtained in similar studies conducted initially in 1954 and later confirmed and expanded in 1962.

EXPERIMENTAL PROCEDURE

Design of experiment. A typical experiment involved the use of 8 groups of 3 females each, chosen at random from a population 3 to 4 months of age. Under our standard mating conditions, these together with 8 males, 3 to 4 months of age, comprised 8 breeding groups. Of these groups, 3 were assigned for unrestricted intake, the remainder were restricted to levels of either 0.5 or 0.75 of the intake of the unrestricted females established by the following technique.

The 9 females in the unrestricted groups were transferred to individual cages at 9:00 AM on day 1 of the breeding period and each offered a measured amount, usually 35 g stock diet. After 8 hours, the unconsumed food plus spillage was measured and the food consumption measured by difference. Each of the females was then returned to the original breeding cage to reconstitute the original groups. On day 2, the average amount of food consumed determined for the 9 females maintained with the unrestricted diet was calculated and quantities corresponding to 0.5 or 0.75 that amount were weighed into individual feed cups and offered to the 15 females in the 5 restricted groups, after they had been distributed among individual cages in the morning. In most instances, these animals consumed the entire ration offered.

Similarly on day 2, the intake of the unrestricted group was measured and thereafter daily. These daily values then constituted the baseline for setting the intakes for the restricted groups for the balance of the experiment. The regimen described was followed in some experiments during the period of gestation and lactation, and in others during gestation only.

After the litters were cast, litter size was arbitrarily adjusted so that no female was left to nurse more than 8 young, regardless of the previous dietary history. In this way such obvious complications as might arise from infection, unequal distribution of milk and other variables that might re-

Received for publication August 5, 1963.
[1] This research was supported by U. S. Public Health Service grants AM 07210-01 and HD 00475-07.

sult from excess litter size, were substantially avoided.

Composition of stock diet. In the first experiments, begun in 1954, a stock diet whose composition is given in table 1 was used. In more recent studies, a commercial preparation[2] was used. Both diets supported satisfactory reproduction and growth of our stock animals. Fresh kale was provided in ample amounts 3 times every week.

Hormone preparations used. One hundred milligrams hog pituitary powder[3] were extracted with 100 cm^3 0.85% NaCl solution, adjusted to pH 5.6 and centrifuged. The clear supernatant was used for subsequent injection. Growth hormone[4] with an activity of 1 USP unit/mg from bovine origin was also used. Fifteen milligrams were weighed on a Roller-Smith balance and dissolved in 50 cm^3 normal saline solution.

Laboratory animals. In both studies, rats from our colony of the original McCollum strain were used. Our standard mating procedure is to house one male rat of established fertility with 3 females in a breeding cage (64 × 32 × 50 cm), with wood shavings. Under ordinary conditions, all our rats become pregnant within 4 weeks. When definite physical signs of pregnancy are established by gross observation or by increase in body weight, the females are then transferred to individual cages of the same size and kept there with their litters until the weanlings are separated at 21 days of age into individual cages of 18 × 18 × 23 cm.

This mating procedure was used in the preceding experiments.

TABLE 1
Stock diet

	kg
Whole wheat	13.5
Maize	9.1
Rolled oats	8.2
Whole milk powder	5.7
Skimmed milk powder	5.7
Casal [1]	0.5
Liver powder	0.9
Sodium chloride	0.23
Calcium carbonate	0.12
Ferric citrate	0.1
Copper sulfate	0.02

[1] A protein preparation of the Crest Foods Company, Ashton, Illinois, containing crude casein and lactalbumin.

RESULTS

Effect of dietary restriction during gestation and lactation periods on reproduction and body weight changes in pregnant rats. Table 2 summarizes the observations of a

[2] Guaranteed analyses: crude protein not less than 23.0%.

[3] Courtesy of Armour and Company, Chicago.

[4] Courtesy of Merck Sharp and Dohme, Rahway, New Jersey.

TABLE 2
Effect of dietary restriction during gestation and lactation periods on reproduction

Treatment [1]	Pregnancies/ female	Young/ litter	Avg fetal wt	Mean Δ in maternal body wt [2]
			g	*g*
UR-1	3/3	10	6.0 ± 0.5 [3]	40
UR-2	3/3	11	7.0 ± 0.7	48
UR-3	3/3	9	7.0 ± 0.7	37
Mean		10	6.7	41.7
R-1	2/3	2	6.0 ± 0.0	5
R-2	2/3	6	4.0 ± 0.5	1
R-3	2/3	6	4.5 ± 0.5	−5
R-4	2/3	7	3.0 ± 1.1	6
R-5	1/3	10	4.5 ± 0.8	−10
R-6	1/3	8	4.0 ± 1.1	−10
Mean		6	4.3	−1.3

[1] UR indicates pregnant rats fed ad libitum during the test periods; R indicates restriction of dietary intake of pregnant rats to one-half that of the unrestricted group.

[2] Mean change in maternal body weight indicates weight after delivery minus the initial body weight before mating.

[3] SE of mean.

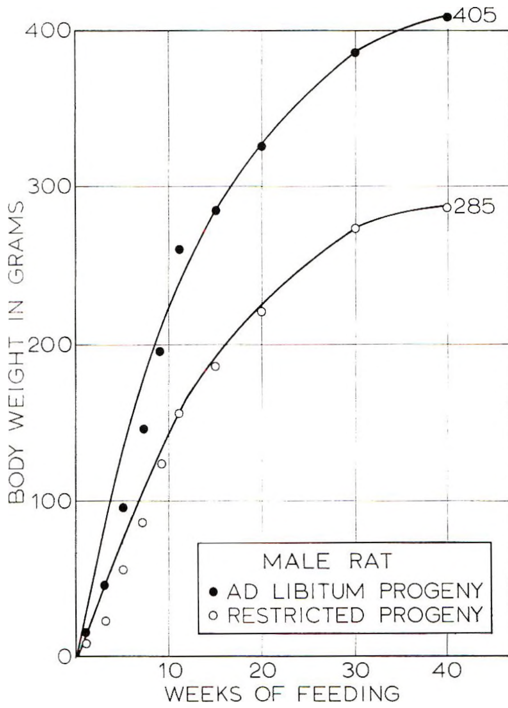


Fig. 1 Growth rates of male rats born to mothers whose dietary intake was unrestricted, or restricted to one-half that of the unrestricted group. Data from study conducted in 1954 with stock diet prepared in our laboratory are plotted.

typical experiment and shows quite clearly the adverse effects of restriction on reproduction as measured by all parameters reported. The levels of restriction chosen allowed the females to go through pregnancy with virtually no weight loss.

Weight gain of the offspring from mothers with or without dietary restriction. In studies conducted in 1954 and 1962, the body weight changes of both male and female offspring from both groups of mothers were recorded weekly during the first 4 weeks after weaning, bi-weekly up to 6 months of age and monthly thereafter. Our routine procedure calls for the removal of feed early in the morning and weighing the animals at least 2 hours later. The results of one of the 1954 studies are shown in figures 1 and 2. Our own stock diet was used. The mean body weights of the offspring in the restricted group were consistently below those of the unrestricted group for both sexes. The lower weight was observed for as long as one year afterwards.[5] The plateau values where the mean body weights finally level off indicate that the offspring of the restricted groups had a distinctly lower body weight; in the case of the male, by as much as 25 to 30% ,

[5] Results after 40 weeks not included in figures 1 and 2 due to differences in mortality rates between the 2 groups.

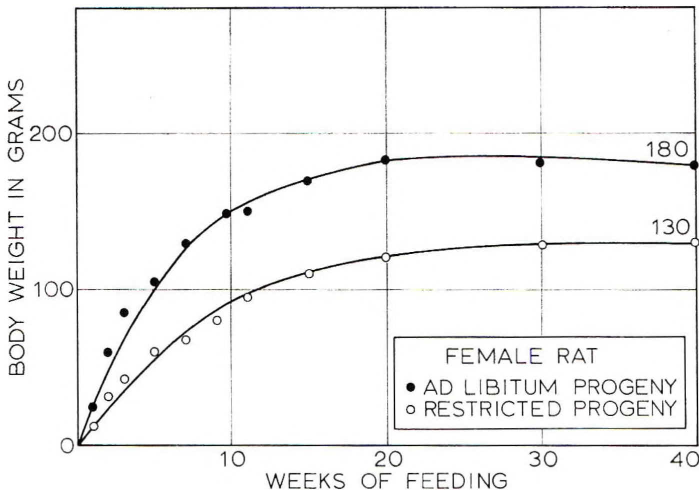


Fig. 2 Growth rates of female rats born to mothers whose dietary intake was unrestricted or restricted to one-half that of the restricted group. Data from study conducted in 1954 with stock diet prepared in our laboratory are plotted.

and in the female rat by 15%. In our 1962 experiment, the commercial laboratory chow diet was used. The results plotted in figure 3 are essentially similar to those just discussed.

Effect of pituitary hormone on the correction of growth retardation. The gonads of the animals in the restricted group appeared to be underdeveloped. We undertook, therefore, to determine whether the animals from the restricted mothers suffered from pituitary atrophy. To this end, weanlings from the unrestricted group and from the restricted group were each subdivided into 2 groups. One of these received an injection of hog pituitary extract 3 times/week for 4 weeks, whereas the other received saline injections as controls. All animals were given the stock diet ad libitum and weighed weekly. The growth rate of these animals is recorded in table 3. The injection of hog pituitary extract promptly corrected the growth retardation of the restricted animals, but had no effect on the unrestricted animals. This corrective effect persisted for at least the 8 months during which we observed these animals. In another experiment similar pituitary extracts were given to young from the unrestricted mothers 3 months after weaning. No corrective effect was observed.

Crude pituitary extract contains a number of factors. To determine whether any growth hormone in the pituitary preparation was responsible for the restoration of growth rate, we injected a highly purified growth hormone preparation 3 times/week for 3 weeks. This preparation was used in place of the crude pituitary extract, and administered to male rats of the restricted and unrestricted animals. The results of this more recent experiment are given in figure 4. Growth hormone in the dosages given had essentially the same effect as crude pituitary extract in stimulating the growth rates of the restricted animals. It is likely that this, then, is the hormone responsible. Data in figure 4 also demonstrate that injection of the growth hormone had no effect on the growth rate of the unrestricted animals.

Anemia. Hematocrit and hemoglobin determinations were made on blood sam-

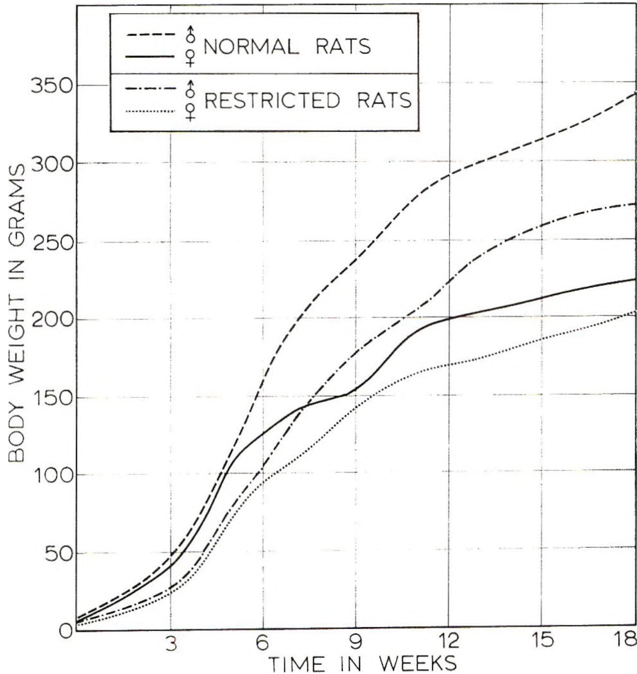


Fig. 3 Growth rates of male and female rats fed unrestricted and restricted diets. The reduction in the dietary intake was 25%. The commercial laboratory chow diet was used.

TABLE 3

Effect of administration of crude hog pituitary extract on body weight of rats fed restricted diet [1]

Weeks after injection	Body weights			
	Male		Female	
	Saline solution	Hog pituitary extract	Saline solution	Hog pituitary extract
	g	*g*	*g*	*g*
0	28.7 ± 1.0 [2]	29.8 ± 1.6	22.7 ± 1.0	22.1 ± 0.7
1	38.8 ± 1.1	43.7 ± 1.4	34.0 ± 0.8	42.0 ± 1.0
2	76.8 ± 2.0	92.7 ± 2.5	62.0 ± 1.4	81.0 ± 1.4
3	118.3 ± 3.0	138.0 ± 3.1	78.0 ± 1.8	111.0 ± 2.0
6	182.4 ± 3.6	231.0 ± 4.8	117.0 ± 1.2	159.0 ± 3.0
9	226.0 ± 4.1	301.0 ± 4.7	134.0 ± 2.7	196.0 ± 5.0

[1] Eight rats (21 to 24 days weanling) were used in each group. Hog pituitary extract or a saline solution was administered subcutaneously 3 times/week for 3 weeks only.
[2] SE of mean.

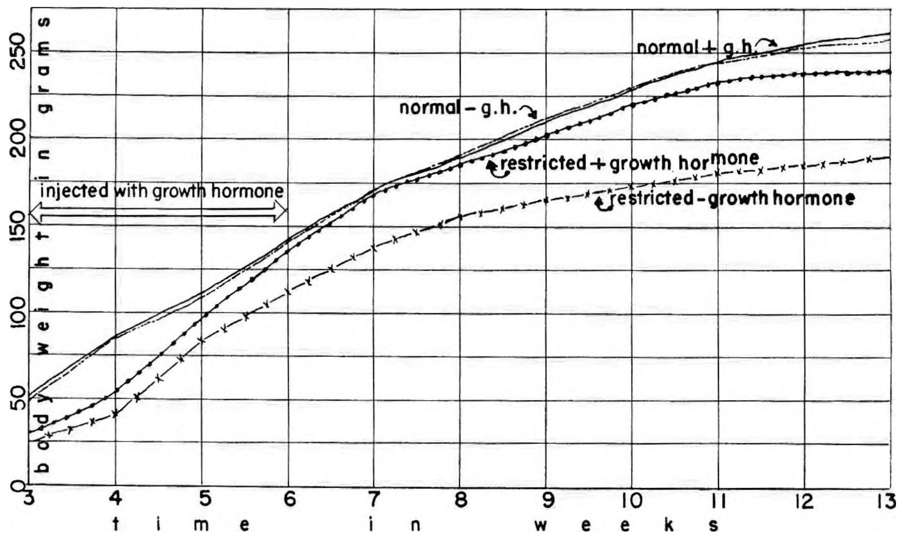


Fig. 4 Effect of growth hormone on growth of normal and restricted rats. Purified growth hormone (0.3 mg) was injected subcutaneously to restricted and unrestricted rats at 3 weeks of age every other day for 3 consecutive weeks, after which no injection was given. The growth rates are plotted.

ples from rats of both groups at varying ages. Table 4 shows the data for females of both groups at 6 and 24 weeks of age. The differences in the hematologic picture which were evident at 6 weeks of age were absent at 24 weeks of age despite the persistence of gross effects as indicated by weight difference.

Effects of hypothermia. To determine the abilities of rats of the 2 groups to withstand a physical stress such as hypothermia, 6-month-old rats of both sexes were placed in individual plastic boxes (perforated for adequate heat transfer). These boxes were small enough to immobilize substantially the animals, yet allowed enough room for limited movement. The boxes were then lowered into a large tank of ice water mixture kept at 0°C. The survival of both groups of animals is recorded in table 5. None of the 10 male animals and one out of the 10 females in the unrestricted group died after 15 minutes of exposure, whereas 6 out of 10 males and

TABLE 4

Hematocrit and hemoglobin analyses of female rats of different ages fed restricted and unrestricted diets

Treatment [1]	Age					
	Six weeks			Twenty-four weeks		
	Body wt	Ht	Hb	Body wt	Ht	Hb
	g	*%*	*g/100 ml*	*g*	*%*	*g/100 ml*
UR	149 ± 5.0 [2]	44 ± 1.1	13.2 ± 0.54	250 ± 6.2	47.0 ± 0.68	14.5 ± 0.22
R	78.6 ± 3.9	38 ± 1.2	11.6 ± 0.47	215 ± 6.4	47.1 ± 0.85	14.5 ± 0.24

[1] UR indicates pregnant rats fed ad libitum during the test periods; R indicates restriction of dietary intake of pregnant rats to one-half that of the unrestricted group.
[2] All results are expressed as averages of the mean ± SE of mean.

TABLE 5

Mortality rate in hypothermia [1]

Sex	UR [2]	R [2]
Male	0/10	6/10
Female	1/10	7/10

[1] Fifteen-minute exposure at 0°C.
[2] UR indicates pregnant rats fed ad libitum during the test periods; R indicates restriction of dietary intake of pregnant rats to one-half that of the unrestricted group.

7 out of 10 females in the restricted group failed to survive.

Effect of duration of restriction period. The previously described experiments were carried out maintaining restricted intake of the mothers in the restricted groups up to the time of weaning. In table 6, data are reported on an experiment in which dietary restriction was stopped at the end of the period of gestation. Again the effects of restriction include some evidence of resorption (few pregnancies), smaller litters, slightly smaller mean fetal weights and persistent failure to attain the same weight as controls from mothers fed ad libitum during gestation.

DISCUSSION

Numerous investigations have reported the effects of dietary restriction on rats and other species of animals of all ages. The major observations of these studies, particularly as they apply to present investigations, are that dietary restriction retards the growth and delays the maturation of animals and that these processes are frequently reversible when ad libitum feeding is reinstated. This reversibility is well exemplified in figure 5 which summarizes a study of the effect of intermittent starvation and ad libitum feeding. Another major observation from studies of this genera

TABLE 6

Effect of dietary restriction during the gestation period only

Treatment [1]	Pregnancy	Young/litter	Avg fetal wt	Body wt of progenies			
				Three months		Eight months	
				Male	Female	Male	Female
	%		*g*	*g*	*g*	*g*	*g*
UR	100 (3) [2]	9	6 ± 0.2 [3]	210 ± 20 (4)	146 ± 7 (4)	380 ± 26	266 ± 27
	100 (3)	10	5.6 ± 0.4	205 ± 10 (5)	132 ± 11 (3)	410 ± 11	218 ± 14
	100 (3)	9	7 ± 0.2	196 ± 5 (5)	150 ± 12 (3)	375 ± 22	250 ± 12
Mean	100	9.3	6.2	203.7	142.7	388	244.7
R	67 (3)	5	5 ± 0.4	135 ± 18 (4)	100 ± 6 (4)	266 ± 14	156 ± 12
	67 (3)	6	4.8 ± 0.2	126 ± 10 (3)	96 ± 12 (5)	284 ± 22	142 ± 18
	67 (3)	6	4.6 ± 0.4	148 ± 21 (2)	110 ± 8 (6)	310 ± 18	176 ± 14
	50 (3)	7	4.2 ± 0.1	138 ± 6 (6)	118 ± 17 (2)	305 ± 30	185 ± 22
Mean	62.8	6	4.6	136.8	106	291.2	164.8

[1] UR indicates pregnant rats fed ad libitum during the test periods; R indicates restriction of dietary intake of pregnant rats to one-half that of the unrestricted group.
[2] Numbers in parentheses denote number of animals/group.
[3] SE of mean.

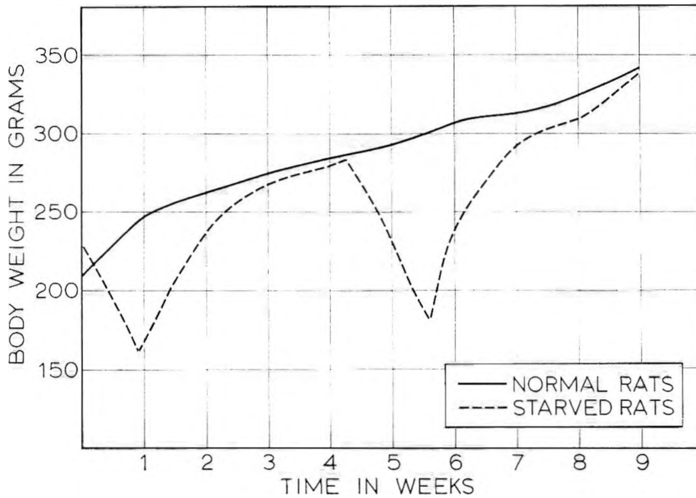


Fig. 5 Growth rate of intermittently starved and ad libitum-fed rats. One group of adult rats (6 months old) were starved until their body weights decreased by one-third and were then fed ad libitum until their body weights reached a plateau. They were then starved again for another period, resulting again in a loss of one-third of their original body weight. They were again fed ad libitum. Their growth rates were compared with those of comparable normal rats on whom no starvation had been imposed.

is the observation that when moderate restriction of dietary intake is imposed and maintained, a very significant prolongation of life span is observed.

Smith (2) has summarized many of these experiments and has discussed at length the various parameters used to measure the effects of dietary restriction, although in many instances no precise quantitative measurements were reported. In virtually all of the studies, dietary restriction was imposed on the young after birth or at varying times after birth. In almost no instance were restrictions imposed *in utero* or for that matter, even during lactation, and only recently did McCance (5) report studies in which stresses were imposed during lactation by the device of controlling the size of the litter being nursed. When litter size ranged from as many as 18 to as few as 3, abnormal situations were induced where limiting factors over and above pure nutritional considerations entered. Where the litter size exceeds the number of teats a "survival of the fittest" situation is induced where the weaker offspring are unable to be accommodated. Moreover, the possibilities of cross infection multiply with larger litter size. McCance recognized these limitations and was appropriately conservative in drawing conclusions from his experiments. Nevertheless, it was demonstrated that under these conditions differences in body weight of groups characterized by small and large nursing litter size persisted for 12 months or more following inauguration of ad libitum feeding at weaning.

It has often been stated that in the earlier stages of gestation the fetus receives an adequate supply of nutrients from the mother. It is only in the later stages that the nutritional status of the mother may become important to the future development of the offspring. Still unproven is the belief that large women tend to give birth to large babies (7,8) and that poor nutrition of the mother towards the end of gestation can reduce the size of the newborn. It has been cited that the weights of twin lambs can be markedly reduced without decreasing the period of gestation (9–11). In addition, it is believed by some obstetricians and veterinarians that the size of a baby or an animal at birth is dependent upon the number of young produced with a single pregnancy. However, this relationship does not necessarily hold with all species of animals. These generalizations have been published, but require

further experimental support from systematic and controlled studies.

In the present investigation we were interested in studying the effect of maternal diet of rats not only on the birth weights, but also on the weight gain of the progeny over a long period of time, extending to 1.5 years or approximately three-fifths of the life span of our stock animals. In our 1954 studies, we observed that progeny from mothers fed a restricted diet weighed less than two-thirds that of the controls even after 1.5 years of ad libitum feeding. It is premature at this time to interpolate our results to human studies, although it is possible also, with some bias, to grope for support from clinical reports. The present studies introduce the stress *in utero* by restricting the food intake of the pregnant rat. The stress, therefore, appears to be fundamentally different and the changes following it significantly prolonged. Over and above the retardation of weight gain, our observations also include marked changes in the hematologic picture during the first 6 months of life and lowered resistance to a specific external stress such as artificially induced hypothermia. Whether the altered resistance to hypothermia can be extrapolated to other stresses, or merely reflects lower body fat, remains to be elucidated.

Similar but somewhat more marked effects appear to follow as restriction of dietary intake of the mother is continued during the lactation period but it is not possible from the data in hand to assess the relative importance of these 2 periods.

Our studies suggest strongly the importance of pituitary growth hormone in the mechanism giving rise to the growth anomaly. Although Hruza et al. (6) have attempted similarly to incriminate growth hormone, the experiments they reported used an excessive amount of preparation and failed to show any responses in less than 6 weeks after administration was started. From general considerations it may well be that their results are better explained on the basis of spontaneous reversal rather than specific growth hormone effects. It is always attractive to assume that effects such as we here described can be explained by invoking the law of the limit. However, it does not appear that our results can be explained satisfactorily by assuming an insufficient intake of a single limiting nutrient. In the first instance we have observed similar effects using 2 types of diets of entirely different composition. Moreover, our stock diet provides at least 2 to 3 times the known requirements of all vitamins and other nutrients and, since the effects were observed with 25% dietary restriction, this level of reduction of intake could hardly have induced specific nutrient lack. Actually, in one of our experiments, additional supplements of known vitamins were given to the unrestricted group and the growth stunting was again observed. Moreover, none of the usual secondary biochemical or clinical signs of known vitamin deficiencies were observed, although we examined for evidence of pyridoxine deficiencies by xanthurenic acid excretion or pyridoxal phosphate level in red cells, and for vitamin B_{12} deficiency by determination of vitamin B_{12} activity in serum or liver.

Dwarfism due to zinc deficiency is known and has been studied extensively by Prasad et al. (12). It has been assumed that zinc deficiency is extremely difficult to achieve experimentally, particularly when the experimental animals are housed in metal cages, as ours were. Moreover, simple calculation from the zinc content of our diets tends to make zinc deficiency an unlikely explanation. Perhaps the observed effects arise from deficiency of one or a combination of amino acids or of some unknown vitamin or other nutritional factor(s). Caloric insufficiency alone is not likely to explain these results since restoration to ad libitum feeding does not achieve prompt reversal. Further studies are projected to explain these alternatives.

LITERATURE CITED

1. Osborne, T. B., and L. B. Mendel 1915 The resumption of growth after long continued failure to grow. J. Biol. Chem., 23: 439.
2. Smith, A. H. 1931 Phenomena of retarded growth (editorial review). J. Nutrition, 4: 427.
3. McCay, C. M. 1935 The effect of retarded growth upon the length of life span and upon the ultimate body size. J. Nutrition, 10: 63.
4. Widdowson, E. M., and R. A. McCance 1960 Some effects of accelerating growth. I. Gen-

eral somatic development. Proc. Royal Soc. Lond. series B., *152:* 188.

5. McCance, R. A. 1962 Food, growth and time. Lancet, *2:* 621.
6. Hruza, Z., P. Fabry, M. Chvapil and E. Holeckova 1962 Medical and Clinical Aspects of Aging — Adaptation to undernutrition and trauma and influence of over-nutrition on biological aging, ed., Herman T. Blumenthal. Columbia University Press, New York and London, p. 42.
7. Widdowson, E. M. 1955 Reproduction and obesity. Voeding, *16:* 94.
8. McKeown, T., R. G. Record 1957 The influence of body weight on reproductive function in women. J. Endocrinol., *15:* 410.
9. Wallace, L. R. 1948 The growth of lambs before and after birth in relation to the level of nutrition, part 1. J. Agr. Sci., *38:* 93.
10. Wallace, L. R. 1948 The growth of lambs before and after birth in relation to the level of nutrition, parts 2, 3. J. Agr. Sci., *38:* 243.
11. Wallace, L. R. 1948 The growth of lambs before and after birth in relation to the level of nutrition, part 3 (continued). J. Agr. Sci., *38:* 367.
12. Prasad, A. S., A. Miale, Jr., Z. Farid, H. H. Sandstead and A. S. Schulert 1963 Zn metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism and hypogonadism. J. Lab. Clin., *61:* 537.

Effect of Degree of Fatty Acid Unsaturation in Tocopherol Deficiency-induced Creatinuria[1]

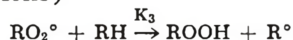
L. A. WITTING AND M. K. HORWITT
L. B. Mendel Research Laboratory, Elgin State Hospital, Elgin, Illinois, and Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

ABSTRACT The rate of development of creatinuria, in the tocopherol-deficient rat proved to be dependent on the degree of unsaturation of the dietary fatty acids. Data were obtained consistent with relative in vivo rates of fatty acid peroxidation of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic fatty acids in the ratios 0.025:1:2:4:6:8, and tocopherol requirements for constant diminution in the rates of peroxidation in the ratios 0.3:2:3:4:5:6. Muscle phospholipid fatty acid compositions were dependent on the compositions of the dietary fats but were independent of the level of dietary fat between 7.5 and 19%. Within this range the rates of development of creatinuria and the tocopherol requirements were also independent of the level of dietary fat. One milligram of *d*-α-tocopheryl acetate per kilogram rat per week delayed the onset of creatinuria by 17 to 20 weeks. When the diet contained suboptimal levels of selenium and sulfur amino acids, creatinuria occurred 30% more rapidly and the tocopherol requirement increased tenfold. Lower levels of the most highly unsaturated fatty acids were noted in the muscle phospholipids of the tocopherol-deficient animals. This is consistent with the concept that susceptibility toward peroxidation increases as the number of double bonds per fatty acid increases.

A variety of pathological conditions arise in experimental animals fed tocopherol-deficient diets. These include encephalomalacia, exudative diathesis, and myopathy (nutritional muscular dystrophy) in the chick; myopathy, testicular degeneration, resorption gestation, and liver necrosis in the rat; steatitis in the cat; and other similar conditions in the rabbit, lamb, monkey, and calf. In many, if not all of these conditions, there exists a complex interrelationship between dietary concentrations of sulfur amino acids, biologically available selenium, unsaturated fat, and tocopherol or other non-toxic, fat-soluble antioxidants (1–4).

All 3 of the deficiency states in the chick may be prevented by supplementation with synthetic fat-soluble antioxidants (5–7) and the rat has been maintained with a tocopherol-deficient diet for 3 generations by supplementation with a synthetic fat-soluble antioxidant (8, 9). Since the major, if not sole, function of α-tocopherol is as a biological fat-soluble antioxidant, the possibility exists that the onset of deficiency symptoms can be related to a specific level of lipid peroxidation in the tissue. In this case, the *rate* of production of deficiency should be related to the degree of unsaturation of the various fatty acids rather than to the total amount of unsaturation in the dietary fat. That is to say, one mole of arachidonate (tetraene) would be expected to give rise to more than twice as much peroxidized material as one mole of linoleate (diene) in the same period of time under identical conditions.

This expectation arises from a consideration of the kinetics of autoxidation in vitro (10). It is known that free radical initiation, $RH \rightarrow R^{\circ}$ and uptake of oxygen $R^{\circ} + O_2 \rightarrow RO_2^{\circ}$ are rapid reactions, not dependent on the nature (degree of unsaturation, monoenoic, dienoic, etc.) of the R group (where R designates the fatty acid). The overall rate of oxidation must therefore be dependent on the rate of the slowest reaction (1/10,000 the rate of the preceding reactions)



$$RO_2^{\circ} + RH \xrightarrow{K_3} ROOH + R^{\circ}$$

which is extremely dependent on the nature of the R group. Although metal complexes and the presence of protein are expected to modify the reaction scheme in vivo, these factors should not affect the specific de-

Received for publication August 8, 1963.
[1] Supported in part by Illinois Mental Health Fund, The National Vitamin Foundation, Inc., and U.S.P.H.S. Grant A-1126 and Grant NB-03523-02.

pendence on degree of unsaturation of the rate-limiting reaction. With a diet sufficiently free of tocopherol or other fat-soluble antioxidants, fatty acid peroxidation in the animal tissues may be inevitable, but the *rate* of peroxidation should be dependent on the fatty acid composition of the tissue as influenced by the dietary fat.

It is known (11) that increasingly greater amounts of α-tocopherol are required to prevent the autoxidation of oleic, linoleic, and linolenic acids in vitro. In the rate equation (10) for the inhibited autoxidation reaction

$$d\,O_2/d\,T = K_1\,K_3\,[RH]/K_4\,[AH],$$

AH designates tocopherol,

K_4 arises from $RO_2^{\circ} + AH \xrightarrow{K_4} ROOH + A^{\circ}$,

and K_1 describes the initiation process.

As the unsaturation of the fatty acid is increased, the ratio K_3/K_4 increases, and must be balanced by the addition of α-tocopherol to decrease the ratio [RH]/[AH]. This phenomenon may also occur in vitro.

The present experiment[2] was undertaken to investigate the quadruple interaction of selenium, sulfur amino acids, tocopherol, and unsaturated fatty acids using an assay based on the occurrence of creatinuria, as a symptom of myopathy, in the tocopherol-deficient rat (12). Various levels of 7 different synthetic fats were fed with graded levels of tocopherol with and without added selenium and methionine.

Muscle lipids were analyzed and the fatty acid composition related to the onset of creatinuria and possibly to the occurrence of lipid peroxidation in vivo.

MATERIALS AND METHODS

Preparation of synthetic fats. Naturally occurring fats, coconut oil, corn oil, linseed oil, and cod liver oil, were saponified, and the nonsaponifiables, including tocopherol, extracted into petroleum ether. The fatty acids obtained after acidification were dried and methylated in the presence of 2,2-dimethoxypropane, methanol, and 2% sulfuric acid. After neutralization, extraction of the product into petroleum ether, and removal of the solvent, the methyl esters were molecularly distilled and then rearranged with glycerol triacetin under vacuum at 80 to 120° in the presence of 0.5% sodium methoxide. When evolution of methyl acetate was complete, a stoichiometric amount of water was added to destroy the catalyst and the resulting soaps were removed by filtration. Double distilled oleic acid, low in linoleic acid, was obtained commercially[3] and used for the preparation of triolein. Synthetic fats were stored until used in filled mason jars under nitrogen at − 5°.

The extent of unsaturation was estimated by determining the Hanus iodine value (13) and also by calculating a theoretical iodine value from the fatty acid composition determined by gas-liquid chromatography (14). The agreement between methods was excellent. The monoenoic fat, largely triolein, with an iodine value of 82 supplied 79, 60, 40 and 22 mmoles of unsaturation (RCH = CHR′) per 100 g of diet when fed at 25, 19, 12.5 and 7.5% levels, respectively. Sufficient saturated fat, essentially palmitoyldistearin (hydrogenated beef fat),[4] was included in the rearrangements so that each of the other synthetic fats (table 1) also had an iodine value of 82. One millimole of R″CH = CHCH$_2$CH = CHR‴ was considered to constitute 2 mmoles of unsaturation. Accordingly, a millimole of trienoic, tetraenoic, pentaenoic, and hexaenoic acid would constitute 3, 4, 5, and 6 mmoles of unsaturation, respectively, and therefore, each synthetic fat also supplied 79, 60, 40 and 22 mmoles of unsaturation per 100 g of diet when fed at the 25, 19, 12.5 and 7.5% levels, respectively. A saturated synthetic fat, based on coconut oil rather than hydrogenated beef fat for reasons of digestibility, was included in the study. This fat supplied small amounts of linoleic and oleic acids, and was included to evaluate the effect of small amounts of the essential fatty acid, linoleic, in a saturated fat as compared with its effect in the almost completely unsaturated monoenoic fat.

Tocopherol determinations. The synthetic fats contained small amounts of Emmerie-Engel reactive material (15), but

[2] Preliminary reports of this work were given at the 1962 and 1963 meetings of the Federation of American Societies for Experimental Biology, Atlantic City, N. J.: L. A. Witting, and M. K. Horwitt 1962 Rat tocopherol needs as a function of n in $(CH_2CH = CH)_n$. Federation Proc., *21:* 474; L. A. Witting, and M. K. Horwitt 1963 The influence of fatty acid structure on relative peroxidizability *in vivo*. Federation Proc., 22: 608.

[3] Emery Industries, Cincinnati, Ohio.

[4] Armour and Company, Chicago, Illinois.

TABLE 1
Fatty acid composition of synthetic dietary fats

Fatty acid type	Dietary fat [1]						
	Saturated [2]	Monoenoic	Dienoic	Trienoic	"Mixed fat" no. 1	"Mixed fat" no. 2	Polyenoic
Saturated, %	87.4 [3]	11.7 [4]	42.5 [4]	58.5 [4]	64.8 [4]	58.8 [4]	62.4 [4]
Monoene, %	9.6	81.1	21.2	10.0	12.0	17.3	20.6
Diene, %	3.0	7.2	36.0	8.4	4.6	13.1	2.2
Triene, %	—	—	0.3	23.1	12.1	0.1	1.7
Tetraene, %	—	—	—	—	1.4	1.9	2.3
Pentaene, %	—	—	—	—	3.2	4.1	5.6
Hexaene, %	—	—	—	—	2.9	5.1	5.2
Iodine value	12.4	82.7	82.0	82.0	82.0	82.0	82.0
mmole unsaturation/ 100 g fat	47	318	316	316	316	316	316
Estimated peroxidizability × 100	3.2	9.2	37.1	54.8	77.1	86.5	91.3
Tocopherol, $\mu g/g$	< 0.1	< 0.1	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25

[1] For size of groups see tables 2 and 3.
[2] Average composition from 0 to 28 weeks. Changed at 18 weeks so that average composition from 0 to 50 weeks was saturated, 90%; monoene, 7.8%; and diene, 2.2%; and estimated peroxidizability (× 100), 2.4.
[3] Largely lauric and myristic acids.
[4] Largely palmitic and stearic acids.

after chromatography on zinc ammine carbonate impregnated paper (16), it could be shown that less than 0.25 µg of tocopherol was present per gram of dienoic, trienoic, and polyenoic fat. The saturated and monoenoic fats were either free of tocopherol or contained less than 0.1 µg/g of fat. When small amounts of α-tocopherol were added to duplicate samples, recoveries were in the 85 to 95% range. Although it could not be proven that they were completely free of tocopherol, these fats probably contained less tocopherol than any others previously reported.

Animals and diets. Male weanling rats of the Sprague-Dawley strain were used. The basal diet fed was similar to that described by Century and Horwitt (17) except that dextrose replaced the cornstarch, since the latter material contains tocopherol. Protein content, as casein, was constant at 21.8% of calories and the carbohydrate content was modified isocalorically to compensate for the various fat concentrations (25, 19, 12.5 and 7.5%). These diets were fed to the animals in the A series. Since casein is known to be suboptimal in sulfur amino acid content (18) and also occasionally to be low in selenium (19), 0.4% DL-methionine and 0.13 ppm of selenium as sodium selenite were added to the rations in the B series. In this case salt mix Illinois 446 (20) replaced salts USP XIV. The *d*-α-tocopheryl acetate in coconut oil was given by mouth, by dropper, 3 times a week. Tocopherol dosage (0.2, 0.4, 0.5, 0.6, 1.0, 1.75, and 15 mg *d*-α-tocopheryl acetate/kg rat body weight/week) was calculated for the average animal weight in 100-g brackets (50 to 150 g; 150 to 250 g; 250 to 350 g, etc.).

Urinary creatine and creatinine. Twenty-four-hour urine samples were collected from the rats every 2 to 4 weeks and creatine and creatinine concentrations were determined by the method of Bonsnes and Taussky (21).

Fatty acid analyses. The gastrocnemius and quadriceps muscles were excised rapidly after death and homogenized in methylal-methanol (14) containing 0.01% α-tocopherol. Neutral lipids and phospholipids were separated by thin layer chromatography (22) and the fatty acid composition determined by gas-liquid chromatography as described previously (14). Tocopherol was present during all stages of sample preparation and storage.

TABLE 2

Time of onset of creatinuria in terms of creatine-to-creatinine ratios; record of initial periods during which significant increases were noted (series A: no added selenium or methionine)

Type of dietary fat	Polyenoic [1]				"Mixed Fat" no. 1		Trienoic														
Tocopherol supplement, mg/kg/rat/week	0		0		0		0		0		0		0	0.2		0.4		0.6		2.4	
% fat in diet	7.5		19		19		7.5		12.5 [2]		19		25	7.5		7.5		7.5		12.5 [2]	
Period during which significant creatinuria appeared, weeks	4	6	4	6	4	6	7	9	6	8	6	9	4 [3]	6	8	5	7	5	7	10	12
Creatine-to-creatinine ratio	0.30 0.31 0.35 0.38 0.54 0.75 1.10	0.67 1.03 1.25 1.36 1.69 1.78 2.35	0.18 0.27 0.30 0.30 0.31 0.35 0.37 0.40 2.54	0.36 0.38 0.39 0.46 0.52 0.85 0.96 1.18 1.32	0.29 0.30 0.31 0.31 0.35	0.35 0.45 0.79 0.95 1.32	0.11 0.17 0.20 0.23 0.31 0.39 0.66	0.78 1.15 1.49 1.71 2.09 2.40 2.62	0.25 0.26 0.34 2.40 3.14	0.56 0.96 2.03 2.57 3.51	0.27 0.27 0.30 0.33 0.33 0.34 0.35 0.39	0.65 1.77 1.82 2.04 2.06 2.36 2.79 2.96	0.77 0.97 0.98 1.28 1.34 1.37 1.42	0.22 0.34 0.38 0.54 0.73	0.50 0.78 0.82 1.04 1.14	0.28 0.35 0.36 0.36 0.41	0.30 0.36 0.44 0.56 0.80	0.26 0.27 0.28 0.29 0.55	0.58 0.69 0.71 0.78 0.87	0.25 0.26 0.27 0.32 0.48	0.21 0.24 0.57 0.57 0.67
Proportion showing positive creatinuria	3/7	7/7	2/9	6/9	0/5	4/5	1/7	7/7	2/5	5/5	0/8	8/8	7/7	2/5	5/5	1/5	3/5	1/5	5/5	1/5	3/5
Breakpoint, weeks	5		5		6		8		7		7		3	7		7		6		12	

Type of dietary fat	Dienoic												Monoenoic				Saturated	
Tocopherol supplement, mg/kg/rat/week	0		0			0	0.2		0.4		0.6		0		0		0	
% fat in diet	7.5		19			25	7.5		7.5		7.5		19		25		19	
Period during which signficant creatinuria appeared, weeks	7	9	6	8	8 [4]	5	6	8	7	9	5	7	15	17	8	10	24	28
Creatine-to-creatinine ratio	0.14	0.28	0.25	0.44	0.30	0.96	0.22	0.31	0.23	0.31	0.33	0.28	0.17	0.15	0.33	0.38	0.12	0.18
	0.20	0.55	0.26	1.63	0.36	1.48	0.22	0.51	0.27	0.33	0.35	0.32	0.21	0.18	0.36	0.54	0.19	0.21
	0.21	0.61	0.34	2.37	0.71	1.51	0.23	0.90	0.28	0.44	0.37	0.37	0.24	0.29	0.37	0.73	0.20	0.28
	0.23	0.64	0.35	2.65	1.44	1.98	0.26	0.98	0.38	0.50	0.39	0.76	0.25	0.47	0.39	1.21	0.25	0.47
	0.26	0.66	0.41	3.09	1.50	2.11	0.86	1.86	0.78	0.51	0.67	0.76	0.87	1.02	0.43	1.67	0.28	0.55
	0.26	0.93	1.15	3.24	1.65	2.43					1.15	0.80	0.97	1.42	0.45	1.73	0.51	0.60
	0.27	0.93	2.36	5.40		2.49						1.12	1.01	1.59	0.82	1.82	0.65	0.65
																	0.93	1.01
Proportion showing positive creatinuria	0/7	6/7	3/8	8/8	4/6	7/7	1/5	4/5	1/5	3/5	2/7	4/7	3/7	4/7	3/7	6/7	3/8	5/8
Breakpoint, weeks	8		7		8	4	8		9		7		17		9		28	

	Polyenoic	Trienoic	Dienoic	Monoenoic	Saturated
Creatine-to-creatinine ratios for tocopherol-supplemented [5] rats	0.248 ± 0.106 [6]	0.193 ± 0.055	0.190 ± 0.077	0.183 ± 0.075	0.183 ± 0.076
No. of determinations	39	44	25	25	19
$P = 0.005$ [7]	0.539	0.329	0.416	0.401	0.407

[1] See table 1 for composition and estimated peroxidizability of fats designated by major source of unsaturation.

[2] Salt mix Illinois 446 (20) without added selenium.

[3] An infantile creatinuria persists for approximately 3 weeks after weaning. During this period deficient animals showed creatine-to-creatinine ratios greater than 0.4 but did not differ significantly from tocopherol-supplemented rats fed the same diet.

[4] Group repeated 3 months after original experiment (creatine-to-creatinine determined at this one time period only).

[5] 15 mg *d-α*-tocopheryl acetate/kg/rat body weight/week.

[6] Average ± SD.

[7] Probability that an animal having a creatine-to-creatinine ratio equaling or exceeding the ratio given is a member of the normal tocopherol-supplemented group.

TABLE 3

Time of onset of creatinuria in terms of creatine-to-creatinine ratios; record of initial periods during which significant increases were noted (series B: selenium and methionine added to basal ration)

Type of dietary fat	Polyenoic [1]																"Mixed fat" no. 2		"Mixed fat" no. 1	
Tocopherol supplement, mg/kg/rat/week	0		0		0.2		0.4		0.5		0.6		1.0		1.75		0		0	
% fat in diet	7.5		19		7.5		7.5		19		7.5		19		19		19		19	
Period during which significant creatinuria appeared, weeks	8	10	6	8	10	12	12	14	15	18	20	22	24	28	35	37	5	7	6	8
Creatine-to-creatinine ratio	0.08	0.24	0.30	0.60	0.23	0.48	0.18	0.19	0.18	0.23	0.17	0.14	0.16	0.10	0.27	0.37	0.27	0.22	0.18	0.29
	0.12	0.31	0.32	0.60	0.23	0.51	0.19	0.20	0.18	0.34	0.23	0.31	0.18	0.17	0.30	0.44	0.31	0.33	0.19	0.31
	0.16	0.38	0.37	0.70	0.25	0.51	0.34	0.40	0.20	0.35	0.38	0.40	0.18	0.41	0.39	0.50	0.32	0.60	0.23	0.49
	0.22	0.47	0.41	0.85	0.27	0.60	0.36	0.46	0.22	0.49	0.41	0.44	0.21	0.48	0.68	0.50	0.36	0.98	0.27	0.93
	0.31	0.84	1.33	1.29	0.31	0.63	0.48	1.87	0.24	0.55	0.50	0.76	0.29	0.49			0.42	1.20	0.31	1.23
	0.37	1.15							0.24	0.57			0.31	0.54						
	0.51	1.44							0.28	0.63			0.34	0.54						
									0.29	0.67			0.36	0.58						
									0.32	0.76			0.87	0.68						
													0.95	1.14						
Proportion showing positive creatinuria	1/7	4/7	2/5	5/5	0/5	5/5	1/5	3/5	0/9	6/9	2/5	3/5	2/10	8/10	1/4	3/4	1/5	3/5	0/5	3/5
Breakpoint, weeks	(10) [2]		7		11		14		17		22		26		37		7		8	

Type of dietary fat	Trienoic												
Tocopherol supplement, mg/kg/rat/week	0	0	0	0	0.2	0.2	0.4	0.4	0.5	0.6	0.6	1.0	1.75
% fat in diet	7.5	12.5 [3]	19	25	7.5	12.5 [3]	7.5	12.5	19	7.5	12.5 [3]	19	19
Period during which significant creatinuria appeared, weeks	7 9	8 10	6 9	4 [4]	10 12	10 12	20 22	14 16	15 18	20 22	19 21	19 22	43 46

Creatine-to-creatinine ratio	0.09 0.10 0.11 0.13 0.16 0.19 0.35	0.18 0.48 0.55 0.63 0.67 1.47 1.68	0.20 0.22 0.26 0.30 0.41	0.25 0.48 0.49 0.58 1.01	0.25 0.28 0.29 0.30 0.33	0.30 0.41 0.52 0.62 1.20	0.37 0.37 0.39 0.41 0.42 0.45 0.52	0.08 0.09 0.16 0.51 0.72	0.27 0.50 0.54 0.55 0.93	0.21 0.21 0.26 0.29 0.35	0.51 0.89 1.00 1.21 1.92	0.17 0.18 0.19 0.24 0.34	0.15 0.42 0.44 0.63 0.88	0.18 0.22 0.26 0.47 0.76	0.23 0.42 0.54 0.65 0.80	0.11 0.13 0.19 0.21 0.70	0.20 0.34 0.43 0.47 0.66	0.16 0.17 0.17 0.21 0.51	0.40 0.41 0.73 0.92 1.05	0.14 0.20 0.24 0.24 0.25	0.65 0.77 0.92 0.97 1.06	0.21 0.22 0.35 0.36 0.67	0.10 0.25 0.42 0.72 1.83	0.17 0.20 0.24 0.65	0.33 0.45 0.50 0.50
Proportion showing positive creatinuria	0/7	6/7	1/5	4/5	0/5	4/5	4/7	2/5	4/5	0/5	5/5	0/5	4/5	2/5	4/5	1/5	3/5	1/5	5/5	0/5	5/5	1/5	3/5	1/4	3/4
Breakpoint, weeks	9		9		9		4	11		12		(22) [2]		15		18		21		21		22		46	

Type of dietary fat	Dienoic																	Monoenoic				Saturated	
Tocopherol supplement mg/kg/rat/week	0		0		0	0.2		0.4		0.5		0.6		1.0		1.75		0		0.5		0	
% fat in diet	7.5		19		25	7.5		7.5		7.5		7.5		19		19		19		19		19	
Period during which significant creatinuria appeared, weeks	7	9	9	14	5 [4]	12	14	20	22	19	22	22	24	24	28	35	38	15	19	35	40	46	49
Creatine-to-creatinine ratio	0.17 0.18 0.20 0.21 0.22 0.24 0.62	0.27 0.64 0.67 0.77 0.78 1.27 1.28	0.25 0.26 0.28 0.29 0.56	0.50 0.54 0.61 0.98 1.09	0.38 0.39 0.44 0.46 0.46 0.46 0.48	0.14 0.18 0.25 0.28 0.82	0.14 0.24 0.43 0.52 0.93	0.09 0.17 0.19 0.20 0.44	0.12 0.27 0.40 0.44 0.56	0.12 0.18 0.19 0.30 0.75	0.20 0.61 0.77 1.63 2.02	0.23 0.24 0.31 0.35 0.57 0.58 1.44	0.13 0.15 0.17 0.42 1.04	0.17 0.24 0.26 0.32 0.58	0.25 0.74 1.95	0.26 0.27 0.28 0.29 0.31	0.12 0.38 0.43 0.57 0.72	0.10 0.14 0.15 0.24 0.29 0.37	0.11 0.46 0.58 0.69 0.86 1.09	0.12 0.16 0.16 0.16 0.19 0.26	0.25 0.26 0.27 0.40 0.51 1.04	0.17 0.18 0.18 0.21 0.22	0.19 0.55 0.95
Proportion showing positive creatinuria	1/7	6/7	1/5	5/5	5/7	1/5	3/5	1/5	3/5	1/5	4/5	3/7	4/7 [5]	1/5	2/3	0/5	3/5	0/0	5/6	0/6	3/6	0/5	2/3
Breakpoint, weeks	(8) [2]		12		5	14		22		22		24		26		38		(18) [2]		40		49	

[1] See table 1 for composition and estimated peroxidizability of fats designated by major source of unsaturation.
[2] The values in parentheses are not consistent with the data in the remainder of the table.
[3] Illinois 446 (20) plus 0.10 ppm selenium rather than 0.13 ppm.
[4] An infantile creatinuria persists for approximately 3 weeks after weaning. During this period deficient animals showed creatine-to-creatinine ratios greater than 0.4 but did not differ significantly from tocopherol-supplemented rats on the same diet.
[5] Two rats with high creatine-to-creatinine ratios killed at 23 weeks.

RESULTS

Onset of creatinuria. The creatine-to-creatinine ratios used in the estimation of the time of onset of creatinuria in the animals fed the various synthetic fats in the series A ration are shown in table 2. Data from animals fed the series B ration are presented in table 3. For a description and examples of complete curves on the course of development of creatinuria see Horwitt et al. (12). To avoid the recording of over 2300 creatine-to-creatinine determinations, only the results of periods just before and just after the onset of significant elevation are included in the tables. From statistical considerations, it was apparent that a creatine-to-creatinine ratio greater than about 0.4 was indicative of positive creatinuria since it could be calculated that the probability of such a value occurring in a tocopherol-supplemented animal fed these diets was only 0.005. The higher urinary creatine-to-creatinine ratios and greater standard deviation of the average ratio found for the tocopherol-supplemented animals fed the polyenoic fat, is an experimental artifact, later related to the frequency with which these animals were supplemented with tocopherol. More frequent supplementation, i.e., daily, instead of 3 times a week, leads to normal ratios.

Effect of degree of unsaturation. The data in tables 2 and 3 indicate that the time required for the production of creatinuria as a symptom of nutritional myopathy in rats fed diets without added vitamin E is dependent on the nature of the dietary fat. The fats that produced the earliest creatinuria (5 to 7 weeks) were those (table 1) in which a given amount of unsaturation was highly concentrated in a relatively small percentage of the fatty acid chains, rather than being distributed evenly as in the case of the monoenoic fat, where the production of creatinuria required 17 to 18 weeks. When the data from the 19% fat diets were plotted against an estimated peroxidizability slightly modified from that previously published (23), (fig. 1) two hyperbolae resulted. Peroxidizability is calculated as percentages of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic, hexaenoic fatty acids in the diet multiplied

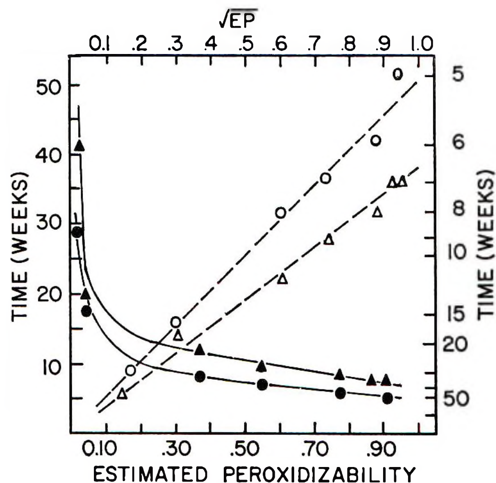


Fig. 1 Time in weeks required to produce creatinuria versus the estimated peroxidizability of the dietary fat (solid line) and time in weeks required to produce creatinuria (reciprocal scale) versus the square root of the estimated peroxidizability of the dietary fat (broken line). Data from 19% fat groups in series A are indicated by circles and comparable data from series B by triangles.

by 0.025, 1, 2, 4, 6, and 8, respectively. These numerical values are based on the in vitro maximal rates of oxidation reported by Holman (24). The question of interaction of rates of autoxidation in a mixture has been explored by Gunstone and Hilditch (25). A straight line was obtained when the rate of production of creatinuria (1/T) was plotted against the square root of the calculated peroxidizability. Addition of selenium and methionine to the ration increased the time required for the development of creatinuria by approximately 40% with any given synthetic fat.

Tocopherol supplementation. When the graded levels of tocopherol used in this study were administered in series A, little protective effect was noted. One milligram of *d-α*-tocopheryl acetate per kilogram rat per week delayed the onset of creatinuria by about 2 weeks. In series B, in which selenium and methionine were added, the onset of creatinuria was delayed by approximately 17 to 20 weeks per milligram of tocopherol when the dienoic, trienoic, and polyenoic fats were fed and approximately 45 weeks when the monoenoic fat was fed (fig. 2). The tocopherol require-

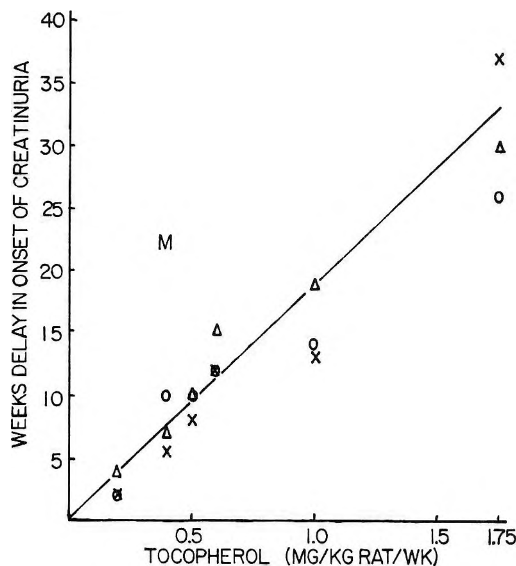


Fig. 2 Delay in onset of creatinuria versus tocopherol dosage. Delay in weeks required to produce creatinuria at a given level of tocopherol supplementation minus weeks required to produce creatinuria without tocopherol supplementation. Data from diets containing polyenoic, dienoic, trienoic, and monoenoic synthetic fats are represented by the symbols: triangle, cross, circle, and M, respectively.

ment appeared to be dependent on total unsaturation except in the case of the monoenoic unsaturation.

Effect of BHA. In an effort to evaluate the effects of possible oxidation of dietary lipid prior to ingestion, butylated hydroxyanisole (BHA) was added to the polyenoic dietary fat at the 0.02% level. Two groups receiving 0.5 and 1.0 mg *d*-α-tocopheryl acetate/kg rat/week were compared to identical groups receiving the same fat not protected by the addition of this antioxidant. The BHA had no effect on the rate of development of creatinuria.

Effect of dietary fat on muscle fat composition. The fatty acid composition of the rat muscle (gastrocnemius and quadriceps) phospholipids and neutral lipids (table 4) were markedly affected by the different dietary lipids. If the tissue compositions reported for the saturated synthetic fat diet are taken as a base line, it will be noted that although the monoenoic fat led to similar linoleic (dienoic) and arachidonic (tetraenoic) acid levels in the phospholipids, there was a marked increase in hexaenoic acid. Furthermore, an increase in the unsaturation of the neutral lipid occurred. With added dienoic fat, there was an increase of tetraene in the phospholipid and large amounts of diene were noted to "overflow" into the neutral lipid. Although the trienoic fat contributed very little linolenate to the phospholipids, and greatly depressed the arachidonate level, an increase in pentaenes and hexaene was noted and the neutral fat was high in linolenate. The polyenoic fat led to an extremely high concentration of the docosahexaenoic acid in the phospholipids but did not furnish large amounts of fatty acids suitable for deposition in the neutral lipids. The extreme degree of unsaturation of these fatty acids, however, made their contribution to the estimated peroxidizability of the neutral fat quite important.

Effect of muscle fat composition on onset of creatinuria. If the suggestion of Century et al. (23) as modified herein, regarding correlation of muscle fatty acid composition to rate of development of creatinuria is applied to the present data (fig. 3) a striking relationship is noted. From the discontinuous nature of the curve in the region where polyunsaturated fatty acids begin to "overflow" into the neutral lipid, it is apparent that peroxidizability of the phospholipid fatty acids is of primary importance, but that neutral lipid fatty acids also affect the rate of development of creatinuria.

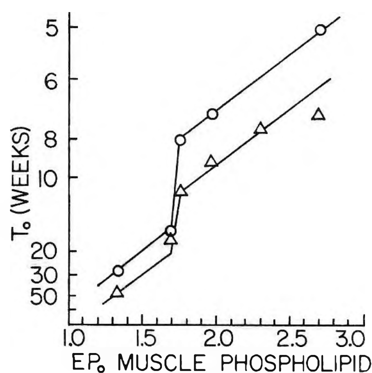


Fig. 3 Time in weeks required to produce creatinuria versus the estimated peroxidizability of the muscle phospholipid fatty acids. Data from 19% fat groups in series A are indicated by circles and comparable data from series B by triangles.

TABLE 4

Effect of level and type of synthetic dietary fat on the fatty acid composition of muscle[1] neutral lipids and phospholipids

Synthetic dietary fat,%	Phospholipid									
	Saturated (19%)	Monoenoic (19%)	Dienoic (7.5%)	Dienoic (19%)	Trienoic (7.5%)	Trienoic (12.5%)	Trienoic (19%)	"Mixed" no. 2 (19%)	Polyenoic (7.5%)	Polyenoic (19%)
Fatty acid composition, %	(3)[2]	(5)	(3)	(9)	(4)	(4)	(6)	(4)	(4)	(8)
Monoenoic	22.0 ± 1.0[3]	23.8 ± 1.0	9.9 ± 0.8	8.7 ± 2.3	11.8 ± 1.7	10.4 ± 0.9	9.7 ± 1.4	9.8 ± 1.5	19.0 ± 2.4	15.0 ± 2.8
Dienoic	18.6 ± 3.8	14.1 ± 0.5	21.0 ± 0.2	21.2 ± 2.5	20.6 ± 2.1	21.3 ± 2.0	21.3 ± 1.1	21.0 ± 1.2	5.3 ± 1.4	8.1 ± 1.9
Trienoic	2.4 ± 0.8	1.9 ± 0.3	0.6 ± 0.1	0.4 ± 0.2	2.8 ± 0.3	2.3 ± 0.9	2.7 ± 1.0	0.5 ± 0.2	0.4 ± 0.2	0.5 ± 0.2
Tetraenoic	15.8 ± 3.2	15.0 ± 1.4	18.3 ± 0.4	21.4 ± 1.0	5.8 ± 1.4	6.6 ± 1.5	4.7 ± 0.9	4.4 ± 1.1	2.8 ± 0.7	4.0 ± 1.4
Pentaenoic	3.5 ± 0.7	2.8 ± 1.0	4.5 ± 0.7	4.7 ± 1.6	10.2 ± 1.7	8.1 ± 1.6	9.1 ± 1.2	5.2 ± 0.8	9.9 ± 4.2	5.4 ± 1.2
Hexaenoic	2.6 ± 0.7	9.0 ± 1.0	5.1 ± 0.5	4.9 ± 1.1	10.4 ± 2.6	9.9 ± 1.1	11.9 ± 2.8	19.8 ± 1.6	26.5 ± 2.7	26.6 ± 2.2
Total PUFA[4]	42.9 ± 2.6	52.8 ± 2.7	49.6 ± 0.6	52.8 ± 1.3	49.8 ± 3.2	48.2 ± 3.5	49.8 ± 2.7	50.9 ± 1.5	45.0 ± 3.7	44.6 ± 2.9
EP_o (× 100)[5]	132 ± 24	169 ± 18	168 ± 9	174 ± 19	199 ± 18	188 ± 12	196 ± 21	229 ± 9	275 ± 30	269 ± 20

Synthetic dietary fat,%	Neutral lipid									
	Saturated (19%)	Monoenoic (19%)	Dienoic (7.5%)	Dienoic (19%)	Trienoic (7.5%)	Trienoic (12.5%)	Trienoic (19%)	"Mixed" no. 2 (19%)	Polyenoic (7.5%)	Polyenoic (19%)
Fatty acid composition, %	(3)	(5)	(1)	(9)	(3)	(5)	(6)	(4)	(4)	(8)
Monoenoic	40.7	65.4	33.2	31.3	44.0	39.3	34.6	41.8	57.7	43.4
Dienoic	2.8	9.0	36.8	36.8	9.7	10.4	9.8	15.4	1.4	2.9
Trienoic	0.2	0.2	0.2	0.5	11.6	12.5	14.3	0.1	0.2	0
Tetraenoic	0.4	0.7	1.7	1.8	0.5	0.3	0.7	0.6	0.3	1.9
Pentaenoic	0.8	0.4	0.9	0.9	1.3	0.4	2.0	0.9	0.8	1.5
Hexaenoic	0.2	0.2	0.4	0.1	0.8	0.2	1.2	0.8	0.3	2.6
Total PUFA[4]	4.4	10.4	40.0	40.1	23.9	23.8	27.9	17.5	3.0	8.7
EP_o (× 100)[5]	12 ± 5	18 ± 17	53	50 ± 5	50 ± 9	42 ± 8	63 ± 6	30 ± 3	12 ± 3	41 ± 2

[1] Gastrocnemius and quadriceps.
[2] Number of samples.
[3] SD.
[4] Polyunsaturated fatty acids.
[5] See page 31 for explanation.

As drawn, the lines are parallel and are of the same slopes on either side of the discontinuity. The tissue lipid fatty acid compositions determined for the groups fed 7.5 and 12.5% fat are comparable to those observed when 19% fat was fed. Data on the rats fed 25% fat are not currently available. Standard errors are not given for the neutral fat values since the possible errors in measuring a series of constituents at the less than 1% level by gas-liquid chromatography are quite large.

Effect of tocopherol deficiency on muscle fat composition. A group of 62 rats receiving stripped corn oil [5] at the 15% level in a series B ration was available for muscle lipid analysis. Although not a part of the study, as tabulated in tables 1–4, the comparison of muscle phospholipid fatty acids of tocopherol-supplemented and tocopherol-deficient rats is of interest, (table 5). The lipids from the tocopherol-deficient group are significantly lower in polyunsaturated fatty acids (PUFA) and the difference occurs mainly in the most highly unsaturated fatty acids. Similar data (table 6) on groups contained in tables 2-4 show a similar trend but demonstration of statistical significance suffers from the smaller group sizes.

Animals were killed within a few weeks of the first significant increase in urinary creatine-to-creatinine ratio. The decrease in polyunsaturated fatty acids was of the order of magnitude of 0.1 μmole/g wet weight of tissue.

DISCUSSION

It seems most practical to discuss the results in terms of 2 distinct but normally simultaneous phenomena. The first is the rate of production of creatinuria with an essentially tocopherol-free diet, and the second is the decrease in this rate brought about by various levels of tocopherol supplementation.

Diets essentially free of tocopherol. Under the specific experimental conditions described herein, it has been possible to relate the degree of unsaturation of various dietary fatty acids to the rate of production of creatinuria as a symptom of myopathy (nutritional muscular dystrophy) in the rat fed a ration essentially devoid of tocopherol. Certain experimental procedures must be emphasized since they are either rather unusual or are of such a nature as to prohibit comparison with various other systems.

The dietary fats used herein were specially formulated and do not correspond to any natural or commercial product. Within the limits of practically available materials, the basic fat (monoenoic, table 1) was tocopherol-free glycerol triolein containing enough linoleic acid to meet the rat's essential fatty acid requirements (approximately 1% of calories as 18:2) (26). The dienoic fat resulted from the substitution of one mole of dienoic acid (linoleic) plus one mole of saturated fatty acid for

[5] Distillation Products Industries, Rochester, New York.

TABLE 5

Comparison of fatty acids percentage composition of muscle phospholipids from tocopherol-deficient and tocopherol-supplemented rats fed 15% stripped corn oil [1]

Fatty acid	Adequately supplemented [2]	Tocopherol-deficient [3]	Significance
18:2 [4]	19.9 ± 0.34 [5]	20.0 ± 0.43	NS [6]
20:4	18.5 ± 0.31	18.1 ± 0.45	NS
22:4	1.7 ± 0.06	1.9 ± 0.08	NS
22:5	5.1 ± 0.19	3.8 ± 0.21	$P < 0.001$
22:5	1.1 ± 0.05	0.8 ± 0.08	$0.005 > P > 0.001$
22:6	5.3 ± 0.18	4.6 ± 0.27	$0.05 > P > 0.025$
PUFA [7]	53.2 ± 0.32	50.6 ± 0.64	$P < 0.001$

[1] Prepared by Distillation Products Industries, Rochester, New York, by molecular distillation.
[2] Thirty-eight rats/group.
[3] Fourteen rats/group.
[4] The first figure represents the number of carbon atoms; the second, the number of double bonds.
[5] SE of mean.
[6] Not significant.
[7] Total polyunsaturated fatty acids.

TABLE 6

Comparison of fatty acid percentage composition of muscle phospholipids from tocopherol-deficient and tocopherol-supplemented rats fed various dietary fats

Dietary fat	Fatty acid	Fatty acids, %		Significance
		Adequately supplemented	Tocopherol-deficient	
Dienoic		(9) [1]	(18)	
	18:2 [2]	20.5 ± 0.90 [3]	18.7 ± 0.33	0.05 > P > 0.025
	20:4	19.9 ± 0.40	21.0 ± 0.52	NS
	22:4	1.5 ± 0.13	1.6 ± 0.12	NS
	22:5	3.4 ± 0.30	2.8 ± 0.24	NS
	22:5	1.2 ± 0.10	0.9 ± 0.07	0.05 > P > 0.025
	22:6	4.9 ± 0.37	4.0 ± 0.21	0.05 > P > 0.025
	PUFA [4]	52.8 ± 0.43	49.5 ± 0.68	0.005 > P > 0.001
Polyenoic		(8)	(22)	
	18:2	7.8 ± 0.88	6.2 ± 0.41	NS
	20:4	4.0 ± 0.50	3.8 ± 0.23	NS
	20:5	2.6 ± 0.32	2.9 ± 0.15	NS
	22:5	2.6 ± 0.39	2.7 ± 0.13	NS
	22:6	26.6 ± 0.81	21.7 ± 1.07	0.02 > P > 0.01
	PUFA	44.6 ± 1.03	38.6 ± 1.43	0.025 > P > 0.020
Trienoic		(6)	(14)	
	PUFA	49.8 ± 1.10	48.0 ± 0.80	NS
Monoenoic		(6)	(7)	
	PUFA	42.9 ± 1.10	39.8 ± 0.72	0.02 > P > 0.01
Saturated		(3)	(7)	
	PUFA	42.9 ± 1.50	36.4 ± 1.40	0.005 > P > 0.001

[1] Number of animals.
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.
[3] SE of mean.
[4] Total polyunsaturated fatty acids.

2 moles of monoenoic fatty acid. Similarly, the trienoic fat was obtained by substituting one mole of trienoic acid (linolenic) plus 2 moles of saturated fatty acid for 3 moles of monoenoic acid. The polyenoic fat contained one mole of tetraenoic acid plus 3 moles of saturated fatty acid in place of 4 moles of monoenoic acid, one mole of pentaenoic acid plus 4 moles of saturated fatty acid in place of 5 moles of monoenoic acid, and one mole of hexaenoic acid plus 5 moles of saturated fatty acids in place of 6 moles of monoenoic acid. Mixed fat no. 1 is practically equivalent to a mixture of the synthetic trienoic and polyenoic fats, whereas mixed fat no. 2 is practically equivalent to a mixture of the synthetic dienoic and polyenoic fats. It should be understood that each fat fed at the same dietary level supplied exactly the same molar quantity of fatty acid unsaturation, but that potential peroxidizability increased as the number of double bonds per molecule increased. Although glyceride structure was "randomized" by rearrangement, it was necessary that at least one-third of the fatty acids be unsaturated to facilitate digestion.

Increasing the number of double bonds per fatty acid molecule while keeping the total number of double bonds in the fat constant increased the rate of development of creatinuria (tables 2 and 3). If this were a simple relation, i.e., if diene had twice the effect of monoene, and tetraene had twice the effect of diene, and hexaene had twice the effect of triene, no difference would be noticed on the present "constant unsaturation" diets.

Another point requires particular emphasis. Data were obtained from young, rapidly growing rats. Tissue lipid fatty acid compositions were markedly influenced by dietary fat (table 4). This experimental condition prohibits direct comparison of the results to data on animals at a stable weight or those with appreciable adipose tissue at the start of the experiment. At 7.5, 12.5 and 19% levels of any given dietary fat, the muscle phospholipid

and neutral fat fatty acid compositions were similar if not practically identical (table 4). For any given dietary fat at any of these 3 levels, the rate of production of creatinuria was probably identical (tables 2 and 3).

At the 25% level of dietary fat, it may be theorized that biosynthesis of saturated and monoenoic fatty acids from carbohydrate was suppressed to a greater extent than with the diets containing less fat. Correspondingly, it would be expected that low fat diets (less than 7.5%) would take longer to produce creatinuria since a greater dilution would occur in the tissues with biosynthetic fatty acids. These points are under investigation.

The best approximation of the relative contribution of the various unsaturated fatty acids to the production of creatinuria appears to be the ratios of maximal rates of oxidation in vitro (24); 0.025:1:2:4:6:8 for monoenoic, dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic acids, respectively. Secondary dietary deficiencies or nonspecific stresses can be expected to change the rate of production of creatinuria without changing the dependence on dietary fatty acids. The time, in weeks, required to produce creatinuria in the A series was approximately 70% of the time, in weeks, required when the same fat was fed in the B series (tables 2, 3). This relation prevailed whether a fat was fed that produced creatinuria in 7 weeks or if one was fed that took 49 weeks to produce creatinuria.

In the course of analyzing the data, an empirical predictive equation was developed which may be useful. T_0, the time in weeks required for the development of creatinuria, could be approximated from $C_1/(EP_0)^{1/2}$ where EP_0, which equals (% monoenoic acid × 0.025) + (% dienoic acid × 1) + (% trienoic acid × 2) + (% tetraenoic acid × 4) + (% pentaenoic acid × 6) + (% hexaenoic acid × 8), describes the dietary fat. With the best diets used herein, which supplied 7.5 to 19% fat, C_1 equaled 7.0. The diets that contained suboptimal levels of selenium and methionine lowered C_1 to 5. High (25%) fat diets lowered C_1 to 2.8 and in combination these stresses, high fat and suboptimal concentrations of selenium and sulfur amino acids, lowered C_1 still further to 2.3. The relation of T_0 to the square root of dietary unsaturated fatty acid percentages may describe the effective entry of these materials into tissue lipids under the present conditions. Tests of the general validity of this relationship might be derived from measurements of essential fatty acid requirements or of the lowering of elevated serum cholesterol levels (27).

Effects of tocopherol supplementation. As might be expected from the tissue lipid analyses (table 4) the tocopherol requirement was found to be independent of dietary fat level from 7.5 to 19% fat. However, at lower or higher dietary fat levels as dilution with biosynthesized saturated and monoenoic fatty acids increased or decreased, respectively, a change in tissue lipid fatty acid composition might be expected to occur and alter the tocopherol requirements accordingly. The protection afforded by tocopherol, expressed in weeks required to produce creatinuria with a given supplement of tocopherol minus weeks required to produce creatinuria with a diet essentially devoid of tocopherol, did not show an apparent dependence on the nature of the dietary unsaturated fatty acids at a constant level of unsaturation (fig. 2) except in the case of monoenoic unsaturation. Relative quantities of tocopherol required to protect one mole of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic acid, respectively, are estimated to be approximately in the ratios 0.3:2:3:4:5:6.

With a diet suboptimal in selenium and sulfur amino acids, one milligram of *d*-α-tocopheryl acetate per kilogram rat per week delayed the onset of creatinuria by approximately 2 weeks, whereas, the same amount of tocopherol with a more adequate ration gave approximately 17 to 20 weeks protection. A more extensive study of the effects of selenium and methionine individually and in combination is in progress. It is not specifically known that this is an alteration in rate without effect on the fatty acid composition dependence since neither the high tocopherol supplements necessary to resolve this question, nor the key nature of high monoenoic acid fats had been anticipated.

Another empirical predictive equation useful under these particular experimental conditions may be of interest. The T_E, the time in weeks to produce creatinuria at a given level of tocopherol supplementation (E_c), can be approximated by

$$T_E = \frac{E_c C_2}{EP_T} + T_o$$

where EP_T which equals (% monoenoic acid × 0.33) + (% dienoic acid × 2) + (% trienoic acid × 3) + (% trienoic acid × 4) + (% pentaenoic acid × 5) + (% hexaenoic acid × 6) describes the percentage composition of the dietary fat. C_2 is about 14 with a good diet but decreases rapidly (approximately tenfold) as the animal is stressed by diets suboptimal in selenium and sulfur amino acids. Given adequate data, the equations suggested might be used to evaluate the apparent relative rates of fatty acid peroxidation and tocopherol destruction in vivo.

At an early stage of tocopherol deficiency, lower percentages of the higher polyunsaturated fatty acids were detected (tables 5 and 6) in the muscle phospholipid fatty acids. Similar observations have been reported by Hove and Seibold (28) in pig liver and adipose tissue, by Bhalerao and Kummerow [6] in chick brain, and by Century and Horwitt [7] in erythrocytes, liver and muscle tissues of the chick. The lower levels of PUFA in the phospholipids of tocopherol-deficient animals are consistent with the concept that the lability to peroxidation of the PUFA increases as the number of double bonds per molecule increases. Although the required oxygen, approximately 0.1 μmole O_2/g wet weight of muscle over a period of 5 to 49 weeks, for lipid peroxidation could easily have been available, there is no direct evidence that this reaction took place. The amount of peroxidized lipid that may be in the tissue at any given time is apparently too small for incontrovertible direct chemical detection.

ACKNOWLEDGMENTS

We wish to thank Dr. Edward R. Perkins, formerly of Armour and Company, for suggestions regarding glyceride synthesis by the rearrangement reaction and Dr. R. J. Vander Wal of the same company for generously providing hydrogenated beef tallow.

The assistance of Donald Koehlert in synthesizing the dietary fats, of Ruth Nelson in preparing lipid extracts, of M. Jane Morton in performing gas-liquid chromatographic analyses and creatine-to-creatinine determinations, of Emmett Harmon in performing tocopherol analyses, and of Joseph Wilkins in caring for the animals is gratefully acknowledged.

LITERATURE CITED

1. Harris, R. S., and I. G. Wool, eds. 1962 Symposium on vitamin E and metabolism. Vitamins and Hormones, 20: 375.
2. Schultz, H. W., E. A. Day and R. O. Sinnhuber, eds. 1962 Lipids and Their Oxidation. The Avi Publishing Company. Inc., Westport, Connecticut.
3. Horwitt, M. K. 1961 Vitamin E in Human Nutrition — An Interpretative Review. Borden's Rev. Nutrition Res., 22: 1.
4. Scott, M. L. 1962 Anti-oxidant, selenium and sulphur amino acids in the vitamin E nutrition of chicks. Nutrition Abstr. Rev., 32: 1.
5. Machlin, L. J., R. S. Gordon and K. H. Meisky 1959 The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken. J. Nutrition, 67: 333.
6. Bunnell, R. H., L. D. Matterson, E. P. Singsen, L. M. Potter, A. Kozeff and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. 3. The influence of feeding or injecting various tocopherols and other antioxidants on the incidence of encephalomalacia. Poultry Sci., 34: 1068.
7. Machlin, L. J., and W. T. Shalkop 1956 Muscular degeneration in chickens fed diets low in vitamin E and sulfur. J. Nutrition, 60: 87.
8. Crider, Q. E., P. Alaupovic and B. C. Johnson 1960 On the function and metabolism of vitamin E. II. The effects of vitamin E on the level of non-vitamin E reducing compounds present in animal tissues. Biochem. Biophys. Res. Comm., 2: 293.
9. Crider, Q. E., P. Alaupovic and B. C. Johnson 1961 On the function and metabolism of vitamin E. III. Vitamin E and antioxidants in the nutrition of the rat. J. Nutrition, 73: 64.
10. Lundberg, W. O., ed. 1961 Autoxidation and Antioxidants, vol. 1. Interscience Publishers, New York.

[6] Bhalerao, V. R., M. G. Kokatnur and F. A. Kummerow 1962 The fatty acid composition of lipids extracted from the brain of chicks with symptoms of encephalomalacia. Federation Proc., 21: 290 (abstract).

[7] Century, B., and M. K. Horwitt 1963 The relationship of arachidonic acid to nutritional encephalomalacia; implications concerning essential fatty acids in the tissues. Federation Proc., 22: 608 (abstract).

11. Lips, H. J. 1957 Stability of *d*-α-tocopherol alone, in solvents, and in methyl esters of fatty acids. J. Am. Oil Chemists' Soc., *34:* 513.
12. Horwitt, M. K., C. C. Harvey, B. Century and L. A. Witting 1961 Polyunsaturated lipids and tocopherol requirements. J. Am. Dietet. A., *38:* 231.
13. Woodman, A. G. 1941 Food Analysis, ed. 4. McGraw Hill Book Co. Inc., New York, p. 185.
14. Witting, L. A., C. C. Harvey, B. Century and M. K. Horwitt 1961 Dietary alterations of fatty acids of erythrocytes and mitochondria of brain and liver. J. Lipid Res., *2:* 412.
15. Emmerie, A., and C. Engel 1938 Colorimetric determination of α-tocopherol. Rec. trav chim. Pays-Bas, *57:* 1351.
16. Green, J., S. Marcinkiewicz and P. R. Watt 1955 The determination of tocopherols by paper chromatography. J. Sci. Food Agr., *6:* 274.
17. Century, B., and M. K. Horwitt 1960 The role of diet lipids in the appearance of dystrophy and creatinuria in the vitamin E-deficient rat. J. Nutrition, *72:* 357.
18. Vars, H. M., and F. N. Gurd 1947 Effect of dietary protein upon the regeneration of liver protein in the rat. Am. J. Physiol., *151:* 399.
19. Bonetti, E., and F. Stirpe 1962 Conditions affecting dietary liver necrosis and liver regeneration in rats. J. Nutrition, *77:* 179.
20. Mameesh, M. S., and B. C. Johnson 1958 The effect of penicillin on the intestinal synthesis of thiamine in the rat. J. Nutrition, *65:* 161.
21. Bonsnes, R. W., and H. H. Taussky 1945 On the colorimetric determination of creatine by the Jaffe reaction. J. Biol. Chem., *158:* 581.
22. Mangold, H. K. 1961 Thin-layer chromatography of lipids. J. Am. Oil Chemists' Soc., *38:* 708.
23. Century, B., L. A. Witting, C. C. Harvey and M. K. Horwitt 1961 Compositions of skeletal muscle lipids of rats fed diets containing various oils. J. Nutrition, *75:* 341.
24. Holman, R. T. 1954 Progress in the Chemistry of Fats and Other Lipids, vol. 2, eds., R. T. Holman, W. O. Lundberg and T. Malkin. Academic Press, Inc., New York, p. 73.
25. Gunstone, F. D., and T. P. Hilditch 1946 The autoxidation of methyl oleate in the presence of small proportions of methyl linoleate. J. Chem. Soc., 1022.
26. Mohrhauer, H., and R. T. Holman 1963 The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. J. Lipid Res., *4:* 151.
27. Kinsell, L. W. 1963 Some thoughts regarding the P:S ratio concept. Am. J. Clin. Nutrition, *12:* 228.
28. Hove, E. L., and H. R. Seibold 1955 Liver necrosis and altered fat composition in vitamin E-deficient swine. J. Nutrition, *56:* 173.

Phosphorus Requirement of the Baby Pig[1]

E. R. MILLER, D. E. ULLREY, C. L. ZUTAUT, BETTY V. BALTZER,
D. A. SCHMIDT, J. A. HOEFER AND R. W. LUECKE
Departments of Animal Husbandry, Veterinary Pathology and Biochemistry, Michigan State University, East Lansing, Michigan

ABSTRACT Studies were made with 32 baby pigs in 2 trials to determine their dietary phosphorus requirement. Using a synthetic milk diet, the calcium level was maintained at 0.8%, and phosphorus concentration varied from 0.2 to 0.8% of dietary solids with dietary casein consistently supplying 0.2% of phosphorus and USP grade $CaHPO_4 \cdot 2H_2O$ supplying the additional phosphorus. A dietary phosphorus level of 0.4% appeared adequate to effect normal growth and economy of food utilization. A dietary phosphorus level of 0.5% was adequate to maintain normal concentrations of serum calcium, inorganic phosphorus and alkaline phosphatase and to provide for an adequate rate of skeletal development. To obtain maximal strength of bone and to insure the absence of rachitic lesions it appears necessary to provide the baby pig with 0.6% of dietary phosphorus.

Reported studies (1–4) have shown phosphorus requirements of growing-finishing pigs fed natural rations to vary from 0.3 to 0.6% of the diet, with the reported requirement differences largely dependent upon the criteria selected. Combs et al. (4) also studied the phosphorus requirement of pigs between the ages of 2 and 7 weeks of age and concluded that 0.44% of dietary phosphorus adequately met the minimal requirement of young pigs fed a fortified corn-soybean meal diet. Zimmerman et al. (5) obtained optimal performance with 3- to 7-week-old pigs when their high milk-product diet contained 0.6% of phosphorus. Freese (6) concluded that the phosphorus requirement of young pigs fed liquid milk diets was 1.1% of the dry matter of the diet. Based upon body composition, Mitchell and McClure (7) estimated that the 13.6-kg pig has a phosphorus requirement of 0.37% of the dry matter in the diet. Lucas and Lodge (8) have chosen as "preferred estimates" of phosphorus requirement 0.9, 0.65 and 0.45% for the 4.5-, 9-, and 13.6-kg pig, respectively.

In the present study an attempt was made to assay the phosphorus requirement of the baby pig reared with a synthetic milk diet using as criteria: growth and food economy; level of serum inorganic phosphorus, calcium and alkaline phosphatase; concentrations of humeral ash, calcium and phosphorus; and measures of femur density and strength.

MATERIALS AND METHODS

Two trials were conducted using 32 Yorkshire-Hampshire crossbred pigs of either sex. Pigs were taken from the sow at one to three days of age and after environmental and dietary adjustment with a low phosphorus diet, were assigned to experimental levels of dietary phosphorus at one week of age. Bases of treatment assignment and environmental conditions were similar to those described by Miller et al. (9). The solids of the basal diet consisted of 30% of casein,[2] 10% of lard, 54% of glucose,[3] 6% of a phosphorus-free mineral mixture (table 1) and vitamins (10). The diet was made into a 20% of solids, homogenized milk and fed 4 times daily for the first 3 weeks of trial 1 and the first 2 weeks of trial 2. During the final 3 weeks of each trial the diet was fed as a dry meal 3 times daily. When fed as a dry meal 5% of cellulose[4] replaced an equal portion of fat in the diet. Experimental levels of phosphorus were 0.2, 0.4 and 0.6% in trial 1 and 0.4, 0.5, 0.6, 0.7 and 0.8% in trial 2, with 4 pigs per level of phosphorus in each trial. All diets contained 1800 IU of vitamin D_3/kg of solids. Calcium level of all diets was maintained at 0.8% and ex-

Received for publication August 5, 1963.
[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal article no. 3145.
[2] Labco, Vitamin-free Casein, The Borden Company, New York.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Solka Floc, Brown Company, Boston.

TABLE 1

Composition of basal mineral mixture

	%
KCl (0.002% I)	10.0
KI	0.002
$FeSO_4 \cdot 7H_2O$	1.0
$CoCl_2 \cdot 6H_2O$	0.1
$CuSO_4 \cdot 5H_2O$	0.1
$MnSO_4 \cdot H_2O$	0.2
$ZnSO_4 \cdot H_2O$	0.4
$MgSO_4 \cdot 7H_2O$	4.0
$NaHCO_3$	25.0
$CaCO_3$	33.3
$CaHPO_4 \cdot 2H_2O$	0.0
Glucose [1]	25.9
Total	100.002

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

perimental levels of phosphorus obtained by adjusting the levels of the last 3 ingredients in table 1, with the casein furnishing 0.2% of phosphorus to each of the experimental diets. Studies (11–14) have indicated excellent usage of dietary phosphorus from dicalcium phosphate by pigs, and the work of Freese (6) and Ludvigson and Thorbek (15) indicate that the phosphorus of casein is well utilized by the young pig. All diets were analyzed periodically by the method of Gomorri (16) to verify the intended levels of phosphorus. Dietary consumption by individual pigs was accurately measured and recorded.

All pigs were weighed weekly. Blood was withdrawn bi-weekly from the anterior vena cava for the determination of levels of serum inorganic phosphorus, calcium and alkaline phosphatase by the methods of Gomorri (16), Mori (17) and Bessey et al. (18), respectively. At the conclusion of each trial all pigs were killed and gross and microscopic pathological observations were made. Various bones, organs and glands were removed and weighed. The methods of Mori (17) and Gomorri (16) were adapted to determine calcium and phosphorus concentration in kidney tissue. Determinations of specific gravity of femur, humerus and eighth rib were made by the method described by Brown et al. (19). Humeral ash, calcium and phosphorus determinations were by AOAC (20) methods. Strength measurements of the femur were made by the methods described by Miller et al. (9). Statistical analyses of data were performed using the multiple range test of Duncan (21).

RESULTS AND DISCUSSION

Trial 1. Growth rate and food consumption were depressed only in those pigs receiving less than 0.4% of phosphorus (table 2) with a significant reduction in rate of growth and feed consumption of pigs receiving 0.2% of phosphorus becoming apparent after only 2 weeks of the experimental period. The degree of growth depression was greater than the degree of reduction in food consumption in these pigs, resulting in a significant reduction in the efficiency of food utilization. A level of 0.4% of dietary phosphorus was sufficient to support maximal growth rate and economy of food utilization.

The initial levels of serum inorganic phosphorus were low in all pigs, indicating that probably some depletion had occurred during the adjustment period. A level of dietary phosphorus of 0.6% rapidly restored the serum phosphorus to a normal level. Pigs receiving the diet containing 0.4% of phosphorus did not possess normal levels of serum phosphorus until the sixth week of the experiment. Serum calcium levels were normal in all pigs, tending to be higher in animals receiving lower levels of dietary phosphorus. Serum alkaline phosphatase concentration became elevated only in the severely phosphorus-deficient pigs. Kidney phosphorus concentration was somewhat increased in phosphorus-deficient pigs. No explanation for this observation is apparent.

Humeral ash, calcium and phosphorus concentrations were maximal in pigs receiving 0.6% of dietary phosphorus. Maximal density of femur and rib were also exhibited by pigs on this level of phosphorus. Furthermore, maximal breaking strength, resistance to bending and stress capacity were exhibited by femurs from pigs receiving the highest level of phosphorus supplementation in this trial. Strength of femur was largely dependent upon the thickness of the compact calcified layer of the diaphysis as shown in figure 1.

Pigs receiving 0.2% of dietary phosphorus exhibited shortened limbs with weakened and bent metatarsals and phalanges (fig. 2). The appearance of the phosphorus-deficient pigs was similar to that observed in calcium deficiency (9) with the exception that phosphorus-defi-

TABLE 2

Growth, blood serum and kidney analyses and skeletal development of baby pigs fed different levels of phosphorus (trial 1)

	Dietary phosphorus level, % of food solids		
	0.2	0.4	0.6
No. of pigs	4	4	4
Initial weight, kg	3.05 ± 0.06 [1]	3.11 ± 0.16	3.03 ± 0.17
Daily gain, kg	0.13 ± 0.01	0.29 ± 0.01 [aa]	0.29 ± 0.03 [aa]
Food solids/day, kg	0.24 ± 0.01	0.38 ± 0.02 [aa]	0.37 ± 0.03 [aa]
Food solids/gain	1.85 ± 0.05	1.31 ± 0.04 [aa]	1.28 ± 0.07 [aa]
Serum organic P, mg/100 ml			
Initial	4.1 ± 0.2	4.2 ± 0.3	3.9 ± 0.3
2 Weeks	5.8 ± 0.5	5.3 ± 0.8	8.8 ± 0.8 [bb]
4 Weeks	2.6 ± 0.4	5.2 ± 0.5 [aa]	9.2 ± 0.6 [bb]
6 Weeks	3.2 ± 0.3	8.4 ± 0.9 [aa]	9.4 ± 0.4 [aa]
Serum Ca, mg/100 ml			
Initial	12.1 ± 0.4	12.3 ± 0.4	12.0 ± 0.4
2 Weeks	9.7 ± 0.3	10.4 ± 0.2	9.3 ± 0.5
4 Weeks	12.8 ± 1.1 [a]	10.7 ± 0.8	9.9 ± 0.6
6 Weeks	12.8 ± 0.2 [aa]	12.2 ± 0.3 [aa]	10.3 ± 0.2
Serum alkaline phosphatase, Bessey-Lowry units			
Initial	11.4 ± 0.8	12.0 ± 1.6	12.2 ± 0.7
2 Weeks	9.4 ± 1.5	14.9 ± 1.1	9.8 ± 1.7
4 Weeks	8.6 ± 1.5	8.4 ± 2.0	4.4 ± 0.4
6 Weeks	20.5 ± 3.5 [bb]	8.3 ± 0.7	4.5 ± 1.4
Kidney analyses			
P, mg/100 g dry tissue	1550 ± 10 [b]	1430 ± 25	1470 ± 14
Ca, mg/100 g dry tissue	51 ± 4	48 ± 1	40 ± 1
Humeral analyses (dry, fat-free basis)			
Ash, %	33.4 ± 1.8	44.1 ± 1.0 [aa]	47.5 ± 0.7 [aa]
Ca, %	11.6 ± 0.7	15.6 ± 0.4 [aa]	16.8 ± 0.3 [aa]
P, %	5.65 ± 0.28	7.91 ± 0.07 [aa]	9.04 ± 0.22 [bb]
Ca/P	2.04 ± 0.02 [aa]	1.98 ± 0.02 [aa]	1.87 ± 0.02
Specific gravity			
Femur	1.11 ± 0.01	1.15 ± 0.01 [aa]	1.17 ± 0.01 [aa]
8th rib	1.13 ± 0.01	1.23 ± 0.01 [aa]	1.27 ± 0.02 [aa]
Weight, g			
Femur	40.6 ± 2.0	65.2 ± 2.3 [a]	66.4 ± 3.2 [a]
8th rib	3.30 ± 0.25	4.49 ± 0.39	4.67 ± 0.60 [a]
Femur strength [2]			
Breaking load, kg	24 ± 1	61 ± 6 [aa]	81 ± 2 [bb]
Bending moment, kg-cm	48 ± 3	118 ± 8 [aa]	160 ± 4 [bb]
Moment of inertia, cm^4	0.06 ± 0.00	0.12 ± 0.01 [aa]	0.14 ± 0.01 [aa]
Breaking stress, kg/cm^2	470 ± 16	636 ± 69 [a]	785 ± 40 [b]

[1] SE of mean.
[a] Significantly greater than least value ($P < 0.05$); [aa] ($P < 0.01$).
[b] Significantly greater than both other values ($P < 0.05$); [bb] ($P < 0.01$).
[2] For formulae, see Miller et al. (9).

cient animals did not exhibit the "humped back" or "protruding eye" symptoms exhibited by calcium-deficient pigs. Perhaps failure of the phosphorus-deficient pig to exhibit these symptoms is due to a more uniform cessation of bone and soft tissue growth as compared with the calcium-deficient pig in which bone growth is preferentially interrupted.

Examination at necropsy revealed multiple rib fractures with calluses in the acutely phosphorus-deficient pigs with "beading" apparent at the costochondral junctions (fig. 1). Histopathological findings were similar to those observed in calcium deficiency (9). A single rib fracture was noted in one of the pigs receiving 0.4% of dietary phosphorus. Histopathological studies re-

Fig. 1 Eighth rib and cross-section of femur from littermates receiving different dietary levels of phosphorus (trial 1). Note the enlarged costochondral joint (beading) and the callus (indicating earlier break) in the rib at left and the lack of compact layer in the corresponding femur. Breaking loads were 27, 70 and 84 kg for femurs shown from pigs receiving 0.2, 0.4 and 0.6% of dietary P, respectively.

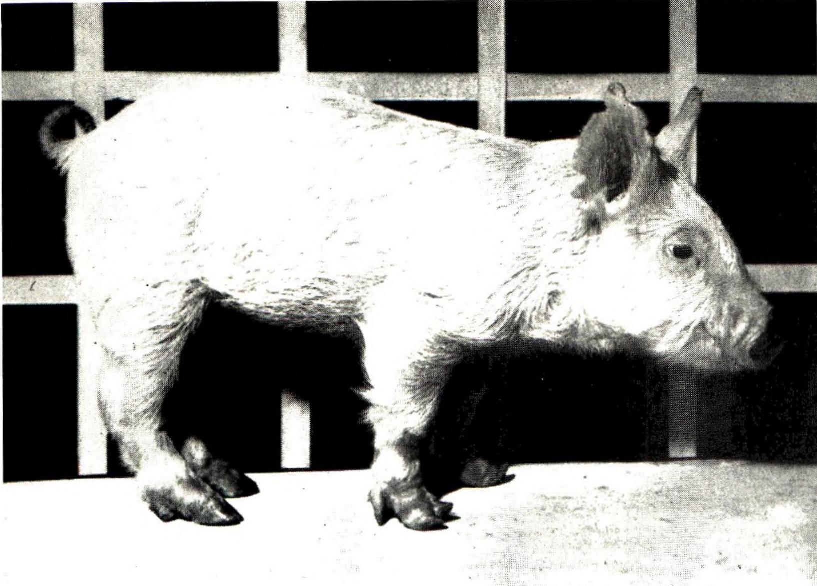


Fig. 2 Phosphorus-deficient pig exhibiting short limbs, weakened particularly in the metatarsal-phalangeal region. This pig from trial 1 received a synthetic milk diet containing 0.2% P, 0.8% Ca and 1800 IU of vitamin D_3/kg.

vealed that the ribs from pigs receiving this level of phosphorus were fairly normal with only borderline rachitic lesions. Ribs from pigs receiving 0.6% of dietary phosphorus appeared normal in every respect.

Judging from the information obtained from trial 1 it can be concluded that 0.4% of dietary phosphorus is adequate to prevent gross rachitic symptoms and to achieve optimal rates of body weight gain and economy of food utilization. However, it can also be concluded that this level of dietary phosphorus is inadequate to support maximal rate of skeletal development. The second trial was designed to determine the level of dietary phosphorus that would support a maximal rate of skeletal development as determined by bone composition, density and strength characteristics.

Trial 2. Results of the second trial are presented in table 3. As in the first trial, 0.4% of dietary phosphorus was adequate also in this trial to support optimal rate of growth and economy of food utilization. Serum inorganic phosphorus levels were significantly lower, however, in pigs receiving 0.4% of dietary phosphorus and their serum alkaline phosphatase concentration was somewhat elevated. Serum calcium level was not significantly affected by dietary phosphorus level. Percentages of humeral ash, calcium and phosphorus were significantly reduced in pigs receiving the lowest level of dietary phosphorus. This reduced level of bone ash was further manifested by the specific gravity of humerus and rib. Femur strength as measured by breaking load, bending moment and breaking stress was significantly greater in pigs receiving the higher levels of dietary phosphorus.

Histopathological studies of rib sections in this trial revealed moderate rachitic lesions in all pigs receiving 0.4% of dietary phosphorus based upon the width and regularity of the zone of proliferating cartilage, the straightness of the line of ossification and the width, straightness and regularity of the bony trabeculae. Pigs receiving 0.5% of dietary phosphorus revealed ribs varying from fairly normal to those with borderline lesions of rickets. Ribs from pigs receiving the higher levels of dietary phosphorus appeared normal in all respects.

The results of trial 2 indicate clearly that 0.5% of dietary phosphorus is adequate to maintain normal concentrations

TABLE 3

Growth, serum analyses and skeletal development of baby pigs (trial 2)

	Dietary phosphorus level, % of food solids				
	0.4	0.5	0.6	0.7	0.8
No. of pigs	4	4	4	4	4
Initial weight, kg	1.82 ± 0.23 [1]	1.91 ± 0.23	1.82 ± 0.12	1.86 ± 0.21	1.91 ± 0.18
Daily gain, kg	0.28 ± 0.01	0.28 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02
Food solids/day, kg	0.85 ± 0.02	0.85 ± 0.01	0.86 ± 0.02	0.86 ± 0.02	0.86 ± 0.01
Food solids/gain	1.40 ± 0.04	1.38 ± 0.08	1.35 ± 0.07	1.37 ± 0.10	1.37 ± 0.06
Serum inorganic P, mg/100 ml					
Initial	9.4 ± 0.1	9.1 ± 0.1	10.1 ± 0.1	9.1 ± 0.6	9.5 ± 0.5
3 Weeks	7.7 ± 0.3	9.6 ± 0.8	10.7 ± 0.8	10.2 ± 0.2	10.3 ± 0.3
5 weeks	8.2 ± 0.5	10.4 ± 0.1 [a]	10.0 ± 0.1 [a]	10.4 ± 0.1 [a]	10.3 ± 0.1 [a]
Serum Ca, mg/100 ml					
Initial	10.4 ± 0.1	10.4 ± 0.1	10.8 ± 0.1	11.0 ± 0.1	11.0 ± 0.1
3 Weeks	11.6 ± 0.1	11.0 ± 0.1	10.1 ± 0.4	10.9 ± 0.1	10.5 ± 0.1
5 Weeks	11.6 ± 0.1	11.0 ± 0.1	11.7 ± 0.1	10.8 ± 0.1	10.9 ± 0.1
Serum alkaline phosphatase, Bessey-Lowry units					
Initial	12.1 ± 1.3	16.0 ± 2.6	15.4 ± 0.8	14.4 ± 2.0	14.9 ± 1.4
3 Weeks	14.1 ± 1.3	12.4 ± 1.2	13.2 ± 1.0	11.5 ± 0.7	9.3 ± 0.8
5 Weeks	11.6 ± 2.2 [a]	8.5 ± 0.7	9.3 ± 0.8	8.4 ± 0.4	7.6 ± 0.4
Humeral analyses (dry, fat-free basis)					
Ash, %	47.5 ± 0.8	52.0 ± 0.3 [aa]	52.1 ± 0.6 [aa]	52.6 ± 0.6 [aa]	53.2 ± 0.3 [aa]
Ca, %	16.3 ± 0.4	17.8 ± 0.1 [aa]	17.8 ± 0.2 [aa]	18.1 ± 0.3 [aa]	18.3 ± 0.2 [aa]
P, %	8.7 ± 0.2	9.4 ± 0.1 [aa]	9.5 ± 0.1 [aa]	9.6 ± 0.2 [aa]	9.7 ± 0.1 [aa]
Ca/P	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0
Specific gravity					
Humerus	1.15 ± 0.02	1.20 ± 0.01 [a]	1.20 ± 0.01 [a]	1.20 ± 0.02 [a]	1.21 ± 0.01 [a]
8th rib	1.24 ± 0.01	1.27 ± 0.01	1.30 ± 0.01 [aa]	1.28 ± 0.01 [a]	1.29 ± 0.01 [a]
Femur strength [2]					
Breaking load, kg	70 ± 10	93 ± 13	99 ± 3	115 ± 16 [a]	115 ± 8 [a]
Bending moment, kg-cm	156 ± 21	193 ± 28	196 ± 25	236 ± 30 [a]	233 ± 16 [a]
Moment of inertia, cm^4	0.10 ± 0.02	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.03	0.12 ± 0.02
Breaking stress, kg/cm^2	990 ± 90	1010 ± 76	1250 ± 145	1240 ± 133	1390 ± 156 [a]

[1] SE of mean.
[a] Significantly greater than least value ($P < 0.05$); [aa] ($P < 0.01$).
[2] For formulae, see Miller et al. (9).

of serum calcium, phosphorus and alkaline phosphatase and to provide for an adequate rate of skeletal development. To obtain maximal strength of bone and to insure the absence of rachitic lesions it appears necessary to provide the baby pig with 0.6% or more of dietary phosphorus. This is in good agreement with the results obtained by Zimmerman et al. (5) and with the recommendations of the National Research Council subcommittee on swine nutrition (22) for the 4.5- and 11-kg pig. Furthermore, results of the phosphorus analyses of sow's milk (23–27) show that post-colostral phosphorus concentration increases during lactation but that for the first 3 weeks of lactation, when sow's milk is usually the sole source of the baby pig's nutrition, the concentration of phosphorus in the dry matter of sow's milk is consistently near this value (0.6%).

LITERATURE CITED

1. Aubel, C. E., J. S. Hughes and H. F. Lienhardt 1936 Phosphorus requirements in the rations of growing pigs. Kansas Agr. Exp. Sta. Tech. Bull. 41., Manhattan, Kansas.
2. Jordan, C. E., J. H. Conrad, M. P. Plumlee and W. M. Beeson 1957 Phosphorus and protein levels for growing-finishing swine on legume pasture. J. Animal Sci., 16: 1077.
3. Chapman, H. L., Jr., J. Kastelic, G. C. Ashton, P. G. Homeyer, C. Y. Roberts, D. V. Catron, V. W. Hays and V. C. Speer 1962 Calcium

and phosphorus requirements of growing-finishing swine. J. Animal Sci., *21:* 122.

4. Combs, G. E., J. M. Vandepopuliere, H. D. Wallace and M. Koger 1962 Phosphorus requirement of young pigs. J. Animal Sci., *21:* 3.
5. Zimmerman, D. R., V. C. Speer, V. W. Hays and D. V. Catron 1963 Effect of calcium and phosphorus level on baby pig performance. J. Animal Sci., *22:* 658.
6. Freese, H. H. 1958 Untersuchungen über den Calcium-, Phosphor-, Magnesium-und Stickstoff-umsatz des Ferkels in Beziehung zum Wachstum bei Muttermilch und Muttermilchersatz. Arch. Tierernährung, *8:* 330.
7. Mitchell, H. H., and F. J. McClure 1937 Mineral nutrition of farm animals. National Research Council Bull., 99. National Academy of Sciences — National Research Council, Washington, D. C.
8. Lucas, I. A. M., and G. A. Lodge 1961 The Nutrition of the Young Pig. Commonwealth Agricultural Bureaux, Farnham Royal, Slough, Bucks, England.
9. Miller, E. R., D. E. Ullrey, C. L. Zutaut, Betty V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1962 The calcium requirement of the baby pig. J. Nutrition, *77:* 7.
10. Miller, E. R., R. L. Johnston, J. A. Hoefer and R. W. Luecke 1954 The riboflavin requirement of the baby pig. J. Nutrition, *52:* 405.
11. Chapman, H. L., Jr., J. Kastelic, G. C. Ashton and D. V. Catron 1955 A comparison of phosphorus from different sources for growing and finishing swine. J. Animal Sci., *14:* 1073.
12. Gobble, J. L., R. C. Miller, G. W. Sherritt and H. W. Dunne 1956 Soft phosphate with colloidal clay as a source of phosphorus for growing and fattening pigs. Pennsylvania Agr. Exp. Sta. Bull. 609, University Park, Pennsylvania.
13. Plumlee, M. P., C. E. Jordan, M. H. Kennington and W. M. Beeson 1958 Availability of the phosphorus from various phosphate materials for swine. J. Animal Sci., *17:* 73.
14. Aldinger, S. M., V. C. Speer, G. C. Ashton, V. W. Hays and D. V. Catron 1959 Effect of feeding different combinations of soft phosphate with colloidal clay and dicalcium phosphate with and without added fluorine for growing-finishing swine. J. Animal Sci., *18:* 555.
15. Ludvigsen, J., and G. Thorbek 1960 Studier over pattegrises raekst of ernaering. II. Tilraekst og foderforbrug same kvaelstof-, fosfor- og kalciumomsaetningen hos kunsfigt ernaerede pattegrise i alderen fra 5. til 35 dag. Beretning fra Forsøgslaboratoriet 320, København.
16. Gomorri, G. 1942 A modification of the colorimetric phosporus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med., *27:* 955.
17. Mori, K. 1959 Direct microcomplexometric analysis of calcium in biological material. Arch. Biochem. Biophys., *83:* 552.
18. Bessey, O. A., O. H. Lowry and M. J. Brock 1946 A method for the rapid determination of alkaline phosphatase with 5 cubic millimeters of serum. J. Biol. Chem., *164:* 321.
19. Brown, C. J., J. C. Hillier and J. A. Whatley 1951 Specific gravity as a measure of fat content of the pork carcass. J. Animal Sci., *10:* 97.
20. Association of Official Agricultural Chemists 1960 Methods of Analysis, ed. 9. Washington, D. C.
21. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
22. National Research Council, Committee on Animal Nutrition 1959 Nutrient requirements of domestic animals. II. Nutrient requirements of swine, pub. 648. National Academy of Sciences — National Research Council, Washington, D. C.
23. Hughes, E. H., and H. G. Hart 1935 Production and composition of sow's milk. J. Nutrition, *9:* 311.
24. Perrin, D. R. 1955 The chemical composition of the colostrum and milk of the sow. J. Dairy Res., *22:* 103.
25. Guegen, L., and E. Salmon-Legagneur 1959 La composition du lait de truie: Variations des teneurs en quelques elements mineraux (P, Ca, K, Na, Mg). C. R. Acad. Sci., *249:* 784.
26. Jylling, B. 1960 Investigations concerning the composition of sow's milk. Royal Veterinary Agricultural College Yearbook, Copenhagen.
27. Travnicek, J. 1960 Chemické slozeni mleziva a mléka prasnic plemene bilého vslechtilého. S. Chek. Acad. Med. Ved., *33:* 497.

Zinc Deficiency Syndrome in the Young Lamb[1]

E. A. OTT, W. H. SMITH, MARTIN STOB AND W. M. BEESON
Purdue University, Lafayette, Indiana

ABSTRACT Eighteen lambs averaging 13.5 kg were divided into 6 comparable groups and fed the following diets: basal, basal + 2% polyethylene, basal + 0.61% phytic acid, basal + 0.61% phytic acid + 2% polyethylene, basal + 100 mg zinc/kg, and basal + 100 mg zinc/kg + 2% polyethylene. The basal diet (2.7 mg zinc/kg) was composed of the following: (in per cent) egg whites, 15.00; cellulose, 18.00; glucose monohydrate, 27.02; starch, 27.02; corn oil, 3.00; minerals and vitamins. The animals fed the diets not supplemented with zinc exhibited the following symptoms: anorexia; depraved appetite (eating wool); reduced growth; reduced feed efficiency; loose wool; swollen hocks; red, wrinkled skin; and open lesions of the skin above the hoof and around the eyes. Tissue changes characteristic of the deficiency were decreased tissue zinc and parakeratotic lesions of the skin. Blood changes typical of the deficient animals were decreased serum zinc, decreased serum albumin and increased globulin levels. Excessive salivation, increased rumen propionic acid production and a corresponding reduction in butyric acid were also associated with the deficiency. The zinc deficiency appeared to be less severe in the lambs fed phytic acid. Feeding 100 mg zinc/kg of the basal diet to deficient lambs for 5 weeks completely alleviated most of the deficiency symptoms. The polyethylene added to the diet had little or no effect on performance but appeared to stimulate rumination.

Since Tucker and Salmon (1) linked the parakeratotic condition in swine described by Kernkamp and Ferrin (2) to a zinc deficiency, a considerable amount of research has been conducted to determine the nutritional requirement of monogastric animals for this element. Knowledge of the ruminant's requirement for zinc, however, has not progressed beyond the point of recognizing that a need exists. This may be due to 2 factors: (a) only recently have reports indicated that natural deficiencies such as those described by Legg and Sears (3) and Haaranen and Hyppola (4) may exist; and (b) the formulation of semi-purified diets for ruminants suitable for trace mineral studies has been far behind corresponding work with monogastrics.

Miller and Miller (5, 6) described the symptoms of zinc deficiency in Holstein calves fed a purified diet containing 3.6 mg zinc/kg, but in later work (7) could not produce the deficiency using a natural diet containing 25 mg zinc/kg. No effect could be found due to high phytic acid levels as have been described for monogastric animals by Plumlee et al. (8), and Oberleas et al. (9).

The objectives of this experiment were: 1) to develop a ration for lambs that would support normal growth and could be rendered very low in zinc; 2) to characterize the symptoms associated with a zinc deficiency in the lamb; and 3) to alleviate the deficiency symptoms by feeding zinc.

METHODS

Eighteen cross-bred native lambs averaging 13.5 kg were divided into 6 comparable groups according to weight, sex and breeding. The age of the lambs ranged from 4 to 8 weeks. Groups were assigned to the following treatments: basal, basal + 2% polythylene; basal + 0.61% phytic acid; basal + 0.61 phytic acid + 2% polyethylene; basal + 100 mg zinc/kg; and basal + 100 mg zinc/kg + 2% polyethylene. The basal diet listed in table 1, which is a modification of the diet used by Lawlor et al. (10), contains 2.7 mg zinc/kg. The polyethylene was added to the diet of one-half of the lambs in the form of 3-mm cubes replacing an equal quantity of cellulose to determine the effect of providing a roughness factor to the diet. The phytic acid was added to 2 of the treatments to test the effect of this chelating agent on the zinc fed to

Received for publication August 5, 1963.

[1] Contribution from the Department of Animal Sciences, Journal paper no. 2179.

TABLE 1

Basal diet [1]

	%
Egg whites [2]	15.00
Cellulose [3]	20.00
Glucose monohydrate [4]	27.02
Starch (corn)	27.02
Corn oil	3.00
$CaHPO_4 \cdot 2H_2O$	3.00
$K_2CO_3 \cdot 1\frac{1}{2}H_2O$	2.00
$MgSO_4 \cdot 3H_2O$	0.70
NaCl	1.00
Trace mineral mix [5]	0.56
Vitamin and antibiotic mix [6]	0.56
Choline chloride	0.14

[1] Diet contained 2.7 mg zinc/kg.
[2] Egg white solids, spray, P-19-S (Henningson Foods, Inc., New York).
[3] Cellulose Flock, K-856 Pulp (International Filter Corporation), North Tonawanda, New York.
[5] Trace mineral mix provided the following in mg/kg of the diet: iron, 70.40; boron, 14.65; manganese, 20.72; copper, 15.86; cobalt, 0.11; molybdenum, 0.33.
[6] Vitamin and antibiotic mix provided the following in mg/kg of the diet: biotin, 0.22; naphthoquinone, 2.20; pyridoxine, 2.20; folic acid, 4.40; thiamine·HCl, 6.60; riboflavin, 8.80; p-aminobenzoic acid, 8.80; vitamin A (362,000 IU/g), 12.21; Ca pantothenate, 22.00; niacin, 33.00; vitamin E, 33.00; vitamin B_{12}, 44.00; ascorbic acid, 110.00; inositol, 110.00; vitamin D_3, (Delsterol, E. I. du Pont de Nemours & Co., Wilmington, Del., 3,000 IU/g), 146.30; and chlortetracycline (Aureomycin, American Cyanamid Co., New York), 22.00.

sheep. The acid was added to the basal at the rate of 1.525% of a solution containing 40% phytic acid.

The experiment was conducted indoors and temperatures were maintained between 23° and 19°C. When the outside temperature exceeded this, the building was allowed to equilibrate with the ambient temperature. Due to the early age at which the lambs were weaned, each group of 3 lambs was placed in a 122- by 244-cm pen for the first 2 weeks of the experiment to allow them to adjust to the new environment. Individual pens were used for the remainder of the experiment. Each pen, except those in which the lambs were to receive added zinc, was lined with wood to deny the animals access to the galvanized pipe frames of the pens. Plastic feeders and waterers were used. Water containing less than 0.1 mg zinc/liter was supplied from a Zeolite water softener. A wooden platform was placed in each pen to eliminate the need for bedding and the pens were washed daily with soft water.

Blood samples were taken at the beginning of the experiment and at the seventh, tenth, and fifteenth week. Hemoglobin and packed cell volumes were determined and the serum was wet-oxidized and analyzed for zinc content by a modification of the dithizone-bis (2 hydroxyethyl) dithiocarbamate method evaluated by Margerum and Santacana (11). This method was used for all subsequent zinc analyses.

The lambs were maintained on the first phase of the experiment for 10 weeks. At the end of the first phase, one lamb from each of the lots not receiving added zinc and 2 lambs from the lots receiving added zinc were killed. Blood samples were taken for serum zinc analysis and since Hoefer et al. (12) had found an increase in serum globulins and a decrease in albumin in zinc-deficient swine, composite samples of blood serum were made for each lot and serum protein fractions determined by electrophoresis.[2] Total serum proteins were determined by the method described by Lowry (13). Tissue samples were taken for zinc analysis and histological examination. Rumen fluid samples were taken from each lamb as it was killed by mixing and sampling the entire reticulorumen contents. The determination of the rumen fluid pH was made within 2 minutes after the animal was killed and the samples were frozen until volatile fatty acid (VFA) analysis could be run. Analysis was by a modification[3] of a procedure described by Erwin (14) using an Aerograph "Hy-Fi" Model 600 gas chromatograph and a 0.3-cm OD by 152-cm stainless steel column packed with 20% LAC 296 and 2% phosphoric acid on 60/80 acid-washed chromasorb W. The procedure readily separates acetic, propionic, butyric, isovaleric and valeric acids.

The 8 lambs remaining from the low zinc lots were divided into 2 comparable groups. One group continued to be fed the basal + 2% polyethylene and the other was given the basal + 100 mg zinc/kg + 2% polyethylene. The 2 lambs remaining from the added zinc lots of the first phase continued to be fed the basal + 100 mg zinc/kg + 2% polyethylene. The repletion phase was continued for 5 weeks. At the end of this

[2] Electrophoresis was as described for procedure B in the Beckman Instruction Manual on the Spinco Model R Paper Electrophoresis system.

[3] Rumsey, T. S. 1963 Corn silage in rations for lactating dairy cows. M.S. Thesis, Purdue University.

phase all lambs were killed and samples comparable to those taken at 10 weeks were taken.

The data were analyzed by analysis of variance techniques as outlined by Snedecor (15). The Duncan multiple range test (16) was used to test for significant differences among the treatment means.

RESULTS AND DISCUSSION

The lambs consumed the ration readily but found some difficulty in adjusting to it. This was evidenced by the variations in the consistency of the feces during the first 3 to 4 weeks; however, the problem did not persist. During the third week, a difference in feed consumption, rate of gain and feed-to-gain ratio became evident between the lots receiving added zinc and the other treatments. These differences became progressively more pronounced throughout the remainder of the experiment. Visible symptoms of the deficiency (fig. 1) were first manifest by the loosening, rubbing and eating of the fleece. These symptoms were noted during the fourth week in 2 lambs and were followed by swelling of the area between the hocks and the hoof and lesions of the skin just above the hoof and around the eyes. The skin areas that were devoid of wool became slightly red and wrinkled. Encrusted areas developed on the ventral surfaces of the body and the skin of the animals showing the most severe conditions was easily damaged. Similar external symptoms have been described by Miller and Miller (5,6) as characteristic of zinc-deficient calves and by Kernkamp and Ferrin (2) as swine parakeratosis. By the eighth week, all the lambs except one were showing some of the symptoms. The symptoms appeared in the remaining lamb very rapidly during the tenth week. Additional symptoms present in the most acute cases include excessive salivation when eating and ruminating, shivering, swollen joints, and a characteristic stance which included

Fig. 1 Left, zinc-deficient lamb after fed basal diet for 15 weeks. Right, lamb of the same age which was fed the basal + 100 mg zinc/kg diet for the same period.

placing the feet very close together and arching the back. The sequence of appearance for the symptoms was not constant between lambs.

Due to the difficulty inherent in describing the progression of a syndrome in a group of animals, a numerical evaluation was made of each lamb's severity of 4 symptoms: fleece condition, general appearance of the skin, lesions around the feet and lesions around the eyes. The score was established by giving each area a zero if normal, and up to a 5 for extreme abnormality. Thus, each animal could have a maximal score of 20 points. The mean value for each lot was called the "gross symptom score." The subjectivity of the score necessitated the exclusion of some symptoms from the evaluation because of the difficulty in assessing their severity. At the end of the first phase the difference in the gross symptom score (table 2) between the lots with and without added zinc was significant ($P < 0.01$). A significantly ($P < 0.05$) more severe condition was also found in the lambs fed the basal diet than in those receiving added phytic acid.

Average daily gain and feed consumption (table 3) were significantly ($P < 0.01$)

TABLE 2

Gross symptoms score [1] for lambs fed basal, basal + phytic acid and basal + 100-mg zinc/kg diets

Treatment	Week of experiment		
	8	9	10
Phase 1 (10 weeks)			
Basal	9.2	11.3	14.3 [2]
Basal + phytic acid	5.8	8.0	10.0
Basal + zinc [3]	0	0	0

Phase 2 (repletion for 5 weeks)

	Week of experiment					
	10	11	12	13	14	15
Basal	10.8 [3]	14.2 [4]	15.5 [5]	15.8 [5]	15.8 [5]	15.8 [5]
Basal + zinc (repletion)	11.2	10.2	8.2	5.0	2.5	0.2
Basal + zinc [6]	0	0	0	0	0	0

[1] Gross symptoms score was calculated by giving each lamb a score of 0 if normal, and up to 5 if extremely abnormal for 4 different symptoms. Mean values were taken for each lot.
[2] Difference between lambs fed the basal and basal + phytic acid was significant ($P < 0.05$).
[3] Value based on lambs remaining after severely affected lambs were killed at the end of phase 1.
[4] Difference between lambs fed basal and basal + zinc repletion diet was significant ($P < 0.05$).
[5] Difference between lambs fed basal and basal + zinc (repletion) diet was significant ($P < 0.01$).
[6] Since the lambs that received added zinc for the entire period did not exhibit any of the symptoms evaluated, they were considered significantly different from the deficient lambs and were not included in the analyses.

TABLE 3

Performance data of lambs fed basal, basal + phytic acid and basal + 100-mg zinc/kg diets

Treatment	No. of animals	Avg daily gain	Daily feed	Feed-to-gain ratio
		kg	*kg*	
Phase 1 (10 weeks)				
Basal	6	0.03	0.45	16.85
Basal + phytic acid	6	0.05	0.51	9.44 [1]
Basal + zinc	6	0.25 [2]	0.97 [2]	3.99 [2]
Phase 2 (repletion for 5 weeks)				
Basal	4	0.02	0.75	31.62
Basal + zinc (repletion)	4	0.29 [3]	1.15	3.99
Basal + zinc	2	0.32 [3]	1.94	6.03

[1] Value significantly different from that for the basal diet ($P < 0.05$).
[2] Value significantly different from the other 2 treatments ($P < 0.01$).
[3] Values significantly different from that for the basal diet ($P < 0.01$).

greater for the zinc-supplemented lambs. Lambs receiving added zinc and added phytic acid were significantly ($P < 0.01$) more efficient than the lambs fed the basal diet, and those receiving the added zinc were more efficient ($P < 0.05$) than those receiving the phytic acid.

Response to the repletion diet was dramatic. Feed consumption increased immediately and the external symptoms started to regress (fig. 2). Lesions healed; skin lost its red, wrinkled appearance; and excessive salivation ceased. The lambs did not lose their appetite for wool for about 2 weeks, but by this time the old wool had tightened and new growth was evident in the denuded areas. Growth response was also immediate. Rate of gain increased each week until it was comparable to that of the lambs receiving the added zinc diet for the entire experiment. Lambs fed the basal diet gained significantly ($P < 0.01$) more slowly than either of the other groups.

Blood analyses showed very little difference in hemoglobin and packed cell volumes at the end of the first phase. Serum zinc levels (fig. 3) decreased to a level of about 20 µg/100 ml. Lambs with low zinc levels at the start of the experiment tended to exhibit the external symptoms first. During the second phase of the experiment there was a tendency for the aforementioned factors in the repleted lambs to approach the values for the lambs maintained with the added zinc diets for 15 weeks. Serum zinc levels in the lambs that had continued to be fed the low zinc diet for 15 weeks remained the same as the 10-week value. Results of serum protein analysis (table 4) indicate that significant ($P < 0.05$) increases in the serum globulin levels occur in the deficient lambs. Fractionation of α_1- and α_2- portions of the globulins was incomplete and since their relative proportion to the β-globulins appeared to be constant the 3 portions were combined. As a group this

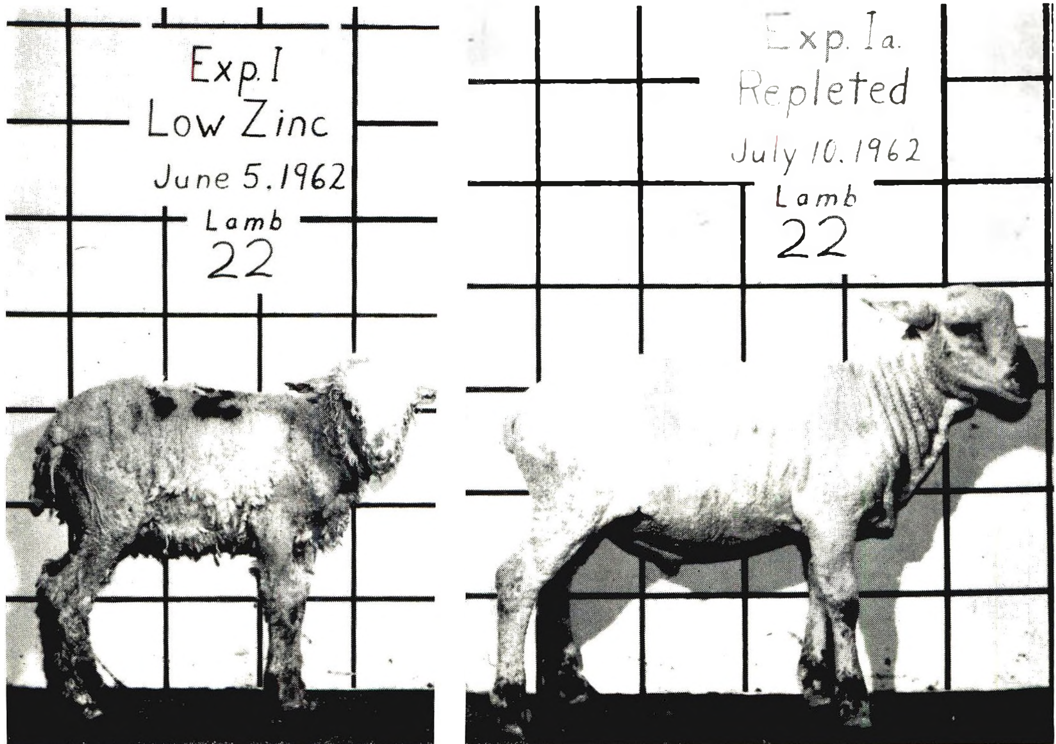


Fig. 2 Left, zinc-deficient lamb after fed basal diet for 10 weeks. Right, same lamb after 5 weeks on the basal + 100 mg zinc/kg diet.

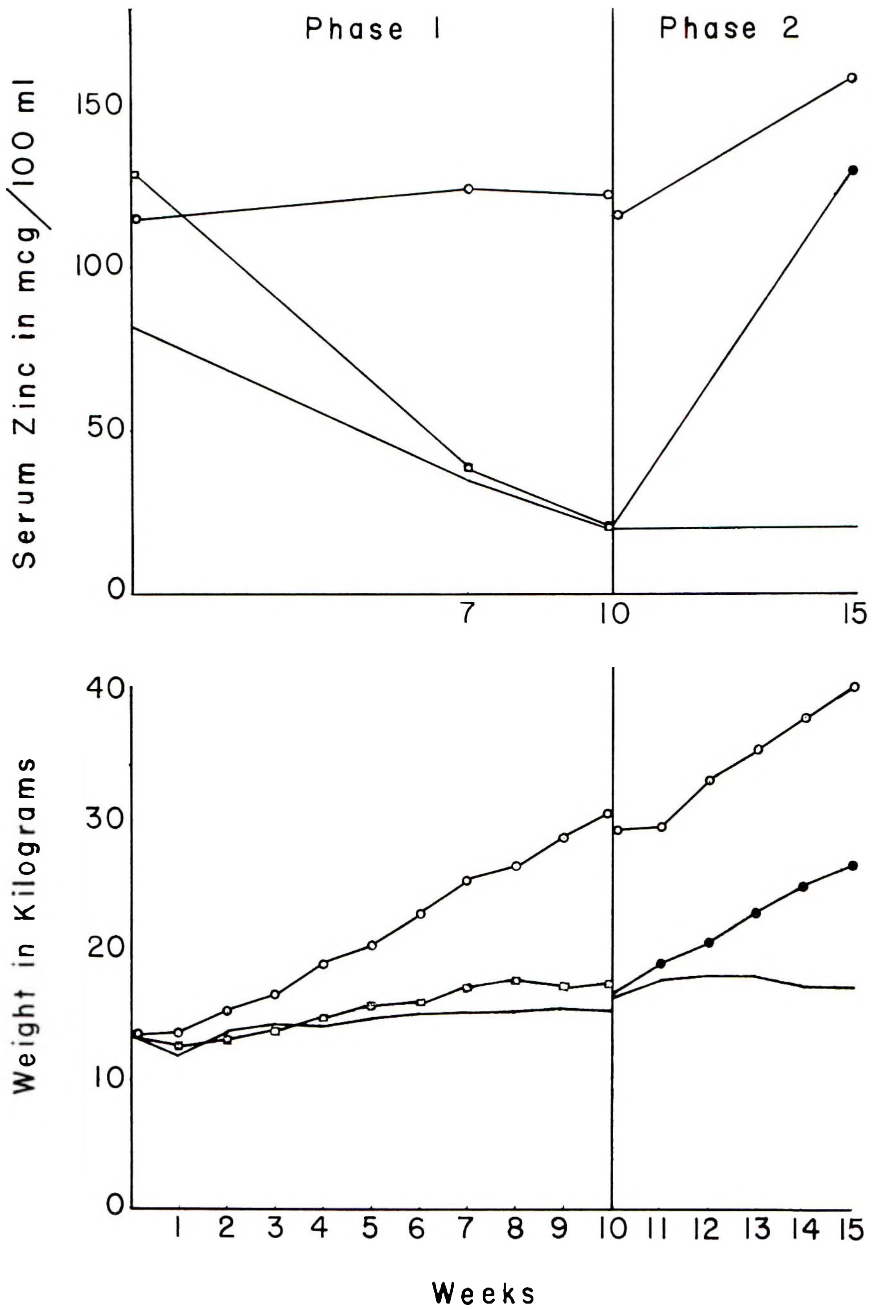


Fig. 3 Growth rate and serum zinc levels of lambs fed deficient and adequate zinc diets. ——, indicates basal diet; —□—, basal plus phytic acid; —○—, basal plus zinc; and —●—, basal plus zinc (repletion).

TABLE 4

Concentrations of albumin and globulins in lamb serum [1]

Treatment	Total protein	Albumin		Total globulins		α- and β-globulins		γ-globulins	
	g/100 ml	*g/100 ml*	*%*	*g/100 ml*	*%*	*g/100 ml*	*%*	*g/100 ml*	*%*
				Phase 1 (10 weeks)					
Basal	10.28	3.92	38.1	6.34	61.8	3.14	30.6	3.20	31.2
Basal + phytic acid	9.88	3.70	37.5	6.17	62.4	3.35	34.0	2.82	28.5
Basal + zinc	7.01	4.33	61.8 [2]	2.68 [2]	38.1 [2]	1.95	27.8	0.72 [3]	10.4 [3]
				Phase 2 (5 weeks)					
Basal	11.05	4.80	43.5	6.24	56.5	2.83	25.6	3.41	30.9
Basal + zinc (repletion)	8.18	4.62	56.5	3.56	43.5	2.05	25.1	1.50	18.4
Basal + zinc	7.65	4.72	61.8	2.92	38.2	1.97	25.8	0.95	12.4

[1] The relative percentages of the various fractions were analyzed by electrophoresis, the total protein concentrations were by the Folin-Lowry method.
[2] Values significantly different from the other 2 treatments ($P < 0.01$).
[3] Values significantly different from the other 2 treatments ($P < 0.05$).

fraction showed a tendency to increase in the deficient lambs but not significantly. The γ- fraction, however, showed a significant ($P < 0.05$) increase in the deficient lambs. Albumin levels tended to decrease in the deficient lambs but the differences were nonsignificant. This decrease in the serum albumin may possibly be a first-order effect of the deficiency; however, the increased globulin level is probably a response to secondary infection resulting from the open lesions of the skin.

The zinc analyses of the tissues, as listed in table 5, indicate some reduction in all tissues taken from the deficient animals at the end of phase 1. Significant reductions were noted in the heart, muscle, pancreas and wool. Similar reductions in tissue zinc were observed in deficient calves by Miller and Miller (6). Samples taken at the end of the second phase showed a general increase in zinc content of the repleted lambs when compared with the deficient lambs. Differences in the kidney, liver, pancreas and spleen were significant. Exceptions to this were the brain and skin. Zinc content of the brain was higher in the lambs not receiving added zinc. The zinc level of the skin did not show a decrease in the deficient animals. This is contrary to what might be expected in observing the other symptoms

TABLE 5

Zinc content of tissue

Tissue	Phase 1			Phase 2		
	Basal	Basal + phytic acid	Basal + zinc	Basal	Basal + zinc (repletion)	Basal + zinc
	μg Zn/g fresh tissue			*μg Zn/g fresh tissue*		
Brain	10.8	10.2	12.7	11.2	10.4	8.5
Heart	15.6 [1]	15.2 [1]	17.5	18.5	21.2	21.2
Kidney	15.6	17.0	17.7	12.1 [2]	15.6	15.8
Liver	24.4	28.4	35.1	27.2 [2]	43.4	39.6 [3]
Muscle	25.7 [4]	21.6 [4]	33.9	— [5]	— [5]	— [5]
Pancreas	11.2 [4]	11.2 [4]	17.4	11.63	16.2	18.0
Skin	5.3	4.0	5.5	7.1	8.8	6.5
Spleen	21.6	19.6	22.3	20.0 [6]	21.6	22.2
Wool	98.8 [1]	101.4 [1]	120.0	— [7]	104.6	121.5

[1] Values significantly different from the basal + zinc diet ($P < 0.05$).
[2] Values significantly different from other 2 treatments ($P < 0.01$).
[3] Value significantly different from the repleted group ($P < 0.05$).
[4] Values significantly different from the basal + zinc diet ($P < 0.01$).
[5] Samples of muscle tissue for lambs on phase 2 were lost during analysis.
[6] Value significantly different from other 2 treatments ($P < 0.05$).
[7] Lambs on the basal diet did not have enough wool for analysis at the end of phase 2.

of the deficiency. A possible explanation may be the variations in the samples analyzed due to varying amounts of hair and wool, which are high in zinc, and varying amounts of subcutaneous fat. These variations in sample uniformity were not a problem with respect to the analysis of other tissues but may have had considerable influence on accuracy of the analysis of the skin.

Histopathological examination of the skin revealed a typical parakeratotic condition. Skin samples taken from the hock and flank areas (fig. 4) showed thickening of the stratum corneum with retention of the nuclei, accumulation of debris, thickening of the stratum germinativum, elongation of the rete pegs and acanthosis in the deficient lambs. Skin from lambs receiving phytic acid appeared to be less severely affected. The lambs that were repleted for 5 weeks lost all excessive stratum corneum; however, the 5-week period appeared to be insufficient for the stratum germinativum to return to normal. Examination of the adrenals, pituitary, thyroid, spleen, pancreas, liver, rumen, reticulum, omasum, abomasum, small intestine, kidney and brain did not reveal any changes which could be associated with the deficiency.

The differences shown by the gross symptoms score, feed efficiency and histological examination appear to indicate that the phytic acid may reduce the severity of the zinc deficiency in sheep. The mechanism of this action is not clear, but may relate to the phosphorus supplied by

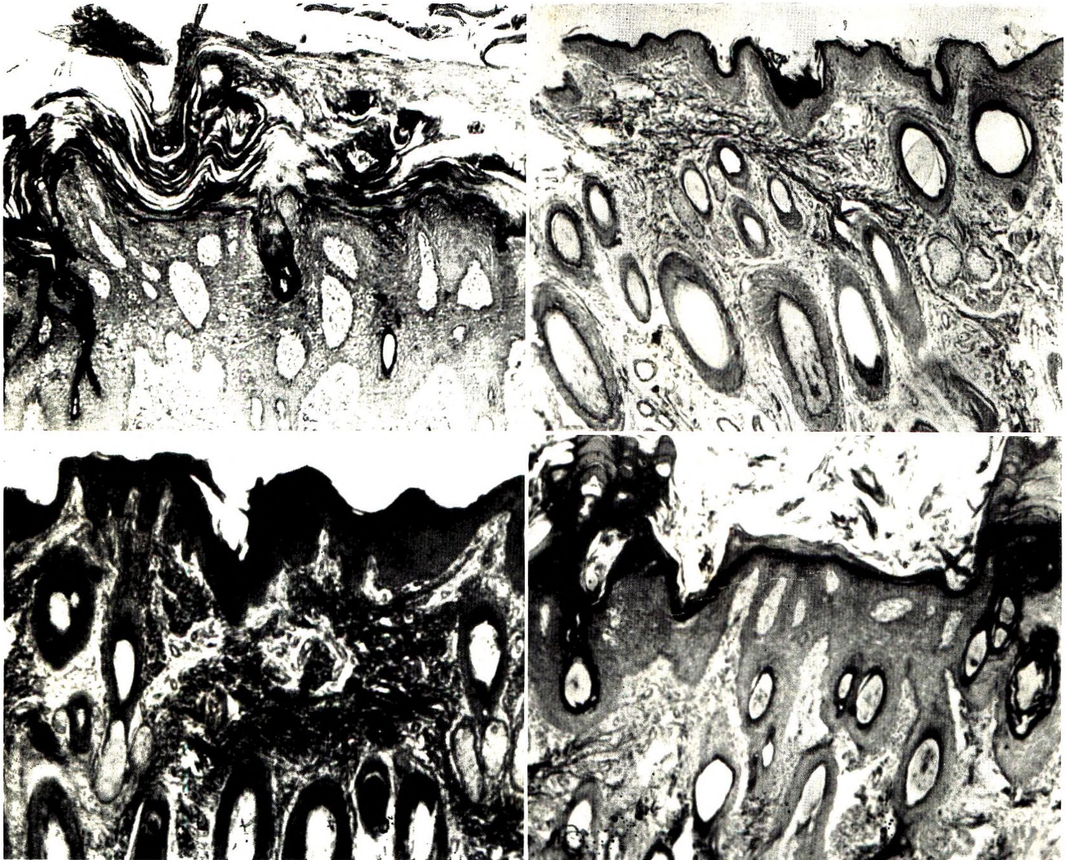


Fig. 4 Photomicrographs of skin sections from lambs receiving different levels of zinc. Upper left, lamb fed basal for 10 weeks, deficient. Upper right, lamb fed basal + 100 mg zinc/kg, normal. Lower left, lamb repleted for 5 weeks. Lower right, lamb fed basal + 1.525% phytic acid solution for 10 weeks.

TABLE 6

Molar percentage of volatile fatty acids and pH of rumen fluid from lambs fed the basal, basal + phytic acid and basal + 100-mg zinc/kg diets with and without polyethylene

Treatment	pH	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid	Valeric acid
Phase 1 (10 weeks)						
Basal	7.13	47.2	30.6	10.3	5.4	6.3
Basal + phytic acid	6.42	60.6	27.2	8.2	1.3	2.6
Basal + zinc	6.30	57.0	24.8	10.9	2.3	6.1
Polyethylene	6.17	49.3	27.6	13.6 [1]	1.4	5.4 [1]
No polyethylene	6.90	61.6 [1]	26.1	6.6	3.2	2.4
Phase 2 (repletion for 5 weeks)						
Basal	7.05	51.3	37.5	5.7	2.3	3.2
Basal + zinc (repletion)	6.45	58.6	21.6 [2]	14.1 [2]	0.7	5.0
Basal + zinc	6.55	54.8	18.7 [2]	19.2 [2]	1.0	6.2

[1] Differences between treatments are significant ($P < 0.05$).
[2] Values significantly different from the basal ($P < 0.01$).

the phytic acid. Tillman (17) showed that the phosphorus in phytic acid is available to the ruminant and Lewis et al. (18) demonstrated that under certain conditions the addition of phosphorus to a low zinc diet for swine reduced the severity of the resulting parakeratosis. Similar conditions may exist in this diet because it supplied calcium and phosphorus at a level of 0.70 and 0.48% respectively, but the added phytic acid resulted in a final phosphorus content of 0.65% or nearly a 1:1 ratio with the calcium.

Differences in the rumen fluid pH were not significant; however, there was a trend toward higher pH values in the deficient lambs (table 6). This may be attributed to the increased salivation which they exhibited. At the end of the first phase no significant differences in VFA proportions could be attributed to the high or low zinc diets. Polyethylene additions resulted in a significant increase ($P < 0.05$) in the percentage of butyric and valeric acids with a corresponding decrease in acetic acid. This variaiton is probably due to the differences in rumination. Since all the lambs were given polyethylene during the second phase, a further check on the polyethylene's effect could not be made. The additional 5 weeks of feeding the basal diet brought about a significant reduction ($P < 0.01$) in butyric acid production and a corresponding increase of propionic acid. The repleted lambs corresponded well with the lambs which had been fed the basal + 100 mg zinc/kg diet for the entire 15-week period.

ACKNOWLEDGMENTS

Our appreciation to R. H. Tetzloff for care of the animals; Mrs. L. Reitz for her assistance and advice on laboratory procedures; Dr. W. R. Featherston for serum protein analysis; and Dr. A. L. Delez for his advice on the histopathological changes in the deficient animals.

LITERATURE CITED

1. Tucker, H. F., and W. D. Salmon 1955 Parakeratosis or zinc deficiency in the pig. Proc. Soc. Biol. Med., *88:* 613.
2. Kernkamp, H. C. H., and E. F. Ferrin 1953 Parakeratosis in swine. J. Am. Vet. Med. A., *123:* 217.
3. Legg, S. P., L. Sears 1960 Zinc sulphate treatment of parakeratosis in cattle. Nature, *186:* 1061.
4. Haaranen, S., and K. Hyppola 1961 Cure, prevention of dairy cattle itch and hair slicking with zinc. Feedstuffs, 33: 46: 38.
5. Miller, J. K., and W. J. Miller 1960 Development of zinc deficiency in Holstein calves fed a purified diet. J. Dairy Sci., *43:* 1854.
6. Miller, J. K., and W. J. Miller 1962 Experimental zinc deficiency and recovery of calves. J. Nutrition, 76: 467.
7. Miller, J. K., W. J. Miller and C. M. Clifton 1962 Calf response to starters of varying zinc contents. J. Dairy Sci., *45:* 1536.
8. Plumlee, M. P., D. R. Whitaker, J. H. Conrad, W. H. Smith, H. E. Parker and W. M. Beeson 1960 The effect of phytic acid and other organic factors on zinc utilization by the growing pig. Feedstuffs, 32: 34: 10.
9. Oberleas, D., M. E. Muhrer and B. L. O'Dell 1962 Effects of phytic acid on zinc availability and parakeratosis in swine. J. Animal Sci., *21:* 57.
10. Lawlor, M. J., W. H. Smith and W. M. Beeson 1963 Semi-purified diets for sheep. J. Animal Sci., 22: 481.

11. Margerum, D. W., and G. Santacana 1960 Evaluation of methods for trace zinc determination. Anal. Chem., *32:* 1157.
12. Hoefer, J. A., E. R. Miller, D. E. Ullrey, H. D. Ritche and R. W. Luecke 1960 Interrelationships between calcium, zinc, iron and copper in swine feeding. J. Animal Sci., *19:* 249.
13. 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.
14. Erwin, E. S., G. J. Marco and E. M. Emery 1961 Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. J. Dairy Sci., *44:* 1768.
15. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State University Press, Ames.
16. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
17. Tillman, A. D., and J. R. Brethour 1958 Utilization of phytin phosphorus by sheep. J. Animal Sci., *17:* 104.
18. Lewis, P. K., Jr., W. G. Hoekstra, R. H. Grummer and P. H. Phillips 1956 The effect of certain nutritional factors including calcium, phosphorus and zinc on parakeratosis in swine. J. Animal Sci., *15:* 741.

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. N. WILLIAMS, JR.

Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, U. S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland

ABSTRACT The influence of including 0.3% DL-methionine in a protein-free ration upon liver succinic oxidase, succinic dehydrogenase, and protein was studied in the adult male rat. The changes were followed as a function of time during protein depletion followed by repletion. Enzyme assay systems were used in which the mitochondria were "damaged" by including calcium ions in the media, thus allowing free access of substrates and dye acceptor to the enzymes. The results with succinic oxidase and succinic dehydrogenase were almost identical, clearly indicating that the rate-limiting step in the succinic oxidase system under these conditions is at the initial dehydrogenase. In the group receiving the protein-free ration without methionine, the activities of the enzymes decreased to 40% of normal after 8 weeks with a slight increase toward normal after about 15 weeks. In the group receiving methionine in the protein-free ration, after an initial decrease to 60% of normal after 3 weeks (which was identical to the response in the group receiving no protein and no methionine), a rapid increase toward normal occurred with normal values for these enzymes being reached after 15 weeks. No significant change occurred in the pair-fed controls throughout the study. Protection of liver protein by methionine in a protein-free ration was also observed. Results from further studies with all other essential amino acids as well as cystine, arginine, and glutamic acid, added individually to the protein-free ration at levels equivalent to 0.3% methionine, indicated that cystine as well as methionine significantly protected the liver cells against loss of succinic oxidase and succinic dehydrogenase. No other amino acid tested showed this effect. Both cystine and methionine also protected liver cells from loss of other protein but to a less extent than succinic oxidase and succinic dehydrogenase.

The initial results of an investigation of cellular changes in the liver of the rat as a function of protein depletion have been presented in previous reports of this series (1–3). In the investigation an attempt was made to define, biochemically and physiologically, the adaptation of liver cells to prolonged protein depletion followed by repletion. In particular, emphasis was placed on those components in the cell most closely related to the maintenance of energy metabolism. To this end a detailed study of the adaptive response of the succinic oxidase system — the initial dehydrogenase and the individual components of the associated electron transport system — as a model mitochondrial system, was carried out.

A few brief reports have appeared in the literature that methionine, when fed at a low level in protein-free rations, offers some as yet unelucidated protection of certain liver components (3, 4). In addition it has been found that methionine or cystine in a protein-free ration causes a several-fold increase in liver lipids.[1] A detailed report of this work will be given separately. Recently Wannemacher and Allison[2] have reported that addition of methionine to a protein-free diet prevented loss of liver ribonucleic acid (RNA) and protein and tended to increase serum albumin levels to above normal levels. In the first 2 publications of the present series

Received for publication July 5, 1963.

[1] Williams, J. N., Jr., and A. D. Jasik 1963 Production of fatty livers by methionine and cystine. Abstracts, Sixth International Congress of Biochemistry, Edinburgh.

[2] Wannemacher, R. W., and J. B. Allison 1963 The effect of methionine upon serum and liver protein metabolism. Federation Proc., 22: 202 (abstract).

(1, 2), 0.3% DL-methionine, which had originally been added to supplement the 20% casein ration, was retained in the protein-free ration. Since this level of methionine, although quite low, might have influenced the results, it was important to re-examine some of the variables studied earlier both with and without methionine included in the protein-free ration. Preliminary results indicated that methionine did influence the results with succinic oxidase and dehydrogenase. Therefore, a study of the effects not only of methionine but also of cystine, arginine, glutamic acid (as control) and each of the other essential amino acids was undertaken. The results of these studies are presented herewith.

In the present paper succinic dehydrogenase has also been re-studied using a system in which the mitochondrial membrane permeability barrier to the electron acceptor in the assay system was eliminated. In the second communication of this series (2), it was pointed out that the method then used for estimating succinic dehydrogenase, which utilized intact mitochondria, probably did not give a true measure of the enzyme activity. Singer and Lusty (5) had reported while the paper was in press that the use of phenazine methosulfate as electron acceptor for assaying succinic dehydrogenase in intact rat liver mitochondria gave erroneously low values since the membrane of undamaged mitochondria exhibits a permeability barrier toward the dye. If the membrane was damaged, for example by including calcium ions in the medium, the barrier could be eliminated.

EXPERIMENTAL

The study was divided into 2 phases. In the first phase the changes in succinic oxidase and succinic dehydrogenase associated with prolonged protein depletion were followed as a function of time of protein depletion followed by repletion. In this phase the effect of including methionine in the protein-free ration was studied. In the second phase the effects of adding individually all other essential amino acids as well as cystine, glutamic acid, and arginine at equimolar levels in a protein-free ration were studied.

Phase 1. Adult male rats of the Sprague-Dawley strain were fed a complete purified diet (diet R1 (1)) for a 3-week adjustment period. The complete diet consisted of: (in per cent) casein, 20; DL-methionine, 0.3; corn oil, 5; salts N plus molybdate (6), 6.5; glucose monohydrate, 63.5; choline chloride, 0.2; *i*-inositol, 0.02; and water soluble-vitamin mix in glucose monohydrate (7), 4.5. Fat-soluble vitamins (1) were given weekly to each rat in 2 drops of corn oil. The rats, which weighed 290 g after the initial adjustment period, were separated into 4 groups. Group 1 received diet R1 ad libitum. Group 2 received diet R1 but was pair-fed with group 4 (average daily food consumption). Group 3 received ad libitum diet R1 from which the casein was omitted. Group 4 received ad libitum diet R1 from which both casein and methionine were omitted. The food consumption of groups 3 and 4 was almost identical.

At zero time and after various time intervals, rats from each group were killed and the livers removed, rapidly weighed, and chilled. Succinic oxidase was determined by the procedure of Potter (8). In this method the mitochondria are damaged by adding calcium to the assay medium. Succinic dehydrogenase was assayed using the same system as for succinic oxidase except for the following changes: 0.1 ml of 3% phenazine methosulfate was included in a side-arm of the flask, and 0.3 ml of 0.01 M KCN was added to the main compartment, both at the expense of water. After temperature equilibration, the phenazine methosulfate and substrate were added to the main compartment and oxygen uptake recorded after 0.5 minute and again after 5.5 minutes. The difference between the 2 readings gave the rate of initial oxidation of succinate *via* the initial dehydrogenase.

Nitrogen was determined in the first phase of the study by a semi-micro Nesslerization procedure as described previously (1). Nitrogen × 6.25 was taken as the value for protein. In the second phase protein was determined directly by the method of Lowry et al. (9). Liver DNA was estimated by using the extraction procedure of Schneider (10) followed by color development with diphenylamine (11).

Since the enzyme assay for only 4 rats could be handled satisfactorily on any one day, the points on the figures in the Results section represent the averages for the animals killed from about the fifth day before to the fifth day after the indicated day. One rat from each of the 4 groups was usually killed on a single day so that rats from the control groups were run simultaneously with experimental rats whenever possible. The animals surviving after 102 days of depletion were re-fed the complete diet ad libitum until nearly normal body weight had been reached. Group 2 was still pair-fed with group 4 during the repletion. The numbers of animals killed for each point were as follows: zero time, 8; 24, 56, and 102 days, 7 to 10 of each group; 3 days post-repletion, 4 to 8 of groups 2–4; 6 days post-repletion, 4 of groups 2–4; 15 days post-repletion, 4 of groups 2–4; 25 days post-repletion, 8 of group 1; 56 and 86 days post-repletion, 6 to 12 of each group.

Phase 2. When it had been ascertained from the first phase of the study that methionine had an effect on the enzymes being studied, another group of 112 rats was set up for an initial adjustment period with the complete diet. After the adjustment period, the rats were separated into 14 groups of 8 rats each (groups 1A–14A) and fed the individual diets for 8 weeks. The diet fed to each group is shown in the legend of figure 4. The purpose of studying all of these amino acids was to ascertain whether the effects observed in phase 1 were specific for methionine or whether other essential amino acids as well as cystine, arginine, or glutamic acid (as control) also showed specific effects.

Enzyme assays and other analyses were carried out as described above in phase 1.

RESULTS AND DISCUSSION

The results are presented as activity or concentration per milligram DNA. Since the concentration of DNA per average liver cell nucleus has been found to be constant during protein deprivation in adult rats (12), activity or concentration of liver cellular components expressed per unit weight of DNA should be directly proportional to the activity or concentration of a component per average liver cell. The results in phase 1 have been analyzed using Student's t test to determine whether the differences between the means of groups 2–4 versus 1, groups 3 and 4 versus 2, and group 4 versus 3 at any one time are significant. The method of noting the significant differences is shown in the legend of the figures. The results in phase 2 are presented as bar graphs for the 14 different groups. The results have been analyzed statistically and the range of 2 standard errors is drawn for each bar to denote significance of difference among the means.

Phase 1. In Figure 1 are presented the results for the response of liver succinic oxidase to protein depletion, with and without added 0.3% DL-methionine. Results for the ad libitum- fed (group 1) and pair-fed controls (group 2) have been separated from the 2 protein-deficient groups (groups 3 and 4) for clarity. The values for the ad libitum-fed controls are presented as microliters of oxygen uptake per hour per milligram of DNA. The results for the other 3 groups are presented as percentage of the ad libitum-fed control values. This was done to negate the influence of the fluctuations in the points for group 1 upon the other groups. Thus a clearer picture of the effects of the diets per se on the variables is obtained.

Three-tenths per cent methionine in the protein-free diet markedly spares succinic oxidase. After the initial loss in both groups 3 and 4 to 60% of normal, the values for group 4 continued to decrease until at 56 days after depletion, the value was only 40% of normal. The presence of methionine, however, enabled group 3 to maintain a significantly higher level of succinic oxidase than that of group 4 after the initial loss. In fact the activity per cell of group 3 returned almost to normal by the one hundred second day of depletion. It cannot be decided at present whether this return toward normal of group 2 represents an actual resynthesis of the enzyme system from body protein, whether it is a type of enzyme induction, or whether it is a reflection of less loss of the enzyme system with respect to other cellular components. When protein was replaced in the diet of group 4 the succinic oxidase activity rebounded to above normal and then declined slowly

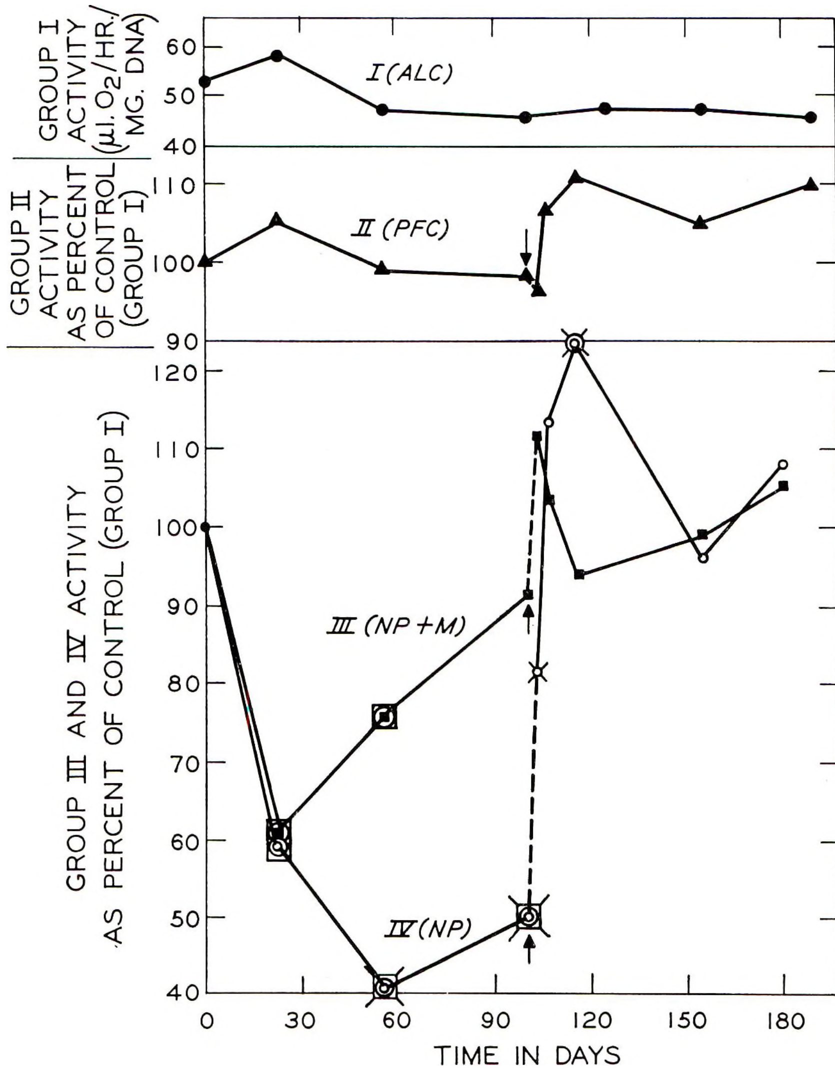


Fig. 1 The response of liver succinic oxidase to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. (I, ALC), ●, indicates ad libitum-fed controls; (II, PFC), ▲, indicates pair-fed controls; (III, NP + M), ■, indicates protein deficient rats supplemented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

Tests for significance of difference between means

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

The numbers of rats at each point are given in the text.

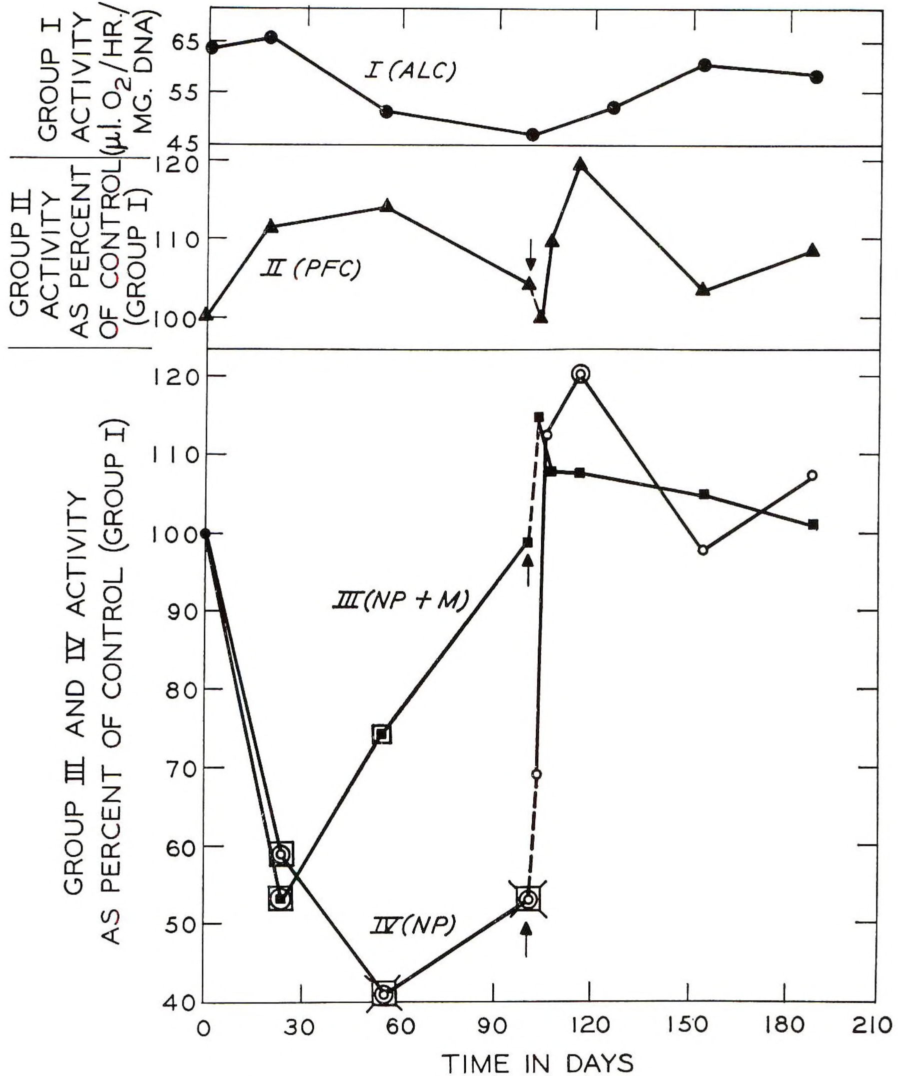


Fig. 2 The response of liver succinic dehydrogenase to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. (I, ALC), ●, indicates ad libitum-fed controls; (II, PFC), ▲, indicates pair-fed controls; (III, NP + M), ■, indicates protein-deficient rats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

Tests for significance of difference between means

Group	versus	Group	Notation for $P < 0.05$ (*see exception below*)
II, III, IV		I	○ (large circles)
III, IV		II	□
IV		III	╳

P was < 0.01 in all cases except for IV versus I at 24 days, and III versus II at 56 days.

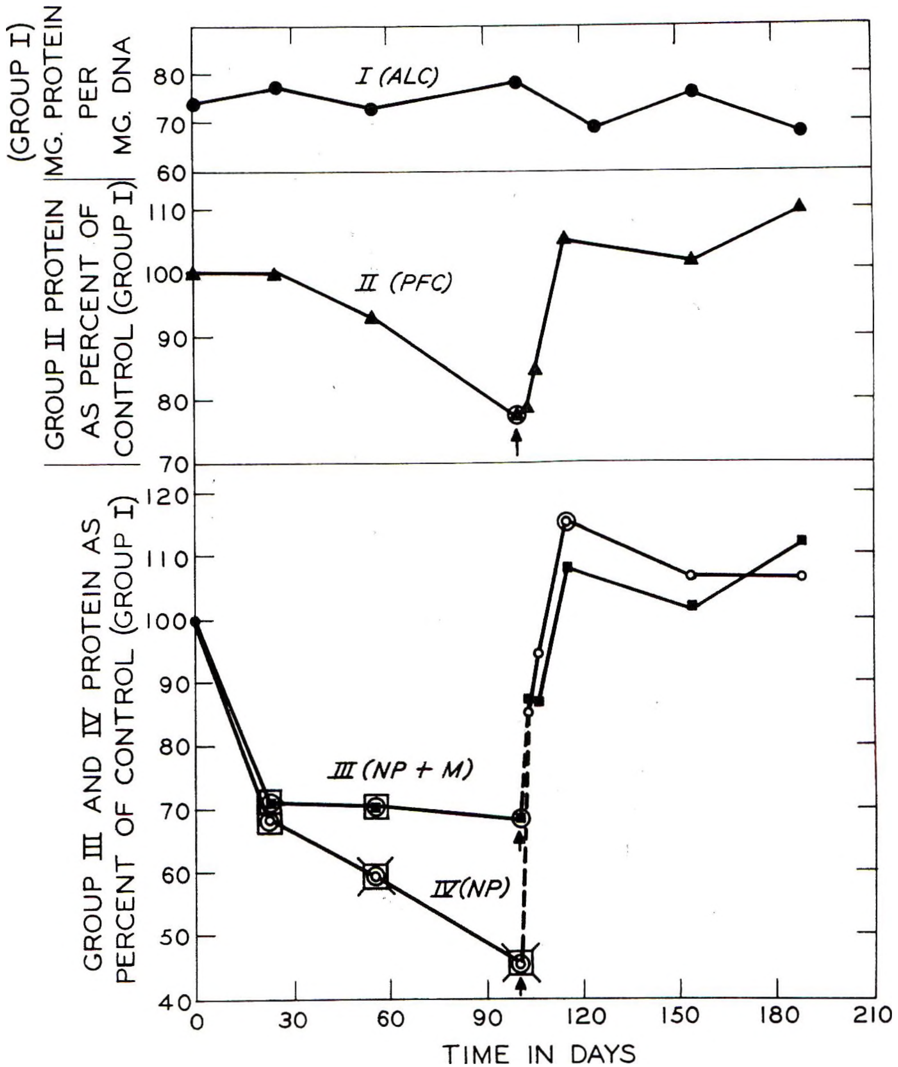


Fig. 3 The response of liver protein (N × 6.25) to prolonged feeding of a protein-free ration with and without 0.30% DL-methionine, followed by repletion. (I, ALC), ●, indicates ad libitum-fed controls; (II, PFC), ▲, indicates pair-fed controls; (III, NP+M), ■, indicates protein-deficient rats supplemented with 0.30% DL-methionine; (IV, NP), ○ (small open circle), indicates protein-deficient rats. Repletion was begun at the arrows. The results for groups II–IV are expressed as percentage of the ad libitum-fed controls (I).

Tests for significance of difference between means

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

The numbers of rats at each point are given in the text.

toward normal. The values for the pair-fed controls did not change significantly throughout the study. Thus the effects of 0.3% methionine in the protein-free ration and of the protein-free ration itself on succinic oxidase are specific effects of the diets and not of changes in food intake.

The results for succinic dehydrogenase (fig. 2) were almost identical to those for succinic oxidase. This indicates clearly that in the succinic oxidase system the rate-limiting step lies in the initial dehydrogenase. In the present studies, the measurement of succinic dehydrogenase was carried out with mitochondria damaged by calcium addition, thus allowing free access of substrate and phenazine methosulfate (the dye acceptor) to the enzyme.

A similar protective effect of methionine was observed in the initial study of this series (2). However, the magnitude of the absolute values was considerably less in the earlier study since the mitochondria were kept intact.

The protection of succinic oxidase by methionine as indicated in figure 1 can thus probably be explained by methionine protection of the initial dehydrogenase. Since succinic dehydrogenase is the rate-limiting step in the system, any changes observed in the succinic oxidase system would be a reflection of changes in succinic dehydrogenase.

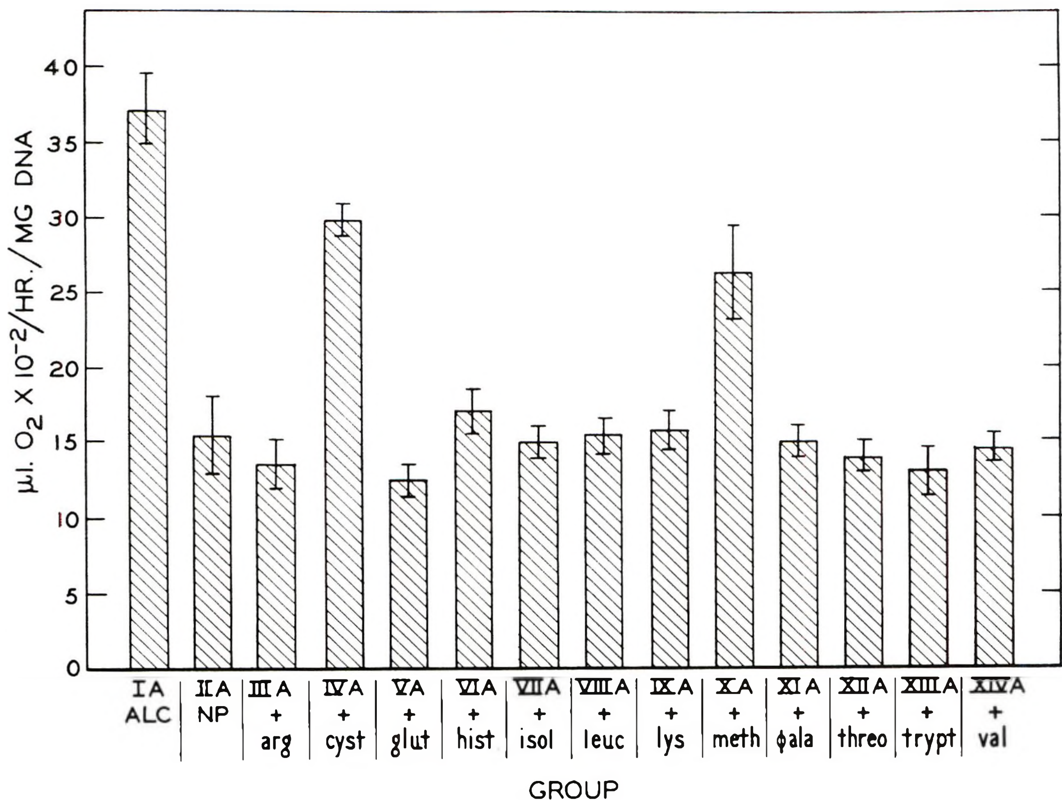


Fig. 4 Succinic oxidase activity per milligram DNA in rats fed a normal control diet (IA), a protein- and amino acid-free diet (IIA) and protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA – XIVA) for 56 days. The meanings of the abbreviations in the figure are as follows: arg, 0.42% L-arginine·HCl; cyst, 0.24% L-cystine; glut, 0.30% L-glutamic acid; hist, 0.42% L-histidine·HCl·H_2O; isol, 0.20% L-isoleucine; leuc, 0.26% L-leucine; lys, 0.37% L-lysine·HCl; meth, 0.30% L-methionine; ϕ ala, 0.33% L-phenylalanine; threo, 0.24% L-threonine; trypt, 0.41% L-tryptophan; val, 0.24% L-valine; ALC = group IA = ad libitum-fed controls; NP = group IIA = rats fed protein- and amino acid-free diet. Twice the standard error of each group is represented by the vertical line through the mean. Each mean is obtained from 7 to 8 rats.

For comparing the enzyme responses with changes in other liver protein, liver protein data from the same animals from which the enzyme results were obtained are presented in figure 3. Although protein was lost from the liver cells in both groups 3 and 4, the specific pattern of loss was different from that for the enzymes. Protein was continuously lost from the liver cells of both groups of rats throughout the depletion in contrast with an elevation of succinic oxidase and dehydrogenase per cell in group 3 after 24 days and in group 4 after 56 days. Nevertheless methionine protected the liver cells to some extent against loss of protein. Also the animals in the pair-fed control group [2] lost significant protein from their liver cells, whereas the levels of the enzymes were unchanged in that group.

Phase 2. In figures 4–6 are presented the results of studies with groups of rats fed the protein-free diets containing other amino acids added individually to the protein-free ration. In addition to methionine, cystine protected succinic oxidase and dehydrogenase (figs. 4, 5). None of the other amino acids tested had any significant effect on these enzymes. The effects of cystine and methionine appear to be equivalent. The effect of these 2 amino acids on liver protein is shown in figure 6. Although both amino acids prevented the loss of protein from the liver cells to some extent, the degree of protection was not as great as with succinic oxidase and dehydrogenase.

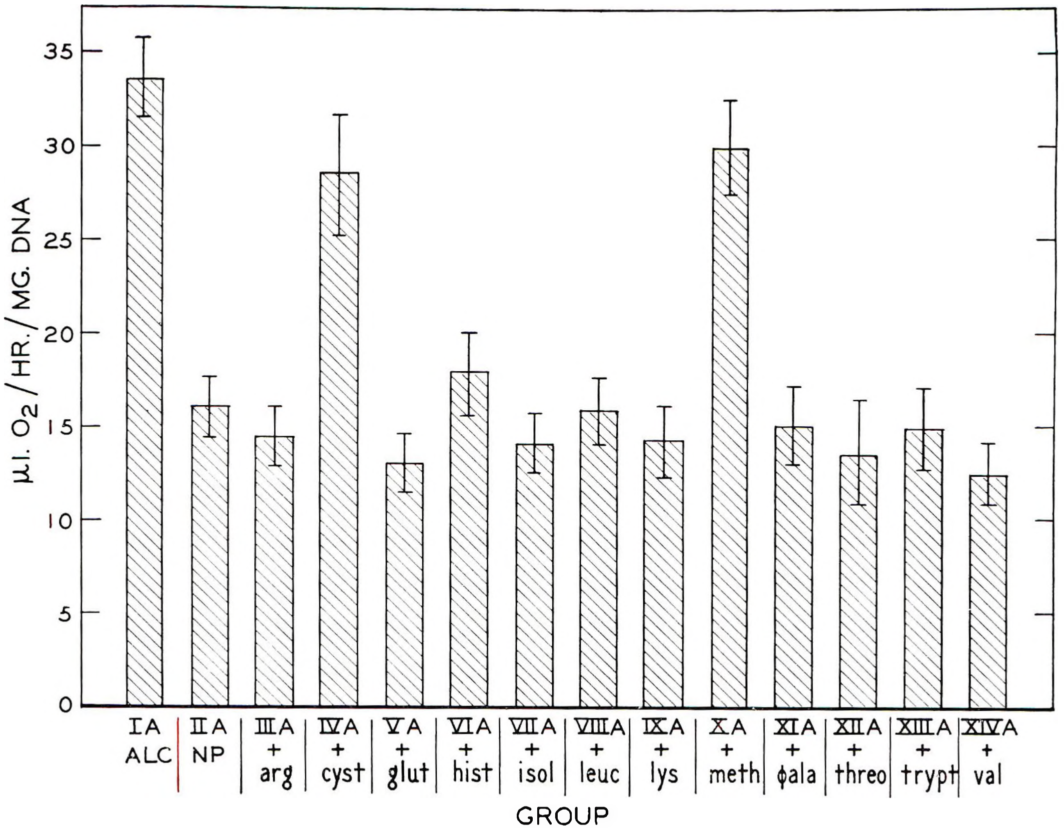


Fig. 5 Succinic dehydrogenase actvity per milligram DNA in rats fed a normal control diet (IA), a protein- and amino acid-free diet (IIA) and protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA–XIVA) for 56 days. The meanings of the abbreviations are given in the legend of figure 4. Twice the standard error of each group is represented by the vertical line through the mean. Each mean is obtained from 7 to 8 rats.

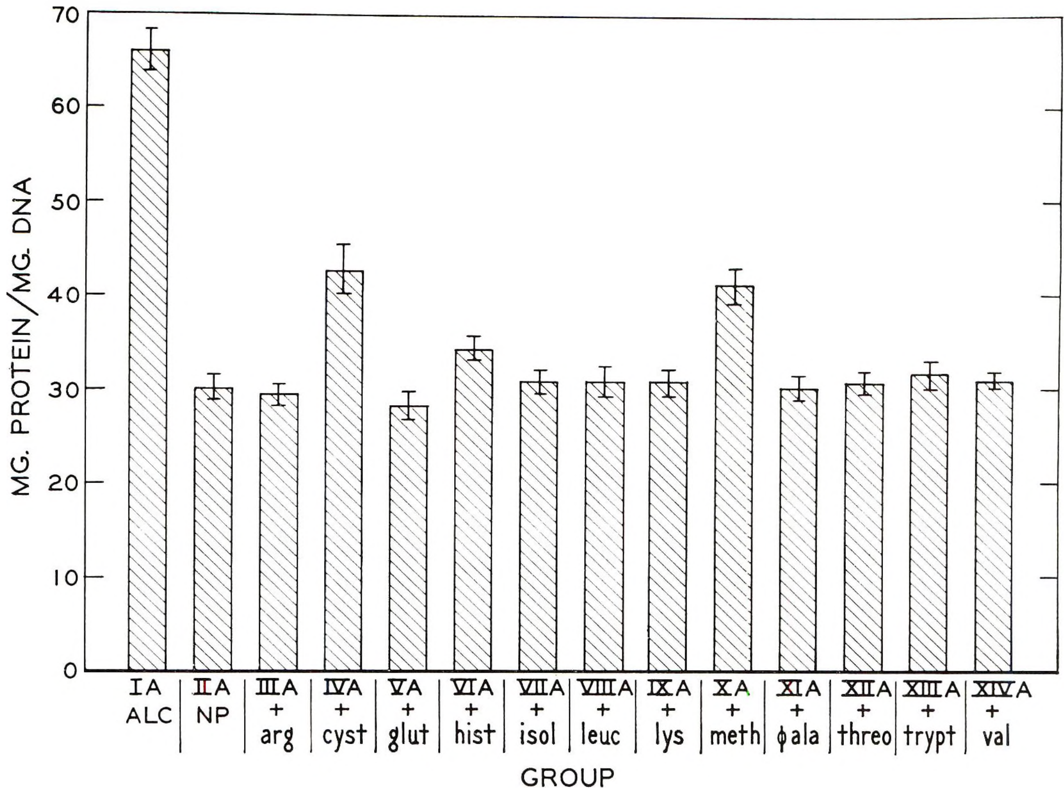


Fig. 6 Liver protein per milligram DNA in rats fed a normal control diet (IA), a protein- and amino acid-free diet (IIA) and protein-free diets containing various amino acids equimolar with 0.30% L-methionine (IIIA – XIVA) for 56 days. The meanings of the abbreviations are given in the legend of figure 4. Twice the standard error of each group is represented by the vertical line through the mean. Each mean is obtained from 7 to 8 rats.

The results with groups 1A, 2A, and 10A corroborate the results in figures 1–3 almost exactly at the 56-day point. Histidine appears to have had a slight effect in preventing loss of liver protein.

The absolute values for succinic oxidase and dehydrogenase activities in phase 1 are somewhat higher on the average than in phase 2 (compare ALC, figs. 1 and 4, and 2 and 5). It is difficult to explain why this occurred. However, it is the author's experience that different groups of rats, although all of the same strain and treated as identically as possible, often show slightly different average enzyme activities from one year to the next. In the present study, phase 1 was carried out approximately a year before phase 2. However, in each phase appropriate controls were included *simultaneously* with the experimental groups to negate this effect.

Since succinic dehydrogenase contains sulfhydryl groups that are necessary for its activity, it is inviting to suggest that methionine and cystine protect the enzyme by protecting the sulfhydryl groups. However, if the only effect of methionine and cystine is to protect activity in this manner, why should they also prevent loss of liver protein? Until more can be learned about amino acid conservation mechanisms and interrelationships among amino acids with respect to protein re-synthesis in protein deficient animals, it would be premature to state with certainty a mechanism for their actions in the present paper. A possible lead for further work is given by the results of Aschkenasy (13), who observed that if low protein diets are supplemented with methionine, the livers of male rats become hypertrophied. If the animals are first adrenalectomized, this effect of methionine

is not obtained. The answers to some of these questions are being sought at present.

ACKNOWLEDGMENTS

The author wishes to thank Alice Jasik for her competent technical assistance in these studies and Mrs. Esther Hurley and Woodrow Duvall for feeding and care of the animals and for preparation of the diets used.

LITERATURE CITED

1. Williams, Jr., J. N. 1961 Response of the liver to prolonged protein depletion. I. Liver weight, nitrogen, and deoxyribonucleic acid. J. Nutrition, *73:* 199.
2. Williams, Jr., J. N. 1961 Response of the liver to prolonged protein depletion. II. The succinic oxidase system and its component enzymes. J. Nutrition, *73:* 210.
3. Williams, Jr., J. N. 1963 Response of the liver to prolonged protein depletion. III. Coenzyme Q. Arch. Biochem. Biophysics, *101:* 512.
4. Boissier, J., and A. Aschkenasy 1950 Effects comparés de l'administration de glycocolle, de thiamine et de méthionine sur les organs de rats privés protéines alimentaires. Bull. Soc. Chemie Biologique, *32:* 942.
5. Singer, T. P., and C. J. Lusty 1960 Permeability factors in the assay of mitochondrial dehydrogenases. Biochem. Biophys. Res. Comm., *2:* 276.
6. Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutrition, *72:* 243.
7. Fox, M. R. S., L. O. Ortiz and G. M. Briggs 1955 Toxicity of ethionine in the young chick. Agr. Food Chem., *3:* 436.
8. Umbreit, W. W., R. H. Burris and J. F. Stauffer 1959 Manometric Techniques. Burgess Publishing Company, Minneapolis, p. 173.
9. 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.
10. Schneider, W. C. 1945 Phosphorus compounds in animal tissue. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.
11. Davidson, J. N., and C. Waymouth 1944 Tissue nucleic acids. 3. The nucleic acid and nucleotide content of liver tissue. Biochem. J., *38:* 379.
12. Thomson, R. Y., F. C. Heagy, 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 the composition of liver tissue. Biochem. J., *53:* 460.
13. Aschkenasy, A. 1955 Suppression par la surrénalectomie de la protection des foie par méthionine contre les effects du régime hypoprotidique. C. R. Soc. Biologique, *149:* 663.

Supplementation of Wheat Gluten Protein[1]

W. L. BANKS, JR.,[2,3] J. B. ALLISON AND R. W. WANNEMACHER, JR.
Bureau of Biological Research, Rutgers — The State University, New Brunswick, New Jersey

ABSTRACT Male weanling Wistar rats were fed diets containing wheat gluten (group 1), wheat gluten supplemented with lysine monohydrochloride (group 2), wheat gluten plus casein (group 3), casein (group 4), and egg albumin (group 5) as the dietary protein sources, as well as a diet free of protein (group 6). The nitrogen growth index of 8.6 for the group 1 animals was improved by either method of supplementation to 16.7, values less than the 20.2 and 27.0 for the group 4 and 5 animals, respectively. Liver protein DNA and RNA/DNA ratios increased with both nitrogen intake and nutritive value of the dietary protein except in those animals in group 2. The values for these ratios in animals in groups 1 and 2 were the same. In the muscle, these ratios increased with both type and quantity of dietary protein without exception. In the brain, these parameters were unaltered by quantity or quality of dietary protein. When these cellular parameters were compared at nitrogen intakes producing animals that gained 50 g over the experimental period of 28 days, considerable difference was noted in these quantities as a result of feeding the various proteins even though the animals would have gained the same weight.

Wheat gluten is an example of an "unbalanced" protein, deficient in the amino acid, lysine (1). Supplementation of wheat gluten with an optimal quantity of this amino acid[4] resulted in an increased rate of growth in young rats, a rate that was almost equal to that associated with rats fed casein. Gain in body weight is only one parameter that may be used to assess the nutritive value of a dietary protein. Recently, more attention has been given to responses of individual cells and tissues to dietary protein changes. Allison et al. (2), for example, examined the response of protein/DNA and RNA/DNA in several tissues to variation in quantity and quality of dietary protein. These investigators found that protein/DNA increased with nitrogen intake in liver, muscle and kidney, but this ratio remained essentially constant in the brain. The ratio increased also in liver and muscle as the nutritive value of the dietary protein was increased. Nutritive value had less effect upon protein/DNA in the kidney and little or no effect upon the ratio in the brain.

The object of the present paper was to study the changes in some of the parameters associated with protein biosynthesis in various tissues which result from supplementation of wheat gluten protein with lysine or casein. These 2 supplemented proteins were compared with the growth promoting qualities of unsupplemented wheat gluten, casein and egg albumin protein.

METHODS

Groups of 10 male weanling rats of the Wistar strain were fed diets containing wheat gluten, wheat gluten supplemented with lysine monohydrochloride (4.7 g/100 g protein), wheat gluten plus casein (2.1 : 1), casein, or egg albumin protein. These diets were prepared in an agar gel base (table 1) (3) and fed ad libitum at several protein levels. Wheat gluten was supplemented with lysine and casein at levels that would produce an equivalent growth response over the experimental period. Daily food consumption and weekly body weight changes were recorded.

After 28 days, the animals were killed and samples were taken of the brain, gastrocnemious muscle, and liver. A 2.0-g sample of the liver was homogenized in 10.0 ml of a 0.35 M sucrose solution buffered with bicarbonate to a pH of 7.4. Weighed samples of the brain and muscle were homogenized in 5.0 ml of the sucrose

Received for publication July 22, 1963.

[1] This work was supported in part by a United States Public Health Service Grant AM-04341.
[2] A Johnson and Johnson pre-doctoral Fellow.
[3] Present address: School of Aerospace Medicine, Brooks AFB, Texas.
[4] Middleton, E. J. 1959 A study on the lysine supplementation of wheat gluten. Ph.D. Thesis, Rutgers University, New Brunswick, New Jersey.

TABLE 1

Basal agar gel diet [1]

	g		*mg/1000 g dry diet*
Protein	180	Thiamine	10
Sucrose	134	Riboflavin	20
Dextrose	212	Pyridoxine	10
Dextrin	140	Menadione	15
Lard	231	Niacin	80
Salt	40	Pantothenic acid	80
Agar	33	Inositol	200
Cod liver oil	20	Folic acid	0.5
α-Tocopherol [2]	10	Biotin	0.5
	1000	*p*-Aminobenzoic acid	80.0
Water	1400	Choline	2000.0
	2400	Ascorbic acid	2.0
			units/1000 g dry diet
		Vitamin A	40,000
		Vitamin D	4,000

[1] The protein content of the diet can be varied by isocaloric replacement of the protein with sucrose and dextrose (3).

[2] 100 mg in 10 g hydrogenated cottonseed oil (Crisco, Procter and Gamble, Cincinnati).

buffer. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined using a modification[5] of the ultra-violet absorption techniques suggested by Hutchison and Munro (4). Total protein was estimated by the Biuret method of Layne (5) on an alkaline hydrolyzate of an acid-insoluble precipitate of the homogenate from which the lipid had been extracted.

Standard errors were determined for all values and a probability value (*P*) of less than 0.01 was taken as the level for statistical significance.

RESULTS

Nitrogen growth index, a measure of the growth promoting quality of a dietary protein (6), was determined for the various diets. The indexes determined for different proteins were wheat gluten, 8.6; casein, 20.2; and egg albumin, 27.0. Supplementing wheat gluten with either the free amino acid, lysine, or casein protein improved the nitrogen growth index to a value of 16.7. Thus, the value of wheat gluten as a dietary protein source for body weight gain was improved equally by either type of supplementation although the amino acid patterns were not the same. The significance of these differences in patterns will be discussed later (table 2).

The data plotted in figure 1 illustrate the effects of nitrogen intake and quality of dietary protein upon RNA/DNA, protein/DNA, and protein/RNA in the liver. The RNA/DNA ratio increased with nitrogen intake, the greatest increase being associated with the feeding of egg albumin and the least with the feeding of wheat gluten. Supplementing wheat gluten with lysine did not result in a greater increase in RNA/DNA ratio than was noted in animals fed unsupplemented wheat gluten. Supplementing with casein, however, improved the RNA/DNA ratio but not to the value observed for casein alone. The protein/DNA ratio followed the same pattern illustrated for RNA/DNA, the most protein being formed in animals fed egg, less in animals fed casein or wheat gluten supplemented with casein, and least in those fed wheat gluten or wheat gluten supplemented with lysine. These data suggest that there is a good correlation between protein in the liver and total RNA, which is confirmed by the constant ratio, protein/RNA, illustrated in figure 1.

These results demonstrate that the maximal protein/DNA, resulting from feeding egg albumin, was not obtained by feeding large intakes of proteins with low nutritive values. Supplementing wheat gluten with lysine did not improve protein biosynthesis in the liver above the results obtained while feeding unsupplemented wheat gluten. It

[5] Banks, W. L., Jr. 1963 Studies on the supplementation of wheat gluten protein in the growing rat. Ph.D. Thesis, Rutgers University, New Brunswick, New Jersey.

TABLE 2

Amino acid intakes producing a body weight gain of 50 g with various dietary proteins

Amino acid	Egg albumin	Casein	Wheat gluten + casein	Wheat gluten + lysine	Wheat gluten	Minimal values [1]
	g	*g*	*g*	*g*	*g*	*g*
Total N	1.9	2.5	3.0	3.0	5.8	1.7
Arginine	0.71	0.61	0.71	0.69	1.33	0.41
Histidine	0.28	0.48	0.45	0.37	0.72	0.25
Isoleucine	0.72	0.99	0.98	0.85	1.65	0.68
Leucine	1.04	1.56	1.53	1.35	2.61	0.93
Lysine	0.86	1.27	0.80	1.26	0.73	0.80
Phenylalanine	0.71	0.85	0.97	0.94	1.82	0.59
Tyrosine	0.40	0.81	0.69	0.52	1.01	0.37
Total aromatic	1.11	1.66	1.66	1.46	2.83	0.96
Cystine	0.35	0.06	0.31	0.44	0.86	—
Methionine	0.50	0.51	0.42	0.31	0.60	—
Total S	0.85	0.57	0.73	0.75	1.46	0.65
Threonine	0.58	0.70	0.64	0.53	1.02	0.51
Tryptophan	0.13	0.15	0.16	0.17	0.28	0.11
Valine	0.89	1.15	1.01	0.80	1.55	0.81
Proline	0.51	2.09	2.66	2.65	5.12	—
Glutamic acid	1.53	3.50	5.70	6.56	12.75	—
Aspartic acid	1.31	1.14	0.95	0.71	1.36	—
Glycine	0.45	0.31	0.53	0.62	1.20	—

[1] Allison et al. (7).

is possible that the free lysine added to the wheat gluten was absorbed and transported in such a way that the improved growth of the rats when fed the supplemented protein was associated primarily with growth of tissue other than the liver, tissue such as muscle. Figure 2 illustrates the increase in protein/DNA in the muscle with nitrogen intake, the greatest increase being associated with the feeding of casein and wheat gluten supplemented with casein. Adding lysine to wheat gluten, however, increased the protein/DNA ratio above that of those fed unsupplemented wheat gluten at the higher intakes.

One way to compare the effect of feeding various dietary proteins upon the variables discussed in this paper would be to make the comparison at some fixed gain in body weight. The amino acid intakes that produced a gain in body weight of 50 g in rats fed various dietary proteins are recorded in table 2. These data suggest that the gain of 50 g in rats fed wheat gluten was associated with excess essential amino acid when compared with minimal values, except for lysine. Thus, lysine could be considered the limiting essential amino acid in wheat gluten. When lysine was added to wheat gluten, however, the essential amino acids were all in excess of minimal values, suggested by Allison et al. (7), except for threonine and valine. Supplementing wheat gluten with casein resulted in lysine as the limiting amino acid, whereas sulfur amino acids were limiting for casein. Thus, each dietary protein presented a different problem in meeting the amino acid requirements for protein biosynthesis.

The data in figure 3 summarize the results obtained during this gain of 50 g. An objection to a comparison at such a low gain in weight is the restriction put upon the proteins of high nutritive value which would, with higher intake and greater growth, produce a cell with more protein/DNA than would be possible with the protein of lower nutritive value. Nevertheless, the difference in nitrogen growth index is well established at this gain of 50 g as illustrated by the data plotted in figure 1. The RNA/DNA and protein/DNA ratios observed in the liver illustrate the poor re-

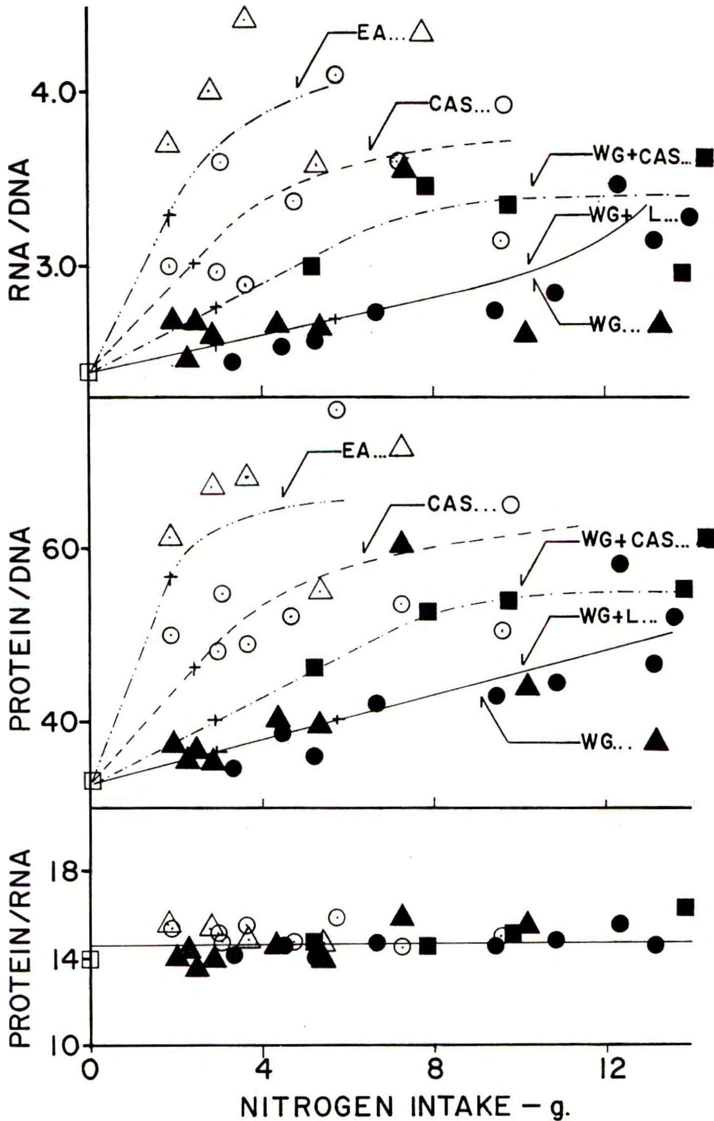


Fig. 1 RNA/DNA, protein/DNA and protein/RNA ratios vs. nitrogen intake for 28 days from the livers of animals fed wheat gluten (solid triangles), wheat gluten supplemented with lysine monohydrochloride (solid circles), wheat gluten plus casein (solid squares), casein (open circles), egg albumin protein (open triangles), and a protein-free diet (PFD) (open squares). All curves were drawn as an approximate fit of the experimental points. Plus signs on curves indicate 50-g gain in body weight.

sponse of the liver-to-lysine supplementation of wheat gluten. The unusually large RNA-to-DNA ratios in the muscles of the animals fed wheat gluten may reflect the relatively large intake of nitrogen in these animals which was necessary to obtain a 50-g weight gain. The low protein/RNA in the muscle of animals fed wheat gluten may reflect a compensating mechanism whereby the muscle protein is catabolized to supply amino acids which could correct the amino acid pattern of wheat gluten. There was little difference in the protein/DNA ratio in the muscles of animals fed

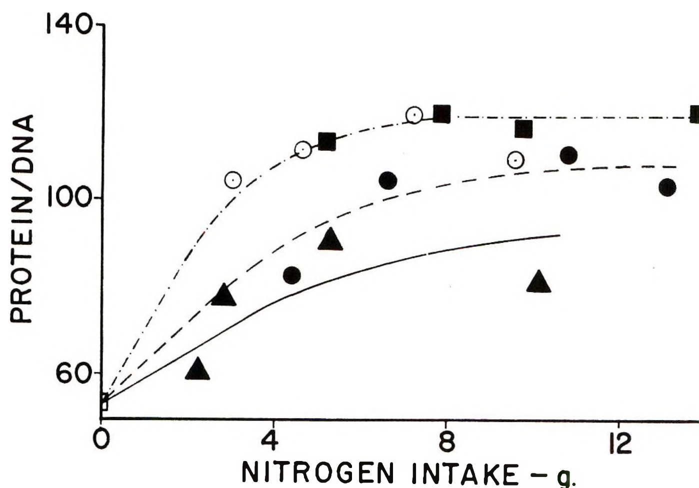


Fig. 2 Protein/DNA vs. nitrogen intake for 28 days of the muscles of animals fed wheat gluten (solid triangles), wheat gluten supplemented with lysine monohydrochloride (solid circles), wheat gluten plus casein (solid squares), casein (open circles), and a protein-free diet (PFD) (open squares).

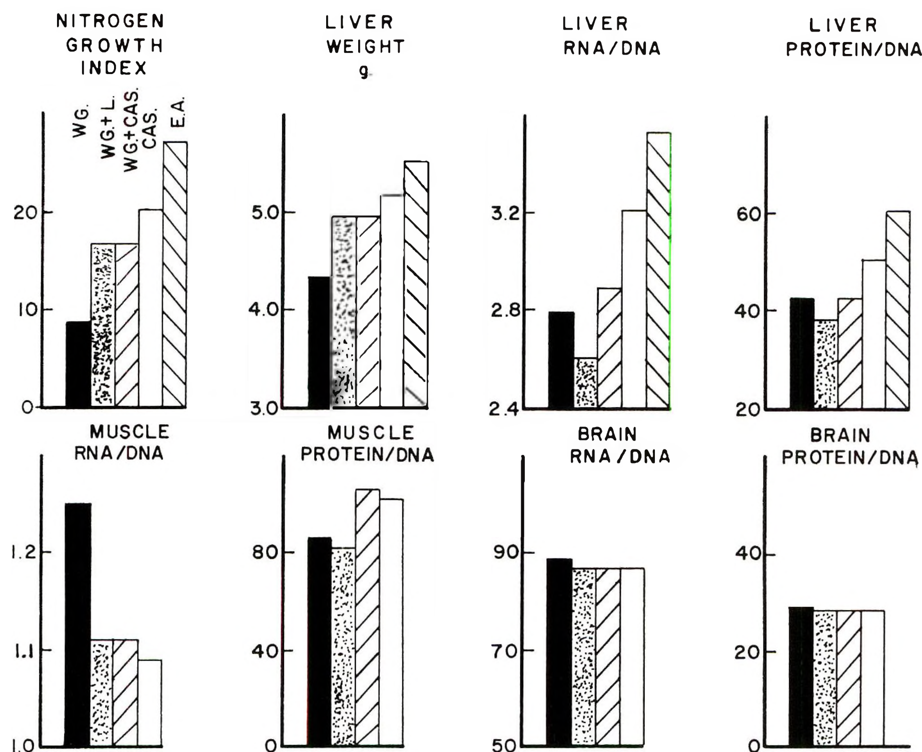


Fig. 3 A comparison of cellular constituents at nitrogen intakes that produced a gain in weight of 50 g. The black bars record data obtained while feeding wheat gluten (WG); the other bars beginning with the stippled bar illustrate data obtained while feeding wheat gluten supplemented with lysine (WG + L), wheat gluten plus casein (WG + CAS), casein (CAS), and egg albumin (EA), respectively.

either wheat gluten or wheat gluten supplemented with lysine. Supplementation of wheat gluten with casein, however, resulted in a higher protein/DNA ratio in the muscle, a ratio that was equivalent to that obtained in animals fed casein. A greater gain in body weight than 50 g in animals fed wheat gluten supplemented with lysine resulted in an improved protein/DNA ratio in the muscle (fig. 2). Thus, nutritive value often can be expressed best at more adequate nitrogen intakes. These data demonstrate also that a nitrogen intake from various dietary protein sources to produce the same gain in weight may result in different biochemical responses in various tissues. A constant response, however, independent of quality of protein is illustrated for the brain in figure 3. The complete significance of a nitrogen growth index, therefore, requires a detailed study of each tissue as it exists in the dynamic metabolic state of the body as a whole.

LITERATURE CITED

1. Grau, C. R., and M. Kamei 1950 Amino acid imbalance and growth requirements for lysine and methionine. J. Nutrition, *41:* 89.
2. Allison, J. B., R. W. Wannemacher, Jr., W. L. Banks, Jr., W. H. Wunner and R. A. Gomez-Brenes 1962 Dietary proteins correlated with ribonuclease, ribonucleic acid and tissue proteins. J. Nutrition, *78:* 333.
3 Allison, J. B., R. W. Wannemacher, Jr., R. Hilf, J. F. Migliarese and M. L. Crossley 1954 Dietary protein and tumor-host relationship in the rat. J. Nutrition, *54:* 593.
4. Hutchison, W. C., and H. N. Munro 1961 The determination of nucleic acid in biological materials. Analyst, *86:* 786.
5. Layne, E. 1957 Spectrophoretic and turbidimetric methods of measuring proteins. III. Biuret method. In Methods in Enzymology, Vol. 3. Academic Press, Inc., New York, p. 450.
6. Allison, J. B., W. H. Fitzpatrick 1960 Dietary Proteins in Health and Disease. Charles C Thomas, Springfield, Illinois.
7. Allison, J. B., R. W. Wannemacher, Jr., E. Middleton and T. Spoerlein 1959 Diet protein requirements and problems of supplementation. Food Technol., *13:* 597.

A Determination of the Essential Amino Acid Proportions Needed to Allow Rapid Growth in Chicks[1]

D. C. DOBSON, J. O. ANDERSON AND R. E. WARNICK
Poultry Department, Utah State University, Logan, Utah

ABSTRACT A series of experiments was conducted to determine how the essential amino acids in a ration should be proportioned to allow the chick to grow at a rapid rate. In the approach used, the level of each essential amino acid in a ration was adjusted until the growth rate was decreased about the same amount when a constant fraction of any essential amino acid was removed. A purified-type ration was fed which contained approximately 9.0% essential amino acids and 2.88% nitrogen provided by a mixture of proteins and amino acids. The ration considered to have the best essential amino acid balance was calculated to contain 1.28% arginine, 0.43% histidine, 1.15% lysine, 1.30% leucine, 0.80% isoleucine, 0.95% valine, 1.33% phenylalanine and tyrosine, 0.20% tryptophan, 0.73% methionine and cystine, and 0.78% threonine. Chicks fed a ration with the natural isomer of these amino acids at these levels gained 25% more weight and their gain/feed ratio was 15% higher than that of chicks fed a ration adjusted to the National Research Council minimum requirement levels. These amino acid levels are not minimum requirements; replacing simultaneously 12.3% of all 12 with an equal level of nitrogen as glutamic acid did not decrease growth rate.

Experiments have been conducted to determine the chick's minimum requirement for each of the essential amino acids. In a typical experiment conducted to define a requirement, chicks were fed graded levels of the amino acid in a ration that contained the proper level of protein and energy, and adequate levels of other required nutrients. The lowest level of the amino acid that would allow the chick to grow at the maximum rate obtained and with the highest feed efficiency, was considered to be the minimum requirement. Members of the National Research Council (1) have reviewed reports of research conducted to define requirements and selected a set of requirements which has been used extensively as a guide in formulating commercial broiler rations.

The requirement for each essential amino acid varies with the protein and energy content of the ration and cannot be expressed as a constant percentage of a ration. In stating the amino acid requirements of the chick, the protein and energy levels of the ration are usually specified. The requirements given by the NRC are for a ration with 20% protein and 900 kcal of productive energy/454 g.

The chick's requirement for arginine is higher when casein provides most of the protein than when commercial-type rations are fed. Anderson and Dobson (2) observed that the high arginine requirement associated with rations based on casein could be reduced by replacing part of the casein with any of the several mixtures of nonessential amino acids. No particular one of the nonessential amino acids was required in the mixtures. Klain et al. (3) reported that chicks fed rations containing amino acids in the proportions occurring in a casein ration, also required a relatively high level of arginine. Casein is an unusually rich source of most of the essential amino acids except arginine. The high arginine requirement associated with the casein rations is apparently related to the relatively high level of one or more of the essential amino acids in the ration.

Experiments have been conducted, using a ration whose protein mixture did not include casein, to study what effect increasing the level of each essential amino acid would have on the arginine require-

Received for publication July 29, 1963.

[1] Approved as Utah Agricultural Experiment Station Journal Paper no. 359.

ment of chicks (2). Increasing the lysine level produced a reduction in growth rate, which was partly overcome by adding more arginine. This indicated that the high lysine level in casein is the factor most responsible for the high arginine requirement of chicks fed rations based on casein. When both lysine and arginine were added, growth rate was further increased by adding a mixture of methionine, threonine, and histidine. The lysine and arginine addition evidently increased the requirement for one or more of the other 3 amino acids.

Anderson and Dobson (2) also reported results of other experiments which indicated that the amount of lysine required to allow the chick to grow at the maximum rate also was increased when the levels of the other 9 essential amino acids in a ration were increased. Under these circumstances the lysine requirement did not appear to be increased as much as the arginine requirement.

A limited number of experiments have been conducted to determine whether the overall essential amino acid level in the ration affects the requirements for individual amino acids other than lysine and arginine.[2] When a ration was fed in which the total essential amino acid/protein ratio was relatively high, comparable to that in a ration based on casein, the amount of leucine recommended by the NRC was adequate. The listed requirements for histidine and threonine proved inadequate in a test of a similar ration. The results indicated that a ration including the essential amino acids at the NRC requirement levels would not constitute the best balanced mixture that might be produced. A variety of diets were fed to chicks to determine the adequacy of requirements adopted by the NRC. These diets varied in their total essential amino acid content and in their balance among the essential amino acids. Requirements determined in this way conceivably would not be the best balanced set that might be developed.

The increases in individual amino acid requirements, as produced by increasing the levels of the other essential amino acids (2),[3] were not associated with significant changes in the gross energy or protein levels of the ration, or in the maximum growth rate obtained. Thus it appeared that even when the protein and energy content of a ration were specified, the essential amino acid content of the mixture that would allow the most rapid growth rate could not be defined on the basis of minimum requirements alone. Apparently a balance among the essential amino acids was also required.

The present paper reports the results of a series of experiments conducted to determine how the essential amino acids should be proportioned in a ration to allow the chick to grow at a rapid rate. In the approach used in these experiments, a ration was said to contain "balanced proportions" of the amino acids when the growth rate obtained with the ration decreased by the same amount when a given fraction of any one of the essential amino acids was removed.

EXPERIMENTAL

Determining the balanced proportions. A series of 36 experiments was conducted to adjust the level of each essential amino acid in a ration until the growth rate obtained with the ration was reduced by the same amount when a given fraction of any one of the essential amino acids was removed. The amino acids considered essential in these experiments are listed in the lower section of table 1.

In the first experiment, a basic ration was formulated which was judged to have a reasonable balance among the essential amino acids. This ration and 10 modifications were fed to chicks in an effort to determine which of the essential amino

[2] The results of these experiments have not been published in detail. They were conducted primarily to determine whether it would be worthwhile to conduct the work reported in this paper. Our previous research indicated that the lysine or arginine requirements increased as the level of the other essential amino acids in the ration increased. The unpublished results suggested that the same was true of the requirements for the other essential amino acids. It appeared unlikely, however, that any arbitrary increase in the total level of essential amino acids in a ration would produce an increase in the requirement for a particular amino acid. Our ability to demonstrate these increases in requirements probably depended on starting with certain proportions among the essential amino acids. After these unpublished experiments were conducted, our ability to judge amino acid balance in these rations appeared somewhat questionable. Further experiments of the same type were not considered worthwhile at that time. The results of the unpublished experiments were presented at the 1959 meeting of the Poultry Science Association. Dobson, D. C., and J. O. Anderson 1959 Effect of essential amino acids in the diet on the chick's requirement for specific amino acids. Poultry Sci., 38: 1199 (abstract).

[3] Unpublished results.

TABLE 1

Composition of the protein and amino acid portion of several rations and their calculated essential amino acid content [1]

Ingredients [2]	Ration						
	1	2	3	4	5	6	7
				g/kg of ration			
Blood fibrin	33.6	31.6	50.0	50.0	50.0	50.0	50.0
Casein	27.2	25.6	56.5	56.5	56.5	56.5	56.5
Isolated soybean protein	69.9	65.8	0	0	0	0	0
Gelatin	39.3	36.9	32.0	32.0	32.0	32.0	32.0
L-Arginine·HCl	2.95	3.69	5.2	6.17	5.04	4.26	3.50
L-Histidine·HCl	1.05	1.30	0	1.60	1.23	0.94	0.68
L-Lysine·HCl	2.26	2.97	0.99	2.86	1.81	1.09	0.38
L-Leucine	1.94	2.60	4.61	3.61	2.66	2.01	1.36
DL-Isoleucine	3.64	4.36	1.36	5.36	4.20	3.40	2.60
DL-Valine	4.36	5.22	2.24	5.24	3.86	2.90	1.96
DL-Phenylalanine	1.14	1.86	4.04	3.44	2.50	1.80	1.12
L-Tyrosine	1.66	1.95	1.93	1.53	1.03	0.72	0.39
L-Tryptophan	0.36	0.46	0.20	0.20	0.05	0	0
DL-Methionine	1.77	2.30	2.01	1.51	1.22	1.02	0.82
L-Cystine	2.11	2.28	2.40	2.20	1.97	1.79	1.63
DL-Threonine	3.44	4.16	0.48	4.08	2.94	2.16	1.38
Glycine	1.92	2.80	4.73	4.73	4.73	4.73	4.73
L-Glutamic acid	10.37	13.76	46.59	27.21	40.42	49.6	58.5
Calculated essential amino acid levels, % of ration							
Arginine	1.28	1.28	1.20	1.28	1.19	1.12	1.06
Histidine	0.43	0.43	0.30	0.43	0.40	0.38	0.36
Lysine	1.15	1.15	1.00	1.15	1.07	1.01	0.95
Leucine	1.30	1.30	1.40	1.30	1.21	1.14	1.08
Isoleucine	0.80	0.80	0.60	0.80	0.74	0.70	0.66
Valine	0.95	0.95	0.80	0.95	0.88	0.83	0.79
Phenylalanine	0.67	0.67	0.70	0.67	0.62	0.59	0.55
Tyrosine	0.66	0.66	0.70	0.66	0.61	0.58	0.55
Tryptophan	0.20	0.20	0.20	0.20	0.19	0.18	0.18
Methionine	0.40	0.40	0.45	0.40	0.37	0.35	0.33
Cystine	0.33	0.33	0.35	0.33	0.31	0.29	0.27
Threonine	0.78	0.78	0.60	0.78	0.72	0.68	0.65
Total essential	8.95	8.95	8.30	8.95	8.31	7.85	7.43

[1] The composition of the remainder of these rations is given in the text of this paper.
[2] Most of the amino acids used in all rations were purchased from Nutritional Biochemicals Corporation, Cleveland.

acids were provided by the basic ration at relatively high levels and which at relatively low levels in relation to the needs of the chick. Using this information a new, and presumably better-balanced basic ration was formulated. The new basic ration was then fed in a similar experiment. This procedure was followed throughout the entire series.

A fraction of each essential amino acid was removed in turn from each basic ration to produce its 10 modifications. The fraction removed was constant for all essential amino acids in any one experiment, but it varied from 10 to 20% in the different experiments conducted. A composite fraction of methionine and cystine was removed from a basic ration to produce one modified ration. The methionine level equaled 50 to 65% of the total level of these 2 amino acids in all rations. A composite fraction of phenylalanine and tyrosine was removed from a basic ration to produce another of the modified rations. The phenylalanine level equaled 50 to 55% of the total level of these amino acids in all rations.

If removing a fraction of an amino acid from the basic ration decreased growth rate more than the average decrease obtained, it was assumed that the basic ration contained a relatively low level of that amino acid. If the decrease in weight gained was less than average, it was as-

sumed that the basic ration contained a relatively high level of that amino acid.

The basic ration fed in each experiment contained a total of 2.88% nitrogen and approximately 9.0% of the essential amino acids provided by a mixture of proteins and amino acids. The 9.0% essential amino acid level was arbitrarily fixed at the beginning of the series of experiments. This level is less than that in rations fed to broilers in commercial production, but more than the sum of the NRC minimium requirements for these amino acids (8.3%). Tables giving the amino acid composition of ingredients used in commercial broiler production indicated that the ratio between the protein content and the total level of essential amino acids in commercial rations would be about two. Therefore, the protein level ($N \times 6.25$) in the experimental rations was 18%.

Glycine, glutamic acid, and proline, sometimes listed as essential amino acids for the chick, were not considered essential in these experiments. It was recognized that chicks grow more rapidly when these amino acids are included in their diet than when they are not, but their levels in a ration were not deemed as critical as those of the amino acids classed as essential in these experiments. All rations fed in these experiments contained these 3 amino acids, but no attempt was made to determine what proportions of each were needed to make a well balanced ration. Limiting these studies to the amino acids considered essential simplified the experiments.

Mixtures of isolated soybean protein,[4] casein,[5] blood fibrin,[6] and gelatin,[7] provided most of the amino acids in the rations used to define the amino acid balance. The defining rations also contained enough of an amino acid mixture to adjust the level of each essential amino acid to the desired level and allow for the desired reductions. L-Glutamic acid was used to adjust the nitrogen level to 2.88%. Kjeldahl determinations were made of the nitrogen content of all protein supplements. DL-Methionine was used and was considered effective as the natural isomer. The racemic form of isoleucine, valine, phenylalanine, and threonine was used but considered only one-half as effective as the natural isomer.

The individual amounts of 16 or 17 amino acids in the proteins used in these rations were determined by ion exchange chromatography after hydrolysis with 6 N HCl, by a procedure adapted from those given by Moore and Stein (4), Moore et al. (5), and Spackman et al. (6). Details on the procedure are given elsewhere.[8] These values, which were used in calculating the amino acid content of the rations fed, are shown in table 2. Since the tryptophan and some of the cystine were destroyed during the acid hydrolysis, the values for these amino acids were taken from the literature, as indicated in table 2.

The amino acid content of each ration was calculated to the nearest 0.01%, but it is realized that the errors involved in determining the balanced proportions do not justify expressing them with this accuracy. The essential amino acid levels in the basic rations were adjusted in increments of 0.025%.

All rations fed in these experiments contained the mineral mixture of Fox and Briggs (8) at a level of 6%, unless they contained amino acid hydrochlorides. If the rations included amino acid hydrochlorides, the chloride level of the ration was adjusted by replacing part of the sodium and potassium chlorides of the mineral mixture with the corresponding bicarbonates. Each kilogram of ration contained 100 g of a vitamin mixture which provided the following: (in mg) thiamine·HCl, 8; riboflavin, 10; pyridoxine, 8; niacin, 100; folic acid, 2; biotin, 0.3; Ca-D-pantothenate, 20; choline chloride, 2000; vitamin B_{12}, 0.01; menadione sodium bisulfite, 5.4; and *dl*-α-tocopheryl acetate, 40. The mixture also provided 10,000 IU of vitamin A and 1500 ICU of vitamin D_3/kg of ration. Glucose monohydrate[9] was used as the vitamin carrier. All rations contained 3% refined corn or cottonseed oil.

[4] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cincinnati, Ohio.

[5] Obtained from Nutritional Biochemicals Corporation, Cleveland.

[6] Obtained from Armour Livestock Bureau, Chicago.

[7] Swift's Superclear Gelatin, Swift and Company, Chicago.

[8] Dobson, D. C. 1961 Essential amino acid composition of a balanced chick diet. Ph. D. Thesis. Utah State University.

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

TABLE 2
Values used in calculating the amino acid content of rations

Amino acid	Isolated soybean protein	Blood fibrin	Casein	Gelatin
		g/16 g N		
Arginine	7.3	6.8	4.2	8.2
Histidine	2.7	2.5	3.2	1.1
Lysine	6.4	8.4	8.7	3.9
Leucine	9.1	7.7	10.3	3.1
Isoleucine	5.2	4.7	5.9	1.2
Valine	5.1	5.4	7.4	2.8
Phenylalanine	5.0	4.3	4.9	2.2
Tyrosine	3.4	5.1	5.4	0.6
Tryptophan	1.0 [1]	2.4 [2]	1.5 [2]	0 [2]
Methionine	1.3	2.1	2.9	0.5
Cystine	0.6 [1]	1.9	0.4 [2]	0.2 [2]
Threonine	4.1	6.6	4.6	1.8
Glycine	4.3	5.4	2.3	24.2
Glutamic acid	18.5	13.2	22.1	11.0
Proline	4.4	12.8	8.2	6.0
Serine	5.2	6.0	6.4	3.2
Aspartic acid	12.5	3.8	3.6	9.1
Alanine	4.9	4.2	8.9	11.8

[1] Value given by Archer-Daniels-Midland, Cincinnati.
[2] Values taken from Block and Weiss (7).

Glucose monohydrate was used to adjust the rations to 100%.

Vantress × Nichol's-108 chicks of both sexes were fed a commercial-type ration for 5 to 7 days. They were then distributed to experimental groups of 6 to 8 chicks each on the basis of their weights. The experimental rations were fed for 9 to 10 days. These periods were the same for all groups in any one experiment, but they varied with the different experiments. The chicks were housed in electrically heated batteries with wire floors. Feed and water were offered ad libitum.

Only the results of the last 4 experiments of the series conducted to determine the balanced proportions are presented in this paper. Rations 1 and 2 (table 1) were the basic rations fed in the 4 experiments reported here. The level of each essential amino acid was reduced by 15% and by 20% of the level in the basic rations in these 4 experiments. The 15% and some of the 20% reductions were made by replacing the proper amount of each essential amino acid in ration 1 with glutamic acid. Where a 20% reduction could not be made by using ration 1, the reduction was made using ration 2.

Comparison of balanced and NRC requirement proportions. The performance of chicks fed a ration with the essential amino acids at the NRC requirement levels was compared with that of chicks fed a ration with these amino acids at the levels which the preceding series of experiments had indicated were well balanced. Results of the last 2 of these experiments are reported in this paper. In the first of these experiments this type of comparison was made by feeding 2 rations in which a mixture of the natural isomer of each of 16 amino acids provided all of the amino acids; another comparison was made in the same experiment using rations that contained proteins and amino acids.

One ration without protein contained the established NRC requirement level of each essential amino acid, although NRC levels are stated to be for a 20% protein ration and the ration fed contained only the equivalent of 18% protein. These levels are the same as calculated for ration 3 as given in table 1. The second ration contained the essential amino acids at the calculated levels of rations 1 or 4 (table 1). Both amino acid rations contained a mixture of essential amino acids, 2% glycine, 0.6% proline, 0.75% asparagine, and enough glutamic acid to adjust the nitrogen content to 2.88%. They also contained 0.6% magnesium trisilicate and

0.3% aluminum hydroxide. The amino acid mixture, the antacids, and the sodium and potassium bicarbonates for a ration were thoroughly mixed in a blender with enough ethanol and water mixture (1:3) to make a slurry. The rations contained 2% cellulose;[10] starch was the carbohydrate except for the glucose monohydrate used as a carrier for the vitamin mixture. The amino acid slurry was dried on the starch and cellulose. These rations contained 3% corn oil and the same vitamin mixture as the rations fed in defining the balanced proportions. The mineral mixture included also provided an identical level of each mineral except for that added by the antacids.

Another comparison of the balanced and the NRC requirement proportions was made by feeding rations 3 through 7 (table 1). Ration 3 was formulated to contain the NRC requirement level of each essential amino acid, whereas rations 4 through 7 contained different total quantities of these amino acids in the proportions used in ration 1. A single mixture of 3 proteins provided most of the amino acids in these rations. Different amino acid mixtures were added to the protein mixture to produce rations with the desired amino acid levels. Rations 4 through 7 contained progressively smaller amounts of each essential amino acid except that rations 6 and 7 contained identical tryptophan levels. All of the tryptophan in ration 6 was provided by the protein mixture and could not be reduced without changing the mixture. Enough glutamic acid was added to each ration to adjust the nitrogen level provided by proteins and amino acids to 2.88%. Glucose monohydrate was used to adjust these rations to 100%. They contained 0.3% magnesium trisilicate, 0.15% aluminium hydroxide, 3% corn oil, and 2% cellulose. The mineral and vitamin mixtures were the same as those added to the amino acid rations.

Three average chicks fed each of the rations in experiment 2 were killed with chloroform after they had fasted for 2 hours. The entire carcass of each bird, including the contents of the gastrointestinal tract, was ground and dried in an oven at 100°C to a constant weight to determine the dry matter. Nitrogen was determined in the dried residue using a Kjeldahl apparatus. Ether extract was also determined in the residue.

When data were analyzed statistically, the analysis of variance was used (9).

RESULTS AND DISCUSSION

Table 3 shows the results of the last 4 experiments of the series conducted to determine how the essential amino acids should be proportioned to make a balanced mixture. The decreases in growth rate, produced by decreasing the level of each essential amino acid by 15 or 20% of the ration 1 level, were not the same for all amino acids at either level of reduction. If ration 1 had balanced proportions of the essential amino acids, equal decreases in growth rate would not be expected in an experiment of this type because of the random variations in growth rate of chicks. These random variations limited our ability to define the balanced proportions. Also, it was realized that this approach might not define the proportions that would allow the fastest growth rate.

The series of experiments was terminated after the 4 experiments reported because further experiments using this approach did not then appear to be justified. The decreases in growth rate produced by removing the same fraction of each essential amino acid in turn were sufficiently similar to negate plans for further experiments. Results presented in table 3 and those of preceding experiments were evaluated in making this decision. The essential amino acid proportions in ration 1 (table 1) are considered well balanced, although it may be possible to achieve a mixture that would allow faster gains. It must be realized that under conditions which deviate from those used in defining these proportions, another set of proportions might be better balanced.

The calculated amino acid levels shown for ration 1 in table 1 reflect errors in the amino acid content assumed for the proteins in the ration. The cystine level for ration 1 in table 1 is 0.01% higher than that calculated for the ration by using the values given in table 2. Cystine in the pro-

[10] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 3

Results obtained when the level of each essential amino acid in rations 1 or 2 was decreased by 15 or 20%

Amino acid decreased	Rations 1, 2	Decreased levels	Avg daily gain [1]	Gain/feed ratio [1]
	%	%	g	
None (ration 1 or 2)	—	—	13.0	0.63
Arginine	1.28	1.09	12.4	0.61
		1.02	12.3	0.60
Histidine	0.43	0.37	12.4	0.62
		0.34	11.9	0.62
Lysine	1.15	0.98	12.0	0.62
		0.92	11.1	0.60
Leucine	1.30	1.11	12.2	0.64
		1.04	11.1	0.59
Isoleucine	0.80	0.68	12.3	0.62
		0.64	10.6	0.59
Valine	0.95	0.81	12.1	0.63
		0.76	11.6	0.61
Phenylalanine } Tyrosine }	0.67 } 0.66 }	0.57 } 0.56 }	12.5	0.63
		0.54 } 0.52 }	11.8	0.62
Tryptophan	0.20	0.17	11.9	0.60
		0.16	13.0	0.62
Methionine } Cystine }	0.40 } 0.33 }	0.34 } 0.28 }	12.5	0.62
		0.32 } 0.26 }	12.1	0.61
Threonine	0.78	0.66	12.7	0.63
		0.62	11.9	0.62

[1] The average gain and gain/feed ratio of chicks fed rations 1 or 2 are based on 12 groups of 6 chicks each; all of the other values are based on 4 groups of 6 chicks each. Approximate significant difference in rate of gain ($P < 0.05$) is 1.1 g/day; in gain/feed ratio, 0.025.

tein mixture was determined after oxidation to cysteic acid by the procedure of Bidmead and Ley (10). The cystine level found in the mixture was higher than that calculated by using the values given in table 2.

When the level of each essential amino acid was reduced by 20% of the ration 1 level, weight gain was reduced about 10% on the average, and the gain/feed ratio was reduced about 4%. A 15% reduction in the level of each essential amino acid reduced weight gain about 5% and the gain/feed ratio about 1.5%. When 10% reductions in the amino acid levels were made in other experiments, the weight gains were decreased about 1%. The 8.95% total level of the essential amino acids must be higher than a chick needs for rapid growth when a ration with 18% protein and the energy levels of these rations is fed.

While the NRC set of minimum requirements and the essential amino acid levels in ration 1 show a high correlation, some important differences may be noted. The 2 sets of values can be compared in table 1. Ration 1 contains a total of 8.95% of these amino acids, whereas ration 5 has the essential amino acids in the same proportions as ration 1 but with a total of 8.3%. Ration 3 has these amino acids in the NRC requirement proportions, also with a total of 8.3%. The balanced pro-

portions defined by these experiments have relatively more histidine, isoleucine, threonine, and valine, and relatively less phenylalanine and tyrosine, leucine, and methionine and cystine.

The balanced proportions defined by our experiments may not be the best balanced values that might be defined. However, chicks fed rations with the essential amino acids in these newly defined proportions have gained weight faster and utilized their feed more efficiently than chicks fed similar rations with the essential amino acids in the proportions given as minimum requirements by the NRC.

Results of the last experiment conducted to compare the 2 sets of values, using rations in which mixtures of the natural isomer of each of 16 amino acids provided all amino acids, are presented in the upper part of table 4. The rate of gain was 26% more and the gain/feed ratio 15% more when the ration contained the essential amino acids in the balanced proportions, than when the ration contained the NRC minimum requirement levels. Both differences were statistically significant ($P < 0.005$). The performance of the chicks fed either ration was as good as that obtained when rations were fed that contained intact protein and the same levels of the essential amino acids (compare results obtained with amino acid rations with those obtained when rations 3 and 4 were fed in experiment 1). Generally, it has been assumed that a high fat level was needed in a diet to allow chicks to grow rapidly when fed rations in which all of the amino acids are provided in the free form. This was not the case with either of the rations fed in these experiments.

TABLE 4

Results obtained when 18% protein rations were fed with the essential amino acids in the NRC (1) requirement proportions or in balanced proportions with different total levels [1]

Essential amino acid proportions	Total essential amino acids	Exp. no.	Avg daily gain	Gain/feed ratio	Carcass content [2]		
					Dry matter	Protein	Ether extract
	% of ration		*g*		*% of live weight*		
	Rations containing the natural isomer of 16 amino acids						
NRC requirement	8.30	1	11.9	0.52			
Balanced	8.95	1	15.0*	0.60*			
	Rations containing proteins and amino acids						
NRC requirement, ration 3	8.30	1	12.0	0.54			
	8.30	2	10.9	0.52	29.4	17.1	2.73
		avg	11.4	0.53			
Balanced, ration 4	8.95	1	14.3	0.58			
	8.95	2	14.0	0.60	30.6	16.4	2.62
		avg	14.1*	0.59*			
Balanced, ration 5	8.30	1	12.9	0.59			
	8.30	2	13.6	0.59	29.3	16.9	2.70
		avg	13.2*	0.59*			
Balanced, ration 6	7.85	1	14.0	0.60			
	7.85	2	13.9	0.60	29.9	16.7	2.67
		avg	13.9*	0.60*			
Balanced, ration 7	7.43	1	13.4	0.60			
	7.43	2	13.2	0.58	31.0	16.8	2.69
		avg	13.3*	0.59*			

[1] Duplicate groups of 5 chicks/treatment in experiment 1, and triplicate groups of 6 in experiment 2. Chicks were 7 days old at the time experiments were started. Their average starting weights were 104 and 99 g in experiments 1 and 2, respectively. The experiments lasted 8 and 9 days, respectively.

[2] Results from analysis of 3 chicks fed each ration in experiment 2.

* Significantly more than gain or gain/feed ratio with the ration containing the essential amino acids at the NRC requirement levels, $P < 0.005$.

Results obtained when the balanced and the NRC requirement proportions were compared by feeding rations 3 through 7 are presented in table 4. Chicks fed the 4 rations with the essential amino acids in the balanced proportions gained significantly more and utilized feed more efficiently than chicks fed the ration adjusted to the NRC requirement levels. The higher total level of essential amino acids in ration 4 evidently was not responsible for the higher growth rate and feed efficiency obtained, as compared with the results with ration 3. The 9.0% total essential amino acid level, which was set somewhat arbitrarily in the series of experiments conducted to determine the balanced proportions, was apparently higher than necessary for chicks to grow rapidly when their ration provided the energy and protein levels fed in these experiments. The level of each essential amino acid in ration 1 or ration 4 is not to be considered as a minimum requiment for chicks fed a ration with 18% protein and the energy level of the rations fed in these experiments. All essential amino acid levels in ration 4 were decreased 12.3% to produce ration 6, but these decreases did not decrease the growth rate or the feed efficiency. Even a simultaneous 17.3% reduction (ration 7) in the level of essential amino acids except tryptophan did not produce a statistically significant reduction in either growth rate or feed efficiency.

At the end of the last experiment, the percentage of dry matter, protein, and ether extract was determined in the carcasses of 3 chicks fed each ration. The averages are shown in table 4. Although small differences in composition were noted, none were statistically significant. Thus, the evidence did not indicate that differences in body composition would explain the differences in weight gain.

The results presented in table 4 are typical of results obtained in other similar experiments conducted near the end of the series of experiments that determined the balanced proportions. The level of each essential amino acid in ration 1 is not a minimum requirement, but the essential amino acid proportions in ration 1 are considered better-balanced than the proportions given as minimum requirements of the chick by the NRC.

LITERATURE CITED

1. National Research Council, Committee on Animal Nutrition 1960 Nutrient requirements of domestic animals, no. 1. Nutrient requirements of poultry. Pub. 827, National Academy of Sciences — National Research Council, Washington, D.C.
2. Anderson, J. O., and D. C. Dobson 1959 Amino acid requirements of the chick. 2. Effect of total essential amino acid level in the diet on the arginine and lysine requirements. Poultry Sci., 38: 1140.
3. Klain, G. J., H. M. Scott and B. C. Johnson 1959 Arginine requirement of chicks fed a crystalline amino acid diet simulating the composition of casein. Poultry Sci., 38: 488.
4. Moore, S., and W. H. Stein 1954 Procedures for the chromatographic determination of amino acids on four per cent cross-linked sulfonated polystyrene resins. J. Biol. Chem., 211: 893.
5. Moore, S., D. H. Spackman and W. H. Stein 1958 Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem., 30: 1185.
6. Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in chromatography of amino acids. Anal. Chem., 30: 1190.
7. Block, R. J., and K. W. Weiss 1956 Amino Acid Handbook. Charles C Thomas, Springfield, Illinois.
8. Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutrition, 72: 243.
9. Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames.
10. Bidmead, D. S., and F. J. Ley 1958 Quantitative amino acid analysis of food proteins on single ion-exchange column. Biochem. Bioph. Acta., 29: 562.

Relative Potency of Diets Containing Amino Acids and Proteins for Promoting Growth of Rats[1,2]

W. D. SALMON

Auburn University Agricultural Experiment Station, Department of Animal Science, Auburn, Alabama

ABSTRACT The necessity for inclusion of certain nonessential amino acids and of the high levels of essential amino acids sometimes used in diets for rats was investigated. The rate of weight gain of rats produced by the best amino acid diets was compared with that produced by diets containing intact protein or combinations of intact protein and amino acids. Data are presented on weight gains of young rats subjected to 23 different dietary treatments for 8 weeks. Accumulated weight gains for the first 4-weeks appeared to be somewhat more dependable than for the first 2-week period; there was no advantage in extending the test over an 8-week period. A mean weight gain of 44+ g/rat/week for a 4-week period was supported by a protein-free diet containing 14 amino acids and 2.16% total nitrogen; of this 0.66% was supplied by L-glutamic acid. A diet containing 19 amino acids and 3.03% total nitrogen was less effective. Similar diets containing 20% of casein, lactalbumen or protein equivalent of dried lean beef supported mean weight gains of 50+ g/rat/week over the same period. This was a significantly higher ($P < 0.005$) rate of gain than was produced by the best amino acid diet. When adequately supplemented with amino acids, 25% of peanut meal, 20% of wheat gluten or 10% of casein in the diet likewise produced mean weight gains of 50+ g/rat/week over a 4-week period. Thus, it was shown that there was a growth-stimulating effect of the intact proteins not observed in any of the amino acid combinations tested.

Beginning with the classic investigations of Rose and associates in 1931, numerous experiments have been conducted on the growth rate of rats or other species fed diets containing amino acids as the sole source of dietary nitrogen. In some experiments with rats the growth rate has been good; generally it has not equaled that produced by a similar diet containing an adequate level of intact proteins of good quality. In a comprehensive review of the literature related to this problem through 1957 by Greenstein and Winitz (1), they stated that "at the present time diets of chemically defined components can equal the nutritive capacity of diets composed of whole protein." Data presented from this laboratory by Sauberlich (2) led to a similar conclusion, but this was not supported by the recent report of Breuer et al. (3).

Additional studies were made to explore the necessity for the high levels of essential amino acids used by Sauberlich and others and the need for inclusion of certain nonessential amino acids in the diet. Diets containing amino acids have been compared wth those containing intact proteins for relative efficacy in supporting weight gains in young rats. Data at significant variance with the above conclusions of Greenstein and Winitz and of Sauberlich are presented in the present paper.

MATERIALS AND METHODS

Weanling male rats of the Charles River CD strain were used in all experiments. They were fed commercial rat breeder diet for 2 days after they were received in the laboratory. They were then weighed and placed in individual, screen-bottom cages suspended on roll-paper type metal racks. Temperature of the animal room was maintained between 22° and 24°C. Each treatment group in an experiment consisted of 5 rats. They were fed the respective experimental diets in glass jars immediately after being placed in individual cages and daily thereafter. Tap water was supplied ad libitum. The rats were

Received for publication August 27, 1963.

[1] Supported in part by PHS Research Grant AM 01175 from National Institute of Arthritis and Metabolic Diseases, Public Health Service.

[2] Some of the data in this paper were presented at the Federation of American Societies for Experimental Biology Meetings in Atlantic City, New Jersey, 1962.

TABLE 1

Amounts of basal components in the test diets

	g/100 g
Amino acids or proteins	[1]
Sucrose	10.0
Lard	20.0
Cornstarch [2]	q.s.
Vitamin premix no. 6 [3]	2.0
Salts no. 8 [3]	5.0
Agar	1.0
Choline chloride	0.3
Ascorbic acid [4]	0.1
Penicillin "G" sodium [5]	0.01
	mg/100 g
Folacin	0.2
Biotin	0.05
	µg/100 g
Vitamin D_2	4
Vitamin B_{12} [6]	5

[1] Shown for each treatment in tables 2 and 3.
[2] In amounts to make 100 g.
[3] For amounts of vitamins and minerals supplied see Materials and Methods in text.
[4] Ascorbic acid was omitted from treatment H.
[5] Penicillin was omitted from treatments A and N.
[6] Vitamin B_{12} was omitted from treatments A, B, and N.

weighed at approximately the same time of day each week, at which time the feed jars were removed and replaced with clean ones. The diets were mixed in quantities of 1 kg and stored at 1° to 2°C until fed.

The basal component composition of the test diets is shown in table 1, except for the individual amino acids and proteins which varied as listed for each treatment in tables 2 and 3. Vitamin premix no. 6 with a sucrose base, supplied the following amounts of vitamins: (in mg/kg of diet)[3] menadione, 50; riboflavin, 20; thiamine·HCl, 20; pyridoxine·HCl, 24; Ca pantothenate, 50; niacin, 100; inositol, 200; D-α-tocopheryl acetate, 55; and vitamin A palmitate, 100,000 USP units. Folacin, vitamin B_{12}, biotin and vitamin D_2 were not included in the premix but were added separately in solution form to each diet. The amounts are shown in table 1. Salts no. 8 supplied the following quantities of minerals: (% of diet) $CaHPO_4 \cdot 2H_2O$, 3.15; KCl, 0.6; $MgSO_4$, 0.4; $NaHCO_3$, 0.7; Fe citrate, 0.123; $MnSO_4 \cdot H_2O$, 0.010; $ZnCO_3$, 0.010; $CuSO_4$, 0.004; KI, 0.003. The amino acids were obtained commercially.[4,5] The casein (no. 1 nutritional grade)[6] was extracted for 48 hours with 82% (vol) methanol and for 48 additional hours with 100% methanol. The extraction was made in a continuous-type extractor with steam-jacketed extraction vessels in which the casein was kept hot while the solvent percolated through. The peanut meal was a high-grade solvent-process product made from shelled peanuts. It was extracted by the same method as the casein. The lactalbumen[7] was obtained commercially, and used as received. The wheat gluten was used as received from the manufacturer.[8]

RESULTS

Mean weight gains of rats by treatments during periods of 2, 4 and 8 weeks are shown in tables 2 and 3. With the exception of treatments A, B, H and N, the only differences in treatments were in the amino acid or protein constituents of the diets and in the levels of corn starch necessitated by such changes. In the formulation of these diets, their adaptability for use in studies of choline deficiency was considered. For this reason cystine was arbitrarily included at a fixed level to keep the level of methionine as low as consistent with maximal growth rate attainable with such diets.

The mean weight gains for the various diet treatments were analyzed by Duncan's multiple range test (4) separately for the 2-, 4-, and 8-week periods. For the 2-week period, a significant decrease in weight gain resulted from omitting vitamin B_{12} (treatment B) or vitamin B_{12} and penicillin (treatment A) from the diet containing 8 nonessential amino acids (treatment C) ($P < 0.05$) or from the diet containing 20% of extracted casein (treatment N vs. treatment O) ($P < 0.01$). At both the 4- and 8-week periods, the deletion of vitamin B_{12} and penicillin from the amino acid diet or from the 20% extracted casein diet decreased weight gains significantly ($P < 0.01$). The results from treat-

[3] Appreciation is expressed to Distillation Products Industries Inc., Rochester, New York, for the dry vitamin A (PG B-250) and the vitamin E supplement (type F-50) and to Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, for the other vitamins used.

[4] Obtained from Nutritional Biochemicals Corporation, Cleveland, or Mann Research Laboratories, New York, except for DL-methionine, N.F. grade, which was provided by the Dow Chemical Company, Midland, Michigan.

[5] Appreciation is expressed to Dow Chemical Company, Midland, Michigan, for the methionine and to General Mills, Minneapolis, for the wheat gluten.

[6] Obtained from A. Adler and Son, Philadelphia.

[7] Obtained from Nutritional Biochemicals Corporation, Cleveland.

[8] Obtained from General Mills, Minneapolis.

TABLE 2

Percentages of test components in diets and mean weight gains of rats by treatments

Treatment	A [1]	B [2]	C	D	E	F	G	H [3]	I	J	K	L
L-Arginine·HCl	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
L-Cystine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.8	0.3	0.3	0.3	0.3
L-Glutamic acid	4.0	4.0	4.0	4.0	8.4	8.4	6.0	6.0	6.0	6.0	5.0	7.0
Glycine	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.7	0.4	0.4	0.4
L-Histidine·HCl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.7	0.7	0.7	0.7
DL-Isoleucine	3.0	3.0	3.0	3.0	3.0	2.0	2.0	2.0	1.5	1.5	1.5	1.5
L-Leucine	2.0	2.0	2.0	2.0	2.0	1.5	1.5	1.5	1.2	1.0	1.0	1.0
L-Lysine·HCl	1.9	1.9	1.9	1.9	1.9	1.5	1.5	1.5	1.5	1.4	1.4	1.4
DL-Methionine	0.8	0.8	0.8	0.8	0.8	0.4	0.4	0.4	0.4	0.4	0.4	0.4
DL-Phenylalanine	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.0	1.0	1.0	1.0
L-Tyrosine	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.36	0.36	0.36	0.36
DL-Threonine	2.0	2.0	2.0	2.0	2.0	1.5	1.5	1.5	1.2	1.2	1.2	1.2
DL-Tryptophan	0.5	0.5	0.5	0.5	0.5	0.3	0.2	0.2	0.2	0.2	0.2	0.2
DL-Valine	2.8	2.8	2.8	2.8	2.8	2.0	2.0	2.0	1.9	1.9	1.9	1.9
DL-Alanine	0.6	0.6	0.6	0.6	—	—	—	—	—	—	—	—
L-Aspartic acid	0.6	0.6	0.6	0.6	—	—	—	—	—	—	—	—
L-Asparagine	0.6	0.6	0.6	0.6	—	—	—	—	—	—	—	—
L-Proline	0.5	0.5	0.5	0.5	—	—	—	—	—	—	—	—
DL-Serine	0.5	0.5	0.5	0.5	—	—	—	—	—	—	—	—
Creatine	0.5	0.5	0.5	—	—	—	—	—	—	—	—	—
$NaHCO_3$	1.6	1.6	1.6	1.6	1.6	1.42	1.42	1.42	1.3	1.24	1.24	1.24
Total nitrogen	3.18	3.18	3.18	3.03	3.03	2.59	2.35	2.35	2.15	2.06	1.97	2.16
No. of rats	5	5	10	15	20	10	10	5	10	10	5	10
Mean wt gain g/rat/2 weeks	65 ± 2.92 [4]	58 ± 4.72	77 ± 4.12	68 ± 3.80	67 ± 3.0	70 ± 4.20	82 ± 1.28	67 ± 5.13	79 ± 3.06	72 ± 2.57	63 ± 2.27	81 ± 2.98
Mean wt gain g/rat/4 weeks	132 ± 9.21	124 ± 4.89	160 ± 7.14	156 ± 2.79	156 ± 3.40	153 ± 6.95	164 ± 4.87	130 ± 5.98	165 ± 5.28	159 ± 3.74	139 ± 8.74	178 ± 5.93
Mean wt gain g/rat/8 weeks	217 ± 15.83	198 ± 6.64	270 ± 7.20	269 ± 3.84	277 ± 6.42	275 ± 6.35	279 ± 7.79	248 ± 11.64	293 ± 7.32	283 ± 4.99	246 ± 22.48	307 ± 7.66

[1] Vitamin B_{12} and penicillin were omitted from diet in treatment A.

[2] Vitamin B_{12} was omitted from diet in treatment B.

[3] Ascorbic acid was omitted from diet in treatment H.

[4] SD.

TABLE 3

Percentages of test components in diets and mean weight gains of rats by treatments

Treatment	M	N [1]	O	P	Q	R	S	T	U	V	W
L-Arginine·HCl	0.8	—	—	0.1	0.33	0.8	0.08	0.08	—	—	—
L-Cystine	0.3	—	—	0.2	0.27	0.3	0.06	0.06	—	—	—
L-Glutamic acid	8.0	—	—	—	2.10	6.0	0.50	3.25	2.6	—	—
Glycine	0.4	—	—	0.12	0.31	0.4	—	—	—	—	—
L-Histidine·HCl	0.7	—	—	0.2	0.34	0.7	0.28	0.28	0.7	—	—
DL-Isoleucine	1.5	—	—	—	0.12	1.5	0.14	0.14	1.7	—	—
L-Leucine	1.0	—	—	—	0.08	1.0	—	—	1.0	—	—
L-Lysine·HCl	1.4	—	—	—	0.50	1.4	1.00	1.00	1.4	—	—
DL-Methionine	0.4	—	—	—	0.08	0.4	—	—	0.6	—	—
DL-Phenylalanine	1.0	—	—	0.13	0.42	1.0	0.11	0.11	0.5	—	—
L-Tyrosine	0.36	—	—	—	—	0.36	0.02	0.02	0.15	—	—
DL-Threonine	1.2	—	—	—	0.38	1.2	0.30	0.30	1.2	—	—
DL-Tryptophan	0.2	—	—	0.02	0.08	0.2	0.08	0.08	0.3	—	—
DL-Valine	1.9	—	—	—	0.50	1.9	0.20	0.20	1.4	—	—
Extracted casein	—	20.0	20.0	15.0	10.0	5.0	—	—	—	—	—
Wheat gluten	—	—	—	—	—	—	20.0	20.0	—	—	—
Extracted peanut meal	—	—	—	—	—	—	—	—	25.0	—	—
Lactalbumin	—	—	—	—	—	—	—	—	—	20.0	—
Dried lean beef	—	—	—	—	—	—	—	—	—	—	21.4
$NaHCO_3$	1.24	—	—	0.12	0.49	1.24	0.6	0.6	0.9	—	—
Total nitrogen	2.25	2.96	2.96	2.35	2.18	2.80	2.94	3.20	3.83	2.90	2.95
No. of rats	5	5	10	5	10	5	10	5	5	5	4
Mean wt gain g/rat/2 weeks	77 ± 2.61 [2]	67 ± 1.70	83 ± 6.03	100 ± 6.88	96 ± 4.76	80 ± 9.76	98 ± 3.79	84 ± 6.89	103 ± 5.51	102 ± 8.30	105 ± 3.78
Mean wt gain g/rat/4 weeks	177 ± 4.12	179 ± 5.93	202 ± 8.15	203 ± 7.87	209 ± 7.62	185 ± 14.34	203 ± 5.34	195 ± 10.89	206 ± 7.26	209 ± 15.01	213 ± 9.39
Mean wt gain g/rat/8 weeks	316 ± 7.26	323 ± 14.30	371 ± 14.58	359 ± 14.49	364 ± 13.99	326 ± 15.34	358 ± 10.44	372 ± 19.09	346 ± 8.52	360 ± 27.16	367 ± 21.58

[1] Vitamin B_{12} and penicillin were omitted from diet for treatment N.

[2] SD.

ments A and B show that the deletion of vitamin B_{12} depressed growth as much as deletion of both vitamin B_{12} and penicillin. Thus, there was no vitamin B_{12}-sparing effect by penicillin in this diet.

When ascorbic acid was omitted from the diet, there was a decrease in mean weight gains (treatment G vs. treatment H). The decrease was significant ($P < 0.05$) at the 2- and 4-week periods and approached significance for the entire 8-week period. The inclusion of 0.50% of creatine in the diet containing the full complement of nonessential amino acids, had no effect on weight gains at any period (treatment C vs. D).

When the essential amino acids were maintained at the high level of treatment D, mean weight gains were not depressed by omission of alanine, aspartic acid, asparagine, proline and serine from the diet, provided that glutamic acid was increased to maintain the same nitrogen level (treatment E). In one experiment, the results of which are not included in the tables, mean weight gains were likewise not depressed by the omission of these 5 amino acids, when the glutamic acid level was held at 4.0% and arginine·HCl was increased to 1.52 or 2.40%. The nitrogen levels of the diets representing these changes were 2.80 and 3.04%, respectively. It was likewise found in this experiment that mean weight gains were not affected by reducing the glutamic acid from 8.4 to 6.0% without increasing arginine; the nitrogen content of this diet was 2.80%. Later tests on levels of glutamic acid in diets containing lower levels of total nitrogen, showed no statistically significant differences in weight gains produced by diets containing 6.0, 7.0, or 8.0% of glutamic acid and 0.80% of arginine·HCl; gains were slightly larger with 7.0 or 8.0% than with 6.0% and significantly larger ($P < 0.05$) than with 5.0% of glutamic acid in the diet.

Numerous tests in which the major essential amino acids were individually decreased stepwise were conducted. The data from these tests are not included in the tables, but data from revised formulations based on these tests are included (treatments F, G, I, L and M). Although mean weight gains supported by the revised formulations were generally higher than those supported by treatments D and E, the differences were statistically significant only for treatments L and M. As compared with treatment D, there was a decrease in the level of 10 amino acids in treatment L. This was exclusive of the 5 nonessential amino acids that were omitted from treatment L and for which there was some compensatory increase in glutamic acid. Of the 10 amino acids that were decreased, 5 were decreased 50% or more; in addition, threonine was decreased 40% and valine 32%. Thus the revised formulations represent a rather substantial reduction in dietary levels of essential amino acids as well as of total nitrogen. Several tests were made to ascertain whether the omission of glycine and tyrosine from the diet would affect the rate of gain. When glycine alone or both glycine and tyrosine were omitted, there was a decrease in mean weight gains. Pending further study, these 2 amino acids were routinely included in all amino acid diets reported in this paper; the level of tyrosine was substantially reduced, however, for treatments I, J, K, L and M.

The best amino acid diets (treatments L and M) were compared with various diets that contained intact protein. These were 20% of extracted casein (treatment O), 15% of extracted casein supplemented with amino acids (treatment P), 10% of extracted casein supplemented with amino acids (treatment Q) 5% of extracted casein superimposed upon the amino acid levels in treatment J (treatment R), 20% of wheat gluten supplemented with amino acids (treatments S and T), 25% of extracted peanut meal supplemented with amino acids (treatment U), 20% of lactalbumen (treatment V) and 21.4% of dried lean beef (treatment W). Analysis of variance was made on the data by periods. At the 2-week period, mean weight gains produced by treatments P, Q, S, U, V, W were significantly greater ($P < 0.005$) than those produced by treatments L and M, but were not significantly greater for treatments O, R and T. The accumulated mean weight gains for the 4-week period were significantly greater than those produced by treatments L and M for treatments O, Q, S, U, V, W ($P < 0.005$) P ($P < 0.01$)

and T ($P < 0.10$). The accumulated mean weight gains for the 8-week period exhibit a pattern similar to that for the 4-week period with minor shifts in rank of some treatments. Significant increases over gains produced by treatments L and M were shown by treatments O, P, Q, S, T, V, W ($P < 0.005$) and U ($P < 0.05$). Treatment R produced no significant increase over treatments L and M at any period. The results show that the intact proteins tested had a growth-stimulating effect greater than that of the best protein-free amino acid diets. The diet containing 10% of casein supplemented with amino acids supported a rate of weight gain comparable to that supported by the diet containing 20% of casein or equivalent levels of lactalbumen or dried lean beef. The addition of 5% of casein to an amino acid diet (treatment R), however, did not produce a statistically significant increase over the weight gain produced by the amino acid treatments L and M.

The length of time required to obtain definitive data from experiments of this nature is of major importance. A breakdown of the mean weight gain for the 23 treatments by periods shows that the maximal rate of gain generally occurred in the 3- to 4-week period and the lowest rate of gain during the 5- to 8-week period. During the first 2-week period the mean weight gain in grams per rat per week was 40, during the second 2-week period 47 and during the final 4-week period only 33. When recorded as accumulated mean weight gains, the differences were masked to some extent but were still obvious. In this case the mean weight gain for the 23 treatments in grams per rat per week for the 2-week period was 40, for the 4-week period 43.5 and for the 8-week period 38; the coefficient of variation was higher for the 2-week period (0.15869075) than for the 4-week period (0.10958502) or the 8-week period (0.10329268). Moreover a treatment, as for example treatment O vs. L and M, occasionally failed to show a difference from another treatment at the 2-week period, but showed a significant difference at the 4- and 8-week periods. It appears that a 2-week test period may be useful for preliminary screening tests, but a 4-week test is more dependable. Such a test will include the period of maximal gain. There appears to be no advantage in extending the test to 8 weeks. Increasing the number of animals in a 4-week test would be preferable.

DISCUSSION

The data presented in this paper are in agreement with reports of other investigators that an excellent rate of growth in rats can result from feeding protein-free diets containing crystalline amino acids. They afford conclusive proof, however, that such diets are not as potent as protein-containing diets for promotion of growth in this species. Diets containing adequate levels of good quality protein produced mean weight gains of 6 to 8 g/rat/week more during a 4-week period than the best amino acid diet tested. The difference was significant ($P < 0.005$). Diets containing incomplete proteins or 10% of casein, when they were adequately supplemented with amino acids produced weight gains comparable to those produced by the diets containing adequate levels of good quality protein. Apparently there was a growth-stimulating substance in the proteins tested that was not present in the amino acid diets. Several sources of unidentified growth factors were tested as supplements to the amino acid diets. These included the dietary levels: (on a % solids basis) fish solubles, 5; distiller's solubles, 5; dried whey, 5; beef extract, 5; liver extract 2 or pepsin, 1. None of these produced any appreciable increase in weight gain. Supplementation with 5% of pancreas powder, whole liver powder or thymus powder produced slight increases but no more than 5% of casein, which was shown in treatment R not to produce a significant increase.

Treatment L represents a simplification of the amino acid mixture and a substantial decrease in the levels of essential amino acids as compared with treatment C or D. It is a less expensive diet without any decrease in effectiveness in supporting weight gains. It may not represent minimal levels of essential amino acids required for maximal weight gains. In most cases the levels are somewhat above the standards proposed by Rose et al. (5, 6). The diets used by those workers, however,

contained only 2 to 4% fat, whereas the diets used in the experiments reported here contained 20% fat. The latter diets represented a considerably higher energy level and supported a substantially higher rate of weight gain. It is questionable whether minimal requirements of essential amino acids or of total nitrogen required for maximal growth of rats can be accurately ascertained until it is possible to include in the diet the growth-stimulating substance that is present in intact proteins.

ACKNOWLEDGMENTS

Appreciation is expressed to A. E. Drake and W. H. Hearn of the Research Data Analysis Laboratory of Auburn University for the statistical analysis of the data presented.

LITERATURE CITED

1. Greenstein, J. P., and W. Winitz 1961 Chemistry of the Amino Acids, vol. 1. John Wiley and Sons, Inc., New York, pp. 245–432.
2. Sauberlich, H. E. 1961 Growth of rats fed protein-free diets supplemented with purified amino acids. J. Nutrition, *74:* 298.
3. Breuer, L. H., Jr., W. G. Pond, R. G. Warner and J. K. Loosli 1963 A comparison of several amino acid and casein diets for the growing rat. J. Nutrition, *80:* 243.
4. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
5. Rose, W. C., L. C. Smith, M. Womack and M. Shane 1949 The utilization of the nitrogen of ammonium salts and certain other compounds in the synthesis of non-essential amino acids in vivo. J. Biol. Chem., *181:* 307.
6. Wretlind, K. A., and W. C. Rose 1950 Methionine requirement for growth and utilization of its optical isomers. J. Biol. Chem., *198:* 697.

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

I. ALTERATIONS IN ENZYME AND COENZYME SYSTEMS INDUCED BY SUPPLEMENTING 40% FAT DIETS WITH 0.1% OF NIACIN[1,2]

LORA LONG RIKANS, DOROTHY ARATA AND DENA C. CEDERQUIST
Department of Foods and Nutrition, College of Home Economics, Michigan State University, East Lansing, Michigan

ABSTRACT Forty male weanling albino rats were divided into 4 groups and fed 20% casein diets of high fat (40%) or low fat (5%) content with adequate or excessive quantities of niacin. The niacin content of both the high and low fat diets was increased by supplementing the diets with 0.1% of niacin. Two control groups, one fed high fat and the other low fat, containing adequate (0.5 mg/100 g) quantities of niacin, were fed the diet simultaneously. Blood samples were collected for pyridine nucleotide determinations on the twenty-first and the forty-second day of the experiment. The animals were killed after 44 days and the livers analyzed for pyridine nucleotide concentration, fatty acid oxidase activity, and proximate composition. Results from this study indicate that excess niacin enters metabolic pathways to produce at least 2 unrelated effects, increased concentrations of pyridine nucleotides in blood and liver and increased levels of fat in the liver. The first effect occurs regardless of the level of fat in the diet, but the second occurs only in conjunction with a high level of dietary fat. The possibility that an increased consumption of niacin and fat increases the animals' requirement for choline is discussed.

Only a few studies have been conducted to determine whether excessive dietary levels of the water-soluble vitamins have any effects on the growth and development of experimental animals. In particular, there is little mention in the literature of hypervitaminosis niacin. The lethal dose of this water-soluble vitamin (2 to 7 g niacin/kg body weight) was determined for the rat by Chen et al. (1), Unna (2), and Brazda and Coulson (3), and for the dog by Elvehjem et al. (4). Sublethal doses were tolerated without gross signs of toxicity or inhibition of growth, and the studies were abandoned because they lacked practical applications.

Now, however, the widespread enrichment programs and the availability of high potency vitamin preparations have made possible the indiscriminate ingestion of large quantities of niacin. One recognized treatment of hypercholesterolemia involves large oral doses of niacin (3 to 7.5 g niacin/day) which often produce a flushing reaction (5) and, possibly, liver dysfunction (6). These factors, along with the development of more sensitive measurements of normal metabolic pathways provide ample justification for a study of the oral consumption of large quantities of niacin.

Recent observations suggest that excess niacin may have some harmful effects in animals. The introduction of 2% of niacin in a 10% fat diet not supplemented with choline, significantly increased the level of fat in the livers of rats. The addition of choline or betaine to the same diet reduced the liver fat level to that of the control group (7). In addition, Gaylor et al. (8) reported that livers from rats fed 10% fat diets containing 1% of niacin (and 0.1% of choline) contained slightly more fat than those from controls, although the level of significance was not reported. Pilot experiments conducted in this laboratory corroborated this observation. Livers from rats fed 0.1% of niacin in a 40%

Received for publication June 12, 1963.

[1] Journal Article no. 3167 from the Michigan Agricultural Experiment Station.

[2] This work was supported in part by a grant from the National Institutes of Health, Project no. A-6093.

corn oil diet contained significantly more fat than those from control animals. The present experiment was undertaken to study the effect produced by feeding male rats rations containing high levels of niacin and fat. An attempt was made to determine the metabolic fate of the excess niacin and the nature of the biochemical lesion(s) associated with fat accumulation in the liver.

METHODS

Forty male weanling albino rats of the Sprague-Dawley strain were divided into 4 groups. Group 1 received a basal diet providing the following in g/100 g diet: casein, 20; salts W,[3] 4; choline, 0.15; vitamin mix, 0.25; corn oil,[4] 5; and sucrose, 70.6. The vitamin mix contained the following in mg/100 g diet: vitamin A powder (20,000 IU/g), 2.5;[5] calciferol, 0.1; thiamine, 0.4; riboflavin, 0.8; niacin, 0.5; vitamin B_{12}, 0.002;[6] pyridoxine, 0.25; Ca pantothenate, 2.0; menadione, 0.4; inositol, 1.0; folic acid, 0.2; biotin, 0.01; *p*-aminobenzoic acid, 0.2; and sucrose to make 0.25 g. The diet for group 2 was identical with that for group 1 except that 0.1% of niacin was added at the expense of sucrose. Animals in groups 3 and 4 received diets identical with diets 1 and 2, respectively, except that 35 additional grams of corn oil replaced 35 g of sucrose. Food and water were provided ad libitum and records were kept of food intake and weight gain throughout the experimental period.

On the twenty-first and forty-second days of the experiment blood samples were taken from the tails of all animals for determination of pyridine nucleotides. On the forty-fourth day the animals were stunned by a sharp blow on the head and decapitated. The livers were removed for the chemical analyses described below.

Blood pyridine nucleotides. Blood pyridine nucleotides (DPN,[7] TPN,[8] and NMN [9]) were determined by the Kring and Williams (9) method with 2 modifications. The sample volume was increased to 0.20 ml and hydrogen peroxide was added to the alcohol solution before the blood sample was introduced. Fluorescence was developed by the method of Carpenter and Kodicek (10). Red cell volumes were determined for each blood sample and results were reported as micrograms pyridine nucleotides per milliliter red blood cells.

Liver pyridine nucleotides. Immediately after the removal of the liver a 0.50-g sample was excised from one lobe for determination of pyridine nucleotides by the method of Robinson et al. (11). Fluorescence was developed by the method of Carpenter and Kodicek (10).

Endogenous oxidation and fatty acid oxidase. Endogenous oxidation and activity of the fatty acid oxidase system were measured by a modification of the manometric procedures described by Lehninger (12) using the Warburg apparatus. A 2.50-g portion of chilled liver was homogenized with 5.0 ml of 0.25 M sucrose. A 1.0-ml aliquot of crude liver homogenate was pipetted into chilled Warburg flasks. All flasks were allowed to equilibrate for 5 minutes at 25.0°C in the Warburg bath, and manometer readings were taken at 5-minute intervals. Calculations of enzyme activity were based upon the 10- or 15-minute interval where activity was highest.

Moisture, lipid, and nitrogen analyses. The remaining portions of the livers that had been stored frozen, were allowed to thaw at room temperature and homogenized with water in a Potter-Elvehjem homogenizer. The homogenates were quantitatively transferred to weighed evaporating dishes and dried at 90°C for 12 hours. Fat was determined by subjecting a 1.000-g sample of the dried ground liver to continuous ether extraction for 3 hours on the Goldfisch apparatus and weighing the ether-soluble lipoidal material. Nitrogen was determined by the macro-Kjeldahl method on 0.250-g samples of the fat-free livers and calculated as percentage of the fresh weight of liver.

---

[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339. Obtained as Salt Mixture-W from Nutritional Biochemicals Corporation, Cleveland.

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

[5] Containing 50 IU vitamin A, slightly exceeding the nutritional requirement for the rat. Brown, R. A. and M. Sturtevant 1949 The vitamin requirements of the growing rat. Vitamins and Hormones, 7: 171.

[6] Two milligrams of a 0.1% trituration of vitamin B_{12} with mannitol.

[7] Diphosphopyridine nucleotide.

[8] Triphosphopyridine nucleotide.

[9] Nicontinamide mononucleotide.

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

RESULTS

Growth and food intake data are presented in table 1. There were no differences in weight gain or food intake between groups fed excess niacin and their respective controls (groups 1 vs. 2 and 3 vs. 4). However, a significant reduction ($P < 0.01$) in both weight and food intake was observed in groups fed 40% corn oil diets (3 and 4) when compared with groups fed 5% corn oil diets whether or not excess niacin was present in the diets (groups 3 vs. 1 and groups 4 vs. 2). Efficiency of food utilization, expressed as g weight gain/100 kcal consumed, did not differ among the 4 groups.

The concentration of pyridine nucleotides in livers was markedly different between groups fed adequate and excessive amounts of niacin (table 2). The addition of 0.1% of niacin to a diet containing either 5 or 40% of fat[10] (groups 2 vs. 1 and 4 vs. 3) resulted in a significant increase ($P < 0.01$) in pyridine nucleotide concentrations in both blood and liver tissues. This increase was apparent at 3 weeks and had not changed appreciably by 6 weeks. Livers from animals fed the 40% corn oil diet (group 3) contained higher levels ($P < 0.01$) of pyridine nucleotides than did those from animals fed the 5% corn oil diet (group 1), although these levels were not as high as in the groups fed excess niacin (2 and 4). Concentrations of blood pyridine nucleotides were comparable in groups 1 and 3.

Endogenous oxidation and the activity of the fatty acid oxidase system were affected by increasing the fat content of the diet (table 3). The endogenous oxidation was significantly lower ($P < 0.05$) and fatty acid oxidase activity higher ($P < 0.01$) in livers from animals fed 40% fat diets (group 3) when compared with those fed 5% fat diets (group 1). These differences were not noted when excess niacin was added to these diets (groups 2 and 4).

Data from analyses of the liver for moisture, fat, and nitrogen are summarized in table 4. The percentage of nitrogen based on wet weight of the liver was not changed by alterations in the diet (table 4). However, differences appeared

[10] Fat was supplied as corn oil in all the diets used in this experiment. A totally different set of results could be obtained with a more saturated fat, a possibility presently under investigation in this laboratory.

TABLE 1

Growth and food intake of albino rats fed diets high in fat or high in niacin, or both

Group [1]	Diet	Weight gain	Food intake	Efficiency [2]
		g/week	*kcal/week*	*g/100 kcal*
1	5% Corn oil	35 ± 1 [3]	326 ± 8 [3]	11 ± 2 [3]
2	5% Corn oil + 0.1% niacin	36 ± 1	355 ± 8	10 ± 2
3	40% Corn oil	23 ± 1	278 ± 10	8 ± 2
4	40% Corn oil + 0.1% niacin	24 ± 1	288 ± 10	8 ± 2

[1] Each group consisted of 10 animals.
[2] Weight gain/100 kcal consumed.
[3] SE of mean.

TABLE 2

Pyridine nucleotides in the livers of rats fed diets high in fat or high in niacin, or both

Group [1]	Diet	Blood		Liver
		3 Weeks [2]	6 Weeks [2]	
		µg/ml RBC	*µg/ml RBC*	*µg/g liver*
1	5% Corn oil	230 ± 25 [3]	280 ± 25 [3]	1260 ± 69 [3]
2	5% Corn oil + 0.1% niacin	408 ± 45	434 ± 41	2557 ± 220
3	40% Corn oil	178 ± 16	235 ± 42	1866 ± 60
4	40% Corn oil + 0.1% niacin	412 ± 70	391 ± 30	2379 ± 152

[1] From 6 to 10 animals used for each analysis.
[2] Length of time fed experimental diet.
[3] SE of mean.

TABLE 3

Endogenous oxidation and fatty acid oxidase activity of rats fed diets high in fat or high in niacin, or both

Group [1]	Diet	Endogenous oxidation	Fatty acid oxidase
		μl O_2/hr/g liver	*μl O_2/hr/g liver*
1	5% Corn oil	1775 ± 73 [2]	331 ± 34 [2]
2	5% Corn oil + 0.1% niacin	1790 ± 56	405 ± 37
3	40% Corn oil	1555 ± 45	500 ± 28
4	40% Corn oil + 0.1% niacin	1750 ± 61	465 ± 41

[1] From 8 to 10 animals used for each analysis.
[2] SE of mean.

TABLE 4

Liver composition of rats fed diets high in fat or high in niacin, or both

Group [1]	Diet	Moisture	Nitrogen wet wt [2]	Fat	
				Wet wt [2]	Dry wt [3]
		%	%	%	%
1	5% Corn oil	71.0 ± 0.4 [4]	3.07 ± 0.08 [4]	3.2 ± 0.1 [4]	10.9 ± 0.5 [4]
2	5% Corn oil + 0.1% niacin	70.1 ± 0.3	3.12 ± 0.06	3.4 ± 0.1	11.3 ± 0.3
3	40% Corn oil	71.2 ± 0.2	3.21 ± 0.06	3.8 ± 0.3	13.1 ± 1.1
4	40% Corn oil + 0.1% niacin	69.3 ± 0.3	3.24 ± 0.04	5.8 ± 0.4	19.0 ± 1.1

[1] Each group consisted of 10 animals.
[2] Calculated on the basis of fresh weight of the liver.
[3] Calculated on the basis of dry weight of the liver.
[4] SE of mean.

in percentage fat[11] and percentage moisture in livers from the high fat groups (3 and 4). Livers from animals in group 4 (excess niacin) contained significantly greater amounts of fat ($P < 0.01$) and less moisture ($P < 0.01$) than those from group 3 (adequate niacin). When livers from animals in the low fat series were compared (groups 1 and 2) no differences were observed in fat or moisture as a result of including excess niacin in the diet.

DISCUSSION

Growth data from this experiment corroborate earlier observations (2, 7) that excessive niacin has no growth inhibiting effect on the rat. The growth depression noted in this study resulting from increasing the fat content of the diet from 5 to 40% probably reflected the lowered food intakes observed in the groups fed high corn oil diets. This is supported by the lack of any significant differences in efficiency of utilization of food between any of the 4 groups.

The effect of excess niacin was shown most dramatically in pyridine nucleotide concentrations which were increased markedly (almost twofold) in rats fed excess niacin. Thus, this study provides strong supportive evidence that at least a portion of the excess niacin present in the diet was absorbed and participated in metabolic reactions, refuting the theory of "inert excretion." These observations are in agreement with those of others (8, 13–15) who reported elevated pyridine nucleotide concentrations when excessive amounts of niacin were administered to rats.

A second effect of consuming excess quantities of niacin was observed in some oxidative processes. Livers from animals fed a 40% fat diet demonstrated an increased activity of the fatty acid oxidase system and a decreased rate of endogenous oxidation when compared with their controls fed 5% fat diets. These changes, coupled with an increase in liver pyridine nucleotides, probably reflect an adaptive response on the part of the rat to the increased fat content of the diet. The animals appeared to have adjusted to the higher level of dietary fat by increasing the rate of fat oxidation in the liver, thus increasing the economy of calorie utilization in the liver. These changes did not

[11] All liver fat data cited in text are based on dry weight of liver.

occur when the diet contained excess niacin, suggesting that the excess niacin interfered in some manner with the adaptive response of the animals to high levels of dietary fat.

The extent to which the metabolism of methyl groups may be involved in the development of fatty livers under these conditions cannot be ignored (7). Prior to its excretion, nicotinamide is methylated by methione (16) which can, in turn, be derived from choline (17). Thus choline is involved in the detoxication of niacin. Moreover, Griffith (18) observed an increased severity of a choline deficiency in young rats by increasing the fat content of the diet.

In the study reported here, increasing the concentration of both niacin and fat in the diet (group 4) would serve to increase the animals' requirement for choline. Thus the fatty livers observed in this group could reflect a mild choline deficiency.

However, the fatty livers observed under conditions reported here did not demonstrate the classical biochemcial symptoms of choline deficiency. The repression in fatty acid oxidation which reportedly occurs in choline-deficient liver slices (19, 20) was not observed in this experiment. That is, oxidation of octanoate was comparable in livers from groups fed high fat diets with normal or excessive amounts of niacin. It is possible that the excess niacin in the biological system may have complicated the classical symptoms of choline deficiency.

The extent to which choline is involved in the effects induced by excess niacin is currently under investigation.

LITERATURE CITED

1. Chen, K. K., C. L. Rose and E. B. Robbins 1938 Toxicity of nicotinic acid. Proc. Soc. Exp. Biol. Med., *38:* 241.
2. Unna, K. 1939 Studies on the toxicity and pharmacology of nicotinic acid. J. Pharmacol. Exp. Therap., *65:* 95.
3. Brazda, F. G., and R. A. Coulson 1946 Toxicity of nicotinic acid and some of its derivatives. Proc. Soc. Exp. Biol. Med., *62:* 19.
4. Elvehjem, C. A., R. J. Madden, F. M. Strong and D. W. Wooley 1938 The isolation and identification of the anti-black tongue factor. J. Biol. Chem., *12:* 137.
5. Parsons, W. B., and J. H. Flinn 1959 Reduction of serum cholesterol levels and beta-lipoprotein cholesterol levels by nicotinic acid. Arch. Int. Med., *103:* 123.
6. Rausen, A. R., and D. Adlersburg 1961 Idiopathic (hereditary) hyperlipemia and hypercholesteremia in children. Pediatrics, *28:* 276.
7. Handler, P., and W. J. Dann 1942 The inhibition of rat growth by nicotinamide. J. Biol. Chem., *146:* 357.
8. Gaylor, J. L., R. W. F. Hardy and C. A. Baumann 1960 Effect of nicotinic acid and related compounds on sterol metabolism in the chick and rat. J. Nutrition, *70:* 293.
9. Kring, J. P., and J. N. Williams, Jr. 1954 Differential determination of pyridine nucleotides and N[1]-methylnicotinamide in blood. J. Biol. Chem., *207:* 851.
10. Carpenter, K. J., and E. Kodicek 1950 The fluorimetric estimation of N[1]-methylnicotinamide and its differentiation from coenzyme I. Biochem. J., *46:* 421.
11. Robinson, J., N. Levitas, F. Rosen and W. A. Perlzweig 1947 The fluorescent condensation product of N[1]-methylnicotinamide and acetone. IV. A rapid method for determination of pyridine nucleotides in animal tissues. The coenzyme content of rat tissues. J. Biol. Chem., *170:* 653.
12. Lehninger, A. L. 1955 Fatty acid oxidation in mitochondria: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, Inc., p. 545.
13. Williams, J. J., Jr., P. Feigelson and C. A. Elvehjem 1950 Relation of tryptophan and niacin to pyridine nucleotides of tissue. J. Biol. Chem., *187:* 597.
14. Williams, J. N., Jr., P. Feigelson, S. S. Shaninian and C. A. Elvehjem 1951 Further studies on tryptophan-niacin-pyridine nucleotide relationships. J. Biol. Chem., *189:* 659.
15. Burch, H. B., C. A. Storvick, R. L. Bicknell, H. C. Kung, L. G. Alejo, W. A. Everhart, O. H. Lowry, C. G. King and O. A. Bessey 1955 Metabolic studies of precursors of pyridine nucleotides. J. Biol. Chem., *212:* 897.
16. Cantoni, G. L. 1951 Methylation of nicotinamide with a soluble enzyme system from rat liver. J. Biol. Chem., *189:* 203.
17. Muntz, J. A. 1950 The inability of choline to transfer a methyl group directly to homocysteine for methionine formation. J. Biol. Chem., *182:* 489.
18. Griffith, W. H. 1940 Choline metabolism. III. The effect of cystine fat and cholesterol on hemorrhagic degeneration in young rats. J. Biol. Chem., *132:* 639.
19. Artom, C. 1953 Role of choline in the oxidation of fatty acids by the liver. J. Biol. Chem., *205:* 100.
20. Rees, D. E., and K. L. Kline 1957 Metabolism of octanoate by fatty liver slices. Am. J. Physiol., *190:* 446.

The Amino Acid Composition and Nutritive Value of Proteins

V. AMINO ACID REQUIREMENTS AS A PATTERN FOR PROTEIN EVALUATION[1]

P. B. RAMA RAO,[2] H. W. NORTON AND B. CONNOR JOHNSON
Division of Animal Nutrition, University of Illinois, Urbana, Illinois

ABSTRACT The pattern of essential amino acid requirements of the growing rat has been used as a reference "protein" for estimating the nutritive value of various proteins. A "requirement index" is proposed as a chemical estimate of the nutritive value of proteins. Its correlation with the biological value of 11 proteins is highly significant ($r = 0.9654$).

Several attempts have been made to evaluate the nutritive value of proteins on the basis of their amino acid compositions. Mitchell and Block (1) used the amino acid composition of whole egg protein as the standard for computing a "chemical score" of protein, based on the most limiting amino acid. Later, Mitchell (2) computed a modified essential amino acid index, modifying that of Kühnau (3), who proposed human milk as the standard, and that of Oser (4), but retaining whole egg protein as the standard. The essential amino acid requirements should serve better as a standard for calculating such amino acid indexes. For this purpose, we redetermined the minimal requirements for the essential amino acids and the optimal protein level for maximal growth and body nitrogen retention of the growing rat (5–7). In the present paper a "requirement index" is proposed which is the geometrical mean of the several amino acids, each expressed as a percentage of its standard value, except that values exceeding 100% are reduced to 100%. The calculated requirement indexes for a number of proteins are compared with published biological values.

The reference pattern of the minimal essential amino acid requirements of the growing rat is shown in table 1. The optimal protein level ($N \times 6.25$) for maximal growth and nitrogen retention was found to be 10% of the diet, with a mixture of nonessential amino acids as in casein providing the nonessential amino acid nitrogen (6).

TABLE 1

Pattern of amino acids required by the growing rat

	% of diet	
L-Histidine	0.25	
L-Lysine	0.90	
L-Tryptophan	0.11	
L-Isoleucine	0.55	
L-Valine	0.55	
L-Leucine	0.70	
L-Threonine	0.50	
L-Methionine	0.16	0.50
L-Cystine	0.34	
L-Phenylalanine	0.42	0.72
L-Tyrosine	0.30	

Plus nonessential amino acid mixture [1] to make a total conventional protein content of 10% (1.6 g N).

[1] 100 g of the nonessential amino acid mixture contains: 12.3 g DL-serine; 41.6 g L-glutamic acid; 12.7 g L-aspartic acid; 5.9 g DL-alanine; 23.7 g L-proline, and 3.8 g glycine.

Rat growth with the minimal amino acid diet. The results of a study of growth with this amino acid mixture and with a 10% protein, whole egg protein diet, are presented in table 2. Growth with the minimal amino acid diet and the whole egg protein diet was almost equal at about 5 g/day, and the efficiency of protein utilization (protein efficiency ratio = 3.9) was the same in the 2 groups.

Received for publication August 19, 1963.

[1] This work was supported in part by a contract with the Surgeon General's Office, Department of the Army, contract no. DA-49-007-MD-544. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.

[2] Present address: Department of Biochemistry, Indian Institute of Science, Bangalore 12, India.

TABLE 2

Rat growth with the minimal amino acid diet

Diet	Avg weight gain [1]	Avg food intake	PER [2]
	g	g	
Whole egg protein [3]	62.6 ± 2.4 [4]	162	3.9 ± 0.19 [4]
Amino acid diet	59.5 ± 2.4	152	3.9 ± 0.26

[1] Average of 6 male rats in each group; average initial weight 56 g; fed ad libitum for 12 days.
[2] Protein efficiency ratio: gram weight gain per gram conventional protein (N × 6.25) intake.
[3] Twelve grams of laboratory-prepared whole egg protein replaced the essential amino acids and the nonessential amino acid mixture in the basal diet to provide a protein level of 10% (N × 6.25); see table 1.
[4] SE of mean.

Correlation between biological values and calculated indexes. To calculate the correlation coefficients between the chemical and biological methods of evaluation of the nutritive value of proteins, the amino acid composition of 15 proteins was taken from the summary tables of Block and Bolling (8). The biological values of these proteins (table 3) were taken from literature (2), except for casein for which our own data were used.[3] The calculated percentage of limiting amino acids in these proteins as compared with the amino acid requirement pattern (table 1) are summarized in table 4 in order from the most limiting to the marginally limiting amino acid. The number of limiting amino acids varies from protein to protein. For example, in the case of whole egg protein, lysine, threonine, and histidine are limiting, whereas in soybean meal methionine + cystine, lysine, threonine, and valine are limiting in that order.

The calculated nutritive values of 15 different proteins and their biological values are shown in table 3. The "chemical score" is the percentage of the most limiting amino acid as compared with the standard. The requirement index is calculated essentially according to Mitchell's (2) method for the calculation of the modified essential amino acid index. For both values the requirement pattern was used as the standard reference "protein."

Figure 1 illustrates the correlation between the chemical scores, calculated ac-

TABLE 3

Biological values of proteins, and scores based on the requirement pattern

	Protein	Biological value	Chemical score [1]	Requirement index [2]
1	Whole egg protein	97	78	95
2	Egg albumin	93	72	94
3	Gelatin	22	0	0
4	Casein	79	78	96
5	Lactalbumin	84	92	99
6	Whole milk	92	84	97
7	Beef liver	77	78	95
8	Whole wheat	67	30	76
9	Wheat flour	52	21	68
10	Whole corn	62	26	77
11	Oats	65	40	82
12	Rice	70	36	81
13	Peanut meal	57	32	67
14	Cottonseed meal	64	39	77
15	Soybean meal	75	72	91

[1] Percentage of the most limiting amino acid in protein as compared with the requirement pattern (table 1).
[2] Calculated according to Mitchell (4) but using the amino acid requirements as standard instead of egg protein.

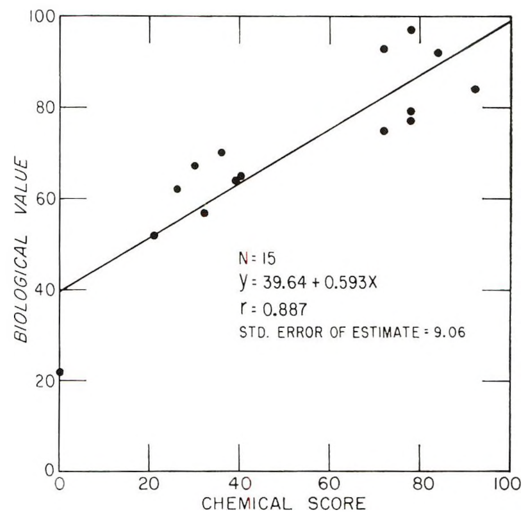


Fig. 1 Relation of biological value to chemical score.

[3] V. C. Metta and B. C. Johnson. Army Report, unpublished.

TABLE 4

Essential amino acids limiting in several proteins as compared with the requirement pattern

	Protein	Limiting essential amino acid (% of requirement levels)
1	Whole egg protein	Lysine (78), threonine (86), histidine (96)
2	Egg albumin	Lysine (72), threonine (84), histidine (96)
3	Gelatin	Tryptophan (0), methionine + cystine (18), isoleucine (31), histidine (36), threonine (38), phenylalanine + tyrosine (39), leucine (50), valine (51), lysine (55)
4	Casein	Methionine + cystine (78), threonine (90), lysine (94)
5	Lactalbumin	Histidine (92)
6	Whole milk	Methionine + cystine (84), threonine (94), lysine (97)
7	Beef liver	Lysine (78), isoleucine (87), methionine + cystine (92)
8	Whole wheat	Lysine (30), threonine (66), isoleucine (73), valine (78), histidine (84), methionine + cystine (86)
9	Wheat flour	Lysine (21), threonine (54), tryptophan (72), valine (75), isoleucine (77), methionine + cystine (78), histidine (88)
10	Whole corn	Lysine (26), tryptophan (55), threonine (74), methionine + cystine (92), valine (97)
11	Oats	Lysine (40), threonine (72), methionine + cystine (76), isoleucine (90), histidine (92), valine (98)
12	Rice	Lysine (36), histidine (68), threonine (76), methionine + cystine (86), isoleucine (95)
13	Peanut meal	Threonine (32), lysine (33), methionine + cystine (52), valine (80), isoleucine (84), histidine (84), tryptophan (91), leucine (96)
14	Cottonseed meal	Lysine (39), threonine (60), isoleucine (73), methionine + cystine (78), leucine (85), valine (88)
15	Soybean meal	Methionine + cystine (72), lysine (76), threonine (78), valine (97)

cording to Mitchell and Block (1) using the requirement pattern as the standard, and the biological values of 15 proteins. The correlation coefficient (0.89) is highly significant and compares well with that (0.846) reported by Mitchell (2) using egg protein as the standard.

Figure 2 illustrates similarly the correlation of the biological value with the requirement index. Obviously 11 samples fit one line and 3 (whole egg, egg albumin, and whole milk) do not. Their departures from the regression line fitting the 11 points are statistically significant, the least exceeding 8 standard deviations. The gelatin point has been omitted from the regression calculation because its requirement index is so extremely uncertain. This uncertainty is traceable mainly to the tryptophan content, reported to be 0%, of which the relative error is extremely large. If the tryptophan content were 1%, the requirement index would be 25, moving from far

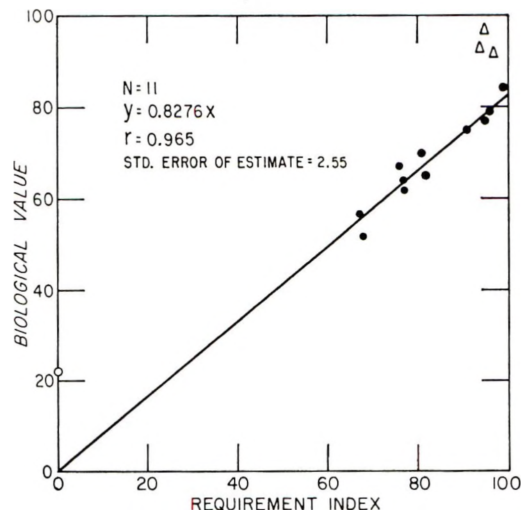


Fig. 2 Relation of biological value to requirement index (whole egg protein, egg albumin, and whole milk protein, designated by △, and gelatin, designated by ○, not included in fitting regression line).

above to somewhat below the line fitted to 11 other proteins. An examination of chart 4 of Mitchell (2), with egg protein used as the standard, shows that most of the points are also on a line of similar position to that plotted here where the pattern of amino acid requirements served as standard.

If no other factor were involved, biological value (which is by definition that percentage of the protein absorbed which is used by the animal) and the degree of correspondence of the amino acid pattern of a protein to the required amino acid pattern should follow a line from (0, 0) to (100, 100). Egg and whole milk proteins appear to fit such a line. All other proteins (except perhaps gelatin) fit very well the straight line $Y = 0.8276X$ with standard error of estimate only 2.5 units of biological value. This slope is significantly ($P < 0.01$) less than unity, the biological values falling about 17% below the 0 to 100 line.

There are 4 obvious possible explanations for this observation, and for egg and milk falling on the theoretical line: 1) If a protein has a deficiency of one amino acid it must have an excess of another. Perhaps this excess depresses the determined biological value. This can apply at most to histidine, lysine, threonine, and methionine plus cystine, because of the excesses of other amino acids in egg and milk, and it is not easy to see that a recalculation of the requirement index allowing somehow for excesses could account for much of the discrepancy. 2) An error in the determination of the amino acid requirements giving too low a value for some one or more amino acid requirements would give erroneously high calculated indexes. However, a regression line calculated using whole egg as the standard (which is significantly higher in several amino acids, but lower in lysine) is not very different in slope. These 2 nitrogen sources give very similar growth rates and nitrogen retentions. The N requirement determinations using the 2 N sources gave 10% protein requirement for the amino acid mix (6) and 10 to 12% for whole egg (9), the egg being limiting in lysine. Assuming a higher value for the requirement for any one amino acid can decrease the requirement index by but a few units, nowhere near enough to bring the points into reasonable agreement with the 0 to 100 line. 3) The relative digestibilities of the different proteins are not considered in the total amino acid analysis. However, it is difficult to believe that lowered digestibilities would release for absorption amino acid mixtures from such divergent proteins as to put them so satisfactorily on one line 17% below theoretical. Sheffner et al. (10) have suggested an assay to include digestibility, and their data do not indicate that the pattern of amino acids released by digestion is of this lower biological value. 4) Another possible explanation involves the so-called nonessential amino acids. If, in fact, one or more of these are essential, as are glutamic acid, arginine, and glycine for the chick (11–13), such requirements have not been used in calculating the requirement index and possibly could account for the excesses over observed biological values.

That the requirement index is a good beginning towards calculating biological value from amino acid composition is indicated by the correlation of 0.9654 for 11 samples. The possibility of multiple regression of biological value on amino acid percentages was considered, but the multiple correlation was only 0.9823 with 9 independent variables, not reaching the 1% significance level.

LITERATURE CITED

1. Mitchell, H. H., and R. J. Block 1946 Some relationships between the amino acid content of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.
2. Mitchell, H. H. 1954 The dependence of biological value of food proteins upon their content of essential amino acids. Wissensch. Abhandl. deutsch. Akad. Landw. Berlin, *2:* 279.
3. Kühnau, J. 1949 Biochemie des Nahrungseiweisses. Angew. Chemie, *61:* 357.
4. Oser, B. L. 1951 Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Dietet. A., *27:* 396.
5. Rama Rao, P. B., V. C. Metta and B. C. Johnson 1959 The amino acid composition and the nutritive value of proteins. I. Essential amino acid requirements of the growing rat. J. Nutrition, *69:* 387.
6. Rama Rao, P. B., V. C. Metta and B. C. Johnson 1960 The amino acid composition and nutritive value of proteins. III. The total protein and the nonessential nitrogen requirement. J. Nutrition, *71:* 361.

7. Rama Rao, P. B., H. W. Norton and B. C. Johnson 1961 The amino acid composition and nutritive value of proteins. IV. Phenylalanine, tyrosine, methionine, and cystine requirements of the growing rat. J. Nutrition, *73:* 38.
8. Block, R. J., and D. Bolling 1951 The amino acid composition of foods and proteins, ed. 2. Charles C Thomas, Springfield, Illinois.
9. Mitchell, H. H., and J. R. Beadles 1952 The determination of the protein requirement of the rat for maximum growth under conditions of restricted consumption of food. J. Nutrition, *47:* 133.
10. Sheffner, A. L., R. Adachi and H. Spector 1956 Measurement of the net utilization of heat-processed proteins by means of the pepsin digest-residue (PDR) amino acid index. J. Nutrition, *60:* 507.
11. Klain, G. J., H. M. Scott and B. C. Johnson 1960 The amino acid requirement of the growing chick fed a crystalline amino acid diet. Poultry Sci., *39:* 39.
12. Greene, D. E., H. M. Scott and B. C. Johnson 1960 A need for glycine in crystalline amino acid diets. Poultry Sci., *39:* 512.
13. Almquist, H. J., and C. R. Grau 1944 The amino acid requirements of the chick. J. Nutrition, *28:* 325.

Effect of Barbituric Acid, Chlortetracycline and Carbohydrates upon Growth and Gastro-intestinal Urease Activity of Chicks[1]

A. P. ALVARES, L. H. HARBERS[2] AND W. J. VISEK
Department of Pharmacology, The University of Chicago, Chicago, Illinois

ABSTRACT Results are presented from 100 lots of 15 chicks/lot fed basal diets containing sucrose, dextrose or starch and supplemented with barbituric acid (1.28g/kg) or chlortetracycline (0.1 g/kg) alone or in combination. These supplements when added alone produced the greatest increase in growth with the sucrose diet and the smallest with the starch diet. The 2 supplements in combination did not show an additive effect. The efficiency of feed utilization followed that of growth. Gastrointestinal ammonia production and ureolytic activity were suppressed only when there was an observed increase in growth with the additives. Ureolytic activity in the small intestine was inversely related to the growth response of the various basal carbohydrate diets; however, lowest activity did not reflect greatest gain for all treatments. The response to barbituric acid, a cyclic urea compound, appears to be dependent on the carbohydrate sources, an observation similar to that noted with antibiotics.

Earlier studies have demonstrated that urea breakdown in the gastrointestinal tract may be altered by dietary anti-microbial agents (1). One of the products, ammonia, has been suggested to be a toxin whose production is suppressed by these agents (2–6). Animals immunized to crystalline jackbean urease have also demonstrated increased growth and efficiency of feed utilization concurrently with depressed urease activity in their gastrointestinal tracts (7, 8). Urease produced by soil bacteria may be inhibited by various cyclic urea compounds (9, 10). Barbituric acid, a suggested anti-metabolite of uracil (11), which is similar in chemical structure to the above compounds, has also been shown to depress urease activity in vitro.[3] More recent work with chicks fed a sucrose-casein diet indicates that barbituric acid will enhance growth concurrently with a reduction in ammonia concentration and urease activity in the gastrointestinal tract (12).

It has been shown that different carbohydrates may alter the requirements for other nutrients (13) and that these differences in requirements are accompanied by changes in the number and type of flora inhabiting the gastrointestinal tract (14). Changes in gastrointestinal flora have also been reported following addition of antibiotics to the diet although these changes have been difficult to reproduce consistently (15). Others have reported differences in utilization of various nutrients in animals fed antibiotics (16). Stokstad et al. (17) have shown that the growth response to antibiotics is dependent upon the type of carbohydrate in diets of chicks.

In the studies reported herein 100 lots of 15 chicks/lot were used to determine their response to barbituric acid and chlortetracycline when either or both were added to purified diets containing various carbohydrates, and to study the effects of these additives on gastrointestinal ammonia concentration and urease activity. An attempt was also made to determine the relationship between growth response and ureolytic activity in the small and large intestine.

EXPERIMENTAL

One-day-old Vantress-Arboracre cockerels having an initial weight of 35 to 45 g were used in all experiments. A 28-day growth period was used as described previously (12). In any set of experiments 3 carbohydrates, sucrose, dextrose or starch,

Received for publication July 29, 1963.

[1] Supported in part by Atomic Energy Commission Contract AT (11-1) 670, and the Herman Frasch Foundation, New York.

[2] Public Health Service Postdoctoral Fellow.

[3] Visek, W. J., A. I. Jacobson, M. E. Iwert and A. P. Alvares 1961 Depression of urease activity in the gastrointestinal tract by chemical agents. Federation Proc., 20: 370 (abstract).

were studied simultaneously. In all studies, experimental diets had the same composition as the basal diet (diet 1, table 1), except for additions in grams per kilogram as follows: diet 2, barbituric acid 1.28; diet 3, chlortetracycline 0.1; and diet 4, barbituric acid 1.28 plus chlortetracycline 0.1. There were several preliminary experiments (table 1) with a total of 52 lots of chicks fed diets containing sucrose, dextrose or starch with and without barbituric acid (1.28 g/kg of diet). These were followed by 2 successive experiments of 24 lots each (table 2).

In the experiments using 24 lots, the chicks were fasted for 4 hours prior to killing at the end of the 28-day experimental period. Ten randomly selected chicks from

TABLE 1

Summary of preliminary investigations with cockerels fed various carbohydrates and supplemented with barbituric acid for 4 weeks

Diet [1]	Weight gain		
	0–2 Weeks	0–4 Weeks	0–4 Weeks
	g	*g*	*g/g feed*
1 Sucrose (12)[2]	75 ± 2 [3]	239 ± 7	0.51 ± 0.02
2 Sucrose + barbituric acid (12)	82 ± 2	250 ± 7	0.56 ± 0.01
1 Dextrose (8)	86 ± 5	236 ± 9	0.47 ± 0.03
2 Dextrose + barbituric acid (8)	88 ± 5	240 ± 7	0.48 ± 0.03
1 Starch (7)	85 ± 5	257 ± 13	0.54 ± 0.01
2 Starch + barbituric acid (5)	89 ± 4	257 ± 18	0.56 ± 0.04

[1] Diet 1, basal: casein (Nutritional Biochemicals Corp., Cleveland), 20; carbohydrate, 61.6; gelatin (Wilson and Co., Chicago), 8; bone ash (Wilson and Co., Chicago), 2; calcium gluconate (Gazzolo Drug and Chemical Co., Chicago), 5; salt mixture, 1.4; choline mixture in casein, 1; and vitamin mixture (17) in casein, 1. Diet 2: basal plus barbituric acid, 1.28 g/kg.
The salt mixture contained: (mg/100 g diet) NaCl, 420; K_2HPO_4, 420; KH_2PO_4, 315; $MgSO_4$, 175; $MnSO_4 \cdot H_2O$, 28; ferric citrate, 35; $CuSO_4 \cdot 5H_2O$, 1.4; KI, 0.42; Zn acetate·$2H_2O$, 1.1725; $Al_2(SO_4)_3 \cdot 18H_2O$, 1.12; $CoCl_2 \cdot 6H_2O$, 0.2675; $NiSO_4 \cdot 6H_2O$, 0.31; KBr, 0.56; sodium molybdate, 0.035.
[2] Figures in parentheses indicate number of lots, with 15 birds/lot.
[3] Mean ± SE.

TABLE 2

Weight gain of cockerels fed basal diets containing various carbohydrates and supplemented with either barbituric acid, chlortetracycline, or both[1,2]

Diet [3]	Weight gain		
	0–2 Weeks	2–4 Weeks	0–4 Weeks
	g	*g*	*g*
Sucrose			
1 Basal	95 ± 7 [4]	170 ± 19	265 ± 14
2 Barbituric acid	116 ± 14 [5]	181 ± 9	297 ± 9
3 Chlortetracycline	120 ± 5 [6]	199 ± 3 [7]	319 ± 8 [6]
4 Barbituric acid + chlortetracycline	123 ± 5 [5,6]	193 ± 6 [7]	316 ± 9 [6]
Dextrose			
1 Basal	123 ± 11	190 ± 7	313 ± 18
2 Barbituric acid	123 ± 9	199 ± 4	322 ± 11
3 Chlortetracycline	131 ± 13 [7]	211 ± 20	342 ± 33
4 Barbituric acid + chlortetracycline	133 ± 7 [7]	201 ± 12	334 ± 19
Starch			
1 Basal	117 ± 2	213 ± 6	330 ± 7
2 Barbituric acid	118 ± 4	208 ± 5	326 ± 5
3 Chlortetracycline	121 ± 3 [7]	214 ± 10	335 ± 8
4 Barbituric acid + chlortetracycline	130 ± 3 [7]	203 ± 8	333 ± 8

[1] Data are averages of 2 successive experiments with 24 lots.
[2] Four lots/treatment, with 15 birds/lot.
[3] Diet 2, basal plus barbituric acid (Eastman Organic Chemicals, Rochester, N.Y.), 1.28 g/kg. Diet 3, basal plus chlortetracycline (Lederle Laboratories, Pearl River, N.Y.), 0.1 g/kg. Diet 4, basal plus barbituric acid, 1.28 g/kg, plus chlortetracycline, 0.1 g/kg.
[4] Mean ± SE.
[5] Barbituric acid vs. no barbituric acid significant at $P < 0.05$.
[6] Chlortetracycline vs. no chlortetracycline significant at $P < 0.01$.
[7] Chlortetracycline vs. no chlortetracycline significant at $P < 0.05$.

each lot were killed. The small and large intestines plus contents were rapidly removed, immediately frozen in dry ice and stored at −10°C. Prior to analyses, the intestines from each lot were thawed at room temperature. The small and large intestines were separated and pooled into respective groups and weighed. The specimens were homogenized with 0.9% NaCl at 4°C in a Waring Blendor for 3 minutes. The total homogenates of the small and large intestines were diluted to 400 and 300 ml, respectively. Ammonia production and urease activity were estimated as described previously (12). Modifications employed were the use of 3-ml homogenate aliquots and determination of liberated ammonia with a Van Slyke-Cullen urea apparatus using 5 ml saturated K_2CO_3 solution with an aeration time of 30 minutes. All of the incubations for ammonia production and ureolytic activity were completed within 0.5 hour after the intestines were thawed. The data from each carbohydrate experiment were subjected to 2-way analysis of variance using orthogonal comparisons as described by Snedecor (18).

RESULTS

Data from the preliminary experiments involving 52 lots suggested that barbituric acid enhanced weight gain and feed efficiency with a sucrose diet, whereas little or no effect was observed with either dextrose or starch (table 1).

Combined growth data from the subsequent experiments are shown in table 2. During the first 2 weeks the addition of barbituric acid to the basal diet containing sucrose increased weight gains by 11% ($P < 0.05$), whereas no significant increases were noted with this agent when added to diets containing dextrose or starch. During this same period chlortetracycline added to the sucrose diet increased growth by 15% ($P < 0.01$), whereas when added to the dextrose or starch diets it increased growth by 7% ($P < 0.05$). Weight gains during the 2- to 4-week period followed those of the first 2-week period but the effects of the additives were less apparent. For the overall experimental period of 4 weeks the difference in growth was greatest with the diet containing sucrose and least with that containing starch. The main effect of barbituric acid with the sucrose diet was an increase in growth of 4%, whereas that of chlortetracycline was 13% ($P < 0.01$). When barbituric acid was the only additive (diet 2), growth was improved by an average of 12% over the basal diet (diet 1) but this difference was

TABLE 3

Efficiency of feed utilization of cockerels fed diets containing various carbohydrates and supplemented with either barbituric acid or chlortetracycline, or both

Diet	Weight gain		
	0–2 Weeks	2–4 Weeks	0–4 Weeks
	g/g feed	*g/g feed*	*g/g feed*
Sucrose			
1 Basal	0.51 ± 0.02 [1]	0.51 ± 0.05	0.52 ± 0.04
2 Barbituric acid	0.63 ± 0.03 [2]	0.52 ± 0.03	0.55 ± 0.01 [4]
3 Chlortetracycline	0.61 ± 0.02 [3]	0.53 ± 0.02	0.55 ± 0.02 [5]
4 Barbituric acid + chlortetracycline	0.67 ± 0.01 [2,3]	0.55 ± 0.01	0.59 ± 0.02 [4,5]
Dextrose			
1 Basal	0.67 ± 0.01	0.54 ± 0.01	0.58 ± 0.01
2 Barbituric acid	0.66 ± 0.02	0.55 ± 0.01	0.59 ± 0.01
3 Chlortetracycline	0.65 ± 0.03	0.56 ± 0.02	0.60 ± 0.02
4 Barbituric acid + chlortetracycline	0.67 ± 0.01	0.54 ± 0.01	0.59 ± 0.01
Starch			
1 Basal	0.63 ± 0.01	0.57 ± 0.01	0.58 ± 0.01
2 Barbituric acid	0.63 ± 0.01	0.56 ± 0.01	0.58 ± 0.01
3 Chlortetracycline	0.65 ± 0.01 [3]	0.57 ± 0.01	0.60 ± 0.01
4 Barbituric acid + Chlortetracycline	0.65 ± 0.01 [3]	0.55 ± 0.01	0.59 ± 0.01

[1] Mean ± SE.
[2] Barbituric acid vs. no barbituric acid significant at $P < 0.01$.
[3] Chlortetracycline vs. no chlortetracycline significant at $P < 0.01$.
[4] Barbituric acid vs. no barbituric acid significant at $P < 0.06$.
[5] Chlortetracycline vs. no chlortetracycline significant at $P < 0.06$.

not statistically significant. Chlortetracycline alone (diet 3) showed an increase of 20% ($P < 0.02$). The combined effects of the agents on growth were not additive, there being only 19% more growth than control with the two in combination. In the dextrose group, chlortetracycline increased body weight gain by 6% which was not statistically significant, whereas barbituric acid treatment showed no effect. For the starch group, neither barbituric acid nor chlortetracycline affected gains in body weight.

The data on efficiency of feed utilization, obtained by dividing the total weight gain per lot by the total feed consumed, are presented in table 3. In general, efficiency of feed utilization paralleled that of growth. Chicks fed starch diets showed the highest efficiency and those fed sucrose the lowest. Both additives influenced utilization of sucrose throughout the experiment, whereas the effects with starch were manifested during the first 2 weeks. No effect was noted with dextrose.

Gastrointestinal ammonia concentration and ureolytic activity, expressed in micrograms per gram wet weight, are shown in table 4. The ammonia concentration and the urease activity were consistently higher in the large intestine than in the small intestine. In the group of chicks fed the sucrose diet, the addition of barbituric acid alone resulted in a depression of ammonia concentration in the small and large intestine by 18 and 13%, respectively, when compared with the basal group. There was a decrease of 10% in the small intestines of chicks fed chlortetracycline. In the dextrose group, addition of barbituric acid resulted in a significant ($P < 0.01$) depression of ammonia in the large intestine. Barbituric acid alone depressed ammonia concentration in chicks fed the starch basal diet, in both the small and large intestine.

The differences in urease activity of the gastrointestinal tracts plus contents of the chicks fed various carbohydrate basal diets were greatest in the small intestine. This activity was highest with the sucrose diet and lowest with the starch diet. Urease activity in the small intestine was lowered by barbituric acid or chlortetracycline either alone or in combination with the

TABLE 4

Ammonia concentration and hydrolysis of urea by gastrointestinal contents of 4-week-old cockerels fed diets containing various carbohydrates and supplemented with barbituric acid or chlortetracycline, or both [1]

Diet	NH_3		Urea split	
	Small intestine	Large intestine	Small intestine	Large intestine
	μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*
Sucrose				
1 Basal	179 ± 16 [2]	450 ± 90	121 ± 18	844 ± 71
2 Barbituric acid	152 ± 16	398 ± 72	77 ± 12 [3]	769 ± 137
3 Chlortetracycline	162 ± 13	445 ± 47	87 ± 25	684 ± 93 [4]
4 Barbituric acid + chlortetracycline	188 ± 9	465 ± 26	62 ± 20 [3]	557 ± 20 [4]
Dextrose				
1 Basal	171 ± 13	446 ± 23	107 ± 18	568 ± 88
2 Barbituric acid	175 ± 13	316 ± 21 [5]	94 ± 18	709 ± 78 [3]
3 Chlortetracycline	187 ± 7	537 ± 9	66 ± 8	542 ± 56
4 Barbituric acid + chlortetracycline	172 ± 12	373 ± 37 [5]	85 ± 40	762 ± 86 [3]
Starch				
1 Basal	205 ± 20	497 ± 77	59 ± 7	737 ± 132
2 Barbituric acid	186 ± 16	409 ± 76	66 ± 23	495 ± 84
3 Chlortetracycline	193 ± 26	452 ± 68	67 ± 16	601 ± 138
4 Barbituric acid + chlortetracycline	195 ± 25	524 ± 67	85 ± 20	669 ± 135

[1] Ten birds/lot; 4 lots/treatment.
[2] Mean ± SE.
[3] Barbituric acid vs. no barbituric acid significant at $P < 0.05$.
[4] Chlortetracycline vs. no chlortetracycline significant at $P < 0.05$.
[5] Barbituric acid vs. no barbituric acid significant at $P < 0.01$.

TABLE 5

Correlation coefficients of growth upon ureolytic activity in the large and small intestines of chicks

Source	Simple	Between treatments	Within treatments
Overall			
Small intestine	−0.345 [1]	−0.720 [2]	+0.325
Large intestine	−0.282 [1]	−0.524	−0.193
Sucrose			
Small intestine	−0.481 [2]	−0.850	−0.192
Large intestine	−0.404	−0.903	−0.185
Dextrose			
Small intestine	−0.401	−0.950 [1]	+0.460
Large intestine	−0.308	+0.116	−0.452
Starch			
Small intestine	+0.143	+0.260	+0.136
Large intestine	+0.054	+0.190	+0.045

[1] Significant at $P < 0.05$.
[2] Significant at $P < 0.06$.

sucrose and dextrose diets. Addition of these agents to the starch diet, however, resulted in a slight increase in ureolytic activity. The urease activity of the large intestine of the chicks fed sucrose was decreased 15% relative to control with the addition of barbituric acid and 30% with chlortetracycline ($P < 0.05$). In the dextrose group, addition of barbituric acid resulted in elevation of urease activity, whereas chlortetracycline showed no effects. With the starch diet, addition of barbituric acid suppressed the ureolytic activity by 15%, whereas chlortetracycline failed to show any effects.

Correlation coefficients for 4-week growth and gastrointestinal ureolytic activity at the time of death were determined overall and with respect to carbohydrate source (table 5). Simple correlations of gain with ureolytic activity in the small and large intestines were negative except for the starch group. Significant negative correlations were found between gain and ureolytic activity in both segments of the gastrointestinal tract over all treatments ($P < 0.05$) and in the small intestine ($P < 0.06$) for the sucrose group. Between treatment analyses followed that of the simple correlations, whereas the within treatments correlations gave no definite pattern.

DISCUSSION

The basal diet containing starch produced the greatest growth in chicks, whereas that containing sucrose produced the least. The sucrose diet supplemented with chlortetracycline led to greater differences in growth and efficiency of feed utilization in chicks than when it was added to a starch diet. These results parallel those obtained by Stokstad et al. (17). Addition of barbituric acid to a sucrose diet resulted in growth differences which were greatest during the first 2 weeks of feeding. Although barbituric acid improved growth concurrently with suppression of ureolytic activity, there was no additive effect with chlortetracycline, suggesting that these exert their effect on the bacterial flora through a common pathway. Barbituric acid, when added to the sucrose diet, improved efficiency of feed utilization with depression of ammonia concentration and ureolytic activity of the gastrointestinal tract. These results are similar to those obtained previously in this laboratory (12).

Chlortetracycline and barbituric acid were not as effective when added to the dextrose diet as when added to a sucrose diet, although the increased gains with the additives were accompanied by a depression of the ureolytic activity in the small intestine. With the starch diet, the additives did not increase the gain in weight of the chicks and there was no significant depression of ureolytic activity in the gastrointestinal tract.

The correlation analyses support the hypothesis that when the addition of chlortetracycline or barbituric acid will enhance

growth, there is a concurrent depression of ureolytic activity. The data show that the type of carbohydrate in the diet influences the response of chicks to barbituric acid. Various carbohydrates have been shown to alter the intestinal flora (14) and the possibility exists that urease-producing bacteria may be suppressed leading to a decrease in production of various enterotoxins including ammonia. Analysis of data within treatment groups, however, does not suggest that lowest ureolytic activity is associated with greatest weight gain.

Although the amount of growth varies with chlortetracycline and barbituric acid, their overall effects on growth, ammonia concentration and urease activity in the gastrointestinal tract are similar. The evidence brought forth in this paper demonstrates that the mode of action of chlortetracycline, an antimicrobial agent, and of barbituric acid, a cyclic urea derivative, when fed to growing chicks, is dependent on the carbohydrate source. The data also suggest that under certain dietary regimens increased growth of chicks may be associated with depressed urease activity of the gastrointestinal tract.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of the American Meat Institute Foundation, Chicago, Illinois, for use of facilities.

LITERATURE CITED

1. Visek, W. J., J. M. Baron and D. M. Switz 1959 Urea metabolism and intestinal ureolytic activity of rats fed antimicrobial agents. J. Pharm. Therap., *126:* 359.
2. Francois, A. C., and M. Michel 1955 Action de la penicillin et de l'aureomycine sur les proprietes de la flore intestinale du porc. C. R. Acad. Sci., *240:* 124.
3. Francois, A. C. 1956 First International Conference on Antibiotics in Agriculture, National Academy Sciences — National Research Council, Washington, D. C., p. 84.
4. Dintzis, R. Z., and A. B. Hastings 1953 The effect of antibiotics on urea breakdown in mice. Proc. Nat. Acad. Sci., *39:* 571.
5. Sherlock, S. 1958 Pathogenesis and management of hepatic coma. Am. J. Med., *24:* 805.
6. Silen, W., H. A. Harper, D. L. Mawdsley and W. L. Weirich 1955 Effect of antibacterial agents on ammonia production within the intestine. Proc. Soc. Exp. Biol. Med., *88:* 138.
7. Dang, H. C., and W. J. Visek 1960 Effect of urease injection on body weights of growing rats and chicks. Proc. Soc. Exp. Biol. Med., *105:* 164.
8. Harbers, L. H., R. F. Hendrickson, H. N. Bass and W. J. Visek 1963 Effect of urease immunity upon rats with controlled vitamin A intakes. Proc. Soc. Exp. Biol. Med., *113:* 420.
9. Gray, C. T., J. C. Gerhart and M. S. Brooke 1959 Inhibition of urease by alloxan and alloxanic acid. Nature (London), *184:* 1936.
10. Gray, C. T., M. S. Brooke and J. C. Gerhart 1961 Metabolism of alloxanic acid in a soil microorganism. J. Bacteriol., *81:* 755.
11. Woods, D. D. 1942 The mode of action of chemotherapeutic agents. Biochem. J., *36:* 3.
12. Harbers, L. H., A. P. Alvares, A. I. Jacobson and W. J. Visek 1963 Effect of barbituric acid and chlortetracycline upon growth, ammonia concentration and urease activity in the gastrointestinal tract of chicks. J. Nutrition, *80:* 75.
13. Harper, A. E., and C. A. Elvehjem 1957 A review of the effects of different carbohydrates on vitamin and amino acid requirements. J. Agr. Food Chem., *5:* 754.
14. György, P. 1961 Recent advances in human nutrition. Little, Brown and Company, Cambridge, Massachusetts, chap. 22.
15. Jukes, T. H. 1957 Antibiotics in nutrition. Medical Encyclopedia Inc., New York, p. 43.
16. Barnes, R. H., E. Kwong, K. Delany and G. Fiala 1960 The mechanism of the thiamine-sparing effect of penicillin in rats. J. Nutrition, *71:* 149.
17. Stokstad, E. L. R., T. H. Jukes and W. L. Williams 1953 The growth promoting effects of aureomycin on various types of diets. Poult. Sci., *32:* 1054.
18. Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames, p. 237.

Absorption of Preformed Vitamin A from Ligatured Poultry Intestinal Sections[1,2]

T. E. SHELLENBERGER,[3] D. B. PARRISH AND P. E. SANFORD
Kansas State University, Manhattan, Kansas

ABSTRACT Absorption of aqueous dispersions of vitamin A was studied, using ligatured intestinal sections as modified in vivo systems. After injecting vitamin A acetate into duodenum, posterior small intestine, or cecum, the vitamin A content of duodenal and intestinal tissues was found to be similar, but that in blood serum was 50% higher after duodenal injections; little vitamin A was absorbed from the cecum. Absorption was better in younger and egg-strain birds than in older and broiler-strain birds. Vitamin A acetate was absorbed faster from the duodenum than from the small intestine, and also resulted in vitamin A increasing to higher levels in blood serum and liver. Vitamin A acetate was absorbed faster than the palmitate and led to higher blood serum and liver vitamin A levels. About 90% of the vitamin A in intestinal tissues was in the ester form, mainly higher fatty acid types, and about 65% of that in blood serum was in the alcohol form, whether vitamin A acetate, palmitate, or alcohol was injected. When serum vitamin A levels reached 11 μg/100 ml from single injections of vitamin A, small, but measurable, quantities of vitamin A were observed in liver. Results of simultaneous injections of ethoxyquin and vitamin A varied. Mechanisms of absorption are discussed.

Vitamin A is more readily absorbed from the intestinal tract when administered as aqueous dispersions or in milk emulsions than as solutions in oil (1–4). There is, however, apparently little published information on absorption of different forms of vitamin A by poultry (5), and none on absorption from ligatured intestinal sections.

This study was made to compare absorption of aqueous dispersions of vitamin A alcohol, acetate, and palmitate from different ligatured intestinal sections of poultry and to compare their effects on intestinal and blood serum vitamin A alcohol and ester levels and ratios. The effect of an antioxidant, ethoxyquin, on the absorption of vitamin A acetate also was investigated. Ligatured intestinal sections were used as modified in vivo systems.

EXPERIMENTAL

Experimental animals. Chicks used were obtained from local hatcheries, or were hatched from eggs laid by hens receiving a carotenoid-free diet.[4] Chicks were fed a diet containing 880 IU vitamin A/kg, composed of carotenoid-free ingredients as follows: (in per cent) white corn, 58.5; soybean oil meal (44%), 31; nonfat dry milk, 2; brewer's yeast, 2; bone meal, 2; $CaCO_3$, 1; NaCl, 0.5; cottonseed oil, 0.65; and vitamin and manganese sulfate premixes in wheat shorts, 2.35. Except for vitamin A, this diet was adequate for normal growth and health. Chicks were raised in electrically heated, wire-floor batteries in rooms at 24 to 28°. Feed and water were given ad libitum.

When 5 to 10 weeks of age, chicks were fed the basal diet, free of vitamin A, for 7 to 10 days to deplete the small pre-experimental reserves. Preliminary results indicated that blood serum vitamin A was less than 5 μg/100 ml and liver vitamin A less than 3 μg/liver after depletion.

Isolation of intestinal sections. Vitamin A-depleted chicks were anesthetized lightly with ether. A lateral incision was made in the abdominal wall so that sections of intestine could be manipulated. Ligatures were applied to duodenal sections, posterior to the ventriculus and anterior to the entrance of the bile and pancreatic ducts, being careful to avoid damage to the pan-

Received for publication August 1, 1963.

[1] Contribution no. 41, Department of Biochemistry, and no. 279, Department of Poultry Science, Kansas Agricultural Experiment Station, Manhattan, Kansas.

[2] Portion of a dissertation presented by the senior author as partial fulfillment of requirements for the Ph.D. degree. For a more extensive treatment and data not presented see University Microfilms, Inc., Ann Arbor, Michigan, no. 61-1002.

[3] Present address: Stanford Research Institute, Menlo Park, California.

[4] Poultry Science Department, Kansas State University.

creas. Posterior small intestine segments[5] were isolated by applying one ligature posterior to the yolk stalk and another anterior to the blind end of the ceca, being careful to avoid rupture of blood vessels. The cecum was ligatured as far from the blind end as possible. Preparations of vitamin A were injected into the ligatured sections using a hypodermic syringe. Ligatured sections were replaced in the abdominal cavity and the incision sutured. Birds were killed at times after injection indicated in tables 1–4, and samples were taken for vitamin A and carotenoid determinations. Sections were washed free of lumen contents at collection time.

Preparation of vitamin A dispersions. Aqueous dispersions of vitamin A acetate, palmitate, and alcohol[6] were prepared prior to each trial by dissolving the vitamin A in 10 ml of ether, adding 25 ml of 20% polyoxyethylene sorbitan monooleate (Tween 40)[7] containing a few drops of 95% ethanol, and removing the volatile solvents under vacuum at 50°.

Analytical methods. Analyses for total vitamin A in tissue, liver, and intestine were made by a modification of the procedure of Neff et al. (6),[8] utilizing the Carr-Price procedure with reagents prepared as described by the AOAC (7). Blood serum vitamin A was determined by the Kimble method (8). Vitamin A alcohol and esters were separated by a modification of the chromatographic procedure of Parrish et al. (9). Vitamin A esters were resolved into low and high molecular weight fatty acid components by the procedure of Kaiser and Kagan (10).

RESULTS

Values for vitamin A content of intestine, blood serum, and liver — 4 hours after injection of vitamin A acetate into intestinal sections — are summarized in table 1. Although vitamin A levels of the duodenal and small intestine tissues were similar, average blood serum and liver vitamin A content was more than 50% larger after vitamin A acetate was injected into the duodenal sections. After the acetate was injected into ligatured cecal sections, only a small quantity of vitamin A was recovered from the tissues (table 1), blood serum vitamin A was much lower than following duodenal or intestinal injections, and there was no increase of vitamin A in livers. Thus, ligatured duodenal, posterior small intestinal, and cecal sections differed markedly in absorption of aqueous dispersions of vitamin A acetate.

Studies were undertaken to test effect of age and type of chick on absorption of vitamin A acetate from duodenal and posterior small intestinal sections. Comparisons were made at 5, 7 and 9 weeks of age, with both broiler strain and egg strain chicks given 740 to 760 units vitamin A/bird. Blood serum vitamin A content was higher in younger and in egg-strain birds,[9] mainly, no doubt, reflecting size of bird in

[5] The segment between yolk stock and ceca, approximately the anterior one-half of the ileum.

[6] Vitamin A acetate was USP vitamin A reference solution. Vitamin A palmitate was 1 million IU/g synthetic product in corn oil, Chas. Pfizer and Company. Vitamin A alcohol was a crystalline product, Distillation Products Industries, Inc., Rochester, New York.

[7] Atlas Powder Company, Wilmington, Delaware.

[8] Peroxide-free ether, and redistilled petroleum solvents were used in extractions.

[9] See footnote 2.

TABLE 1

Absorption of vitamin A acetate from ligatured duodenal and posterior small intestine sections

Site of injection	No. of experimental birds	Vitamin A content					
		Intestinal tissue		Blood serum		Liver	
		Avg	Range	Avg	Range	Avg	Range
		µg/tissue		*µg/100 ml*		*µg/liver*	
None [1]	4	1 [2]		2		2 [3]	
Duodenum	7 [4]	44	27–55	22	12–29	14	5–17
Posterior small intestine	7 [4]	48	35–57	14	10–16	8	5–11
Cecum	12 [5]	5		6		2	

[1] Received 0.5 ml carrier only.

[2] Cecum tissue.

[3] Only 2 values in this average.

[4] Birds received 600 or 620 USP units preformed vitamin A acetate in 1 ml 20% Tween 40. All birds killed 4 hours after injection of vitamin A.

[5] 385 or 500 USP units vitamin A acetate in 0.5 ml 20% Tween 40; liver data only from birds injected with higher level vitamin A.

relation to quantity of vitamin A injected. Differences in duodenal and posterior small intestinal absorptions were, in general, as illustrated by table 1.

To determine the time required for vitamin A to appear in intestinal tissue, blood serum, and liver, samples were taken for analyses at definite intervals following injection of vitamin A acetate into ligatured duodenal sections (table 2). Considerable vitamin A was observed in duodenal tissue within 0.25 hour, increased to about 70 μg/tissue at 0.5 hour, then decreased irregularly. Blood serum vitamin A was 9 μg/100 ml within 0.5 hour after duodenal injection, and increased further during the next 3 to 4 hours. Liver vitamin A increases lagged only slightly, being 8 μg 0.75 hour after injection, and increasing substantially in the next 1 to 3 hours.

Vitamin A was observed in ligatured posterior small intestinal tissue also within 0.25 hour after vitamin A acetate was injected, and it increased further in 2 hours (table 2). Blood serum vitamin A content increased slowly, but within 2 hours it was some 10 times that of those in the initial samples. Vitamin A deposition increased even more slowly in the livers; 2 hours after injection, liver vitamin A was only 4 times greater than that in the initial samples. Thus aqueous dispersions of vitamin A acetate injected into ligatured duodenal sections were absorbed into the blood and deposited in the liver faster, and reached higher levels within 2 to 3 hours than that injected into ligatured posterior small intestine.

Comparisons were made also of the relative duodenal absorptions of vitamin A palmitate and acetate as dispersions with Tween 40. After acetate was injected, duodenal vitamin A content was more than twice that after palmitate was injected (table 3). Blood serum vitamin A was 3 times higher in the acetate-treated than in the palmitate-treated birds, and liver vitamin A content was 5 times greater in the former.

To study the role of esterification and hydrolysis during absorption and the effect on the state of vitamin A in blood, analyses were made of the vitamin A ester and alcohol content and ratios in intestinal tissues and blood serum following injections of aqueous dispersions of vitamin A acetate, palmitate, and alcohol into ligatured duodenal sections. Four hours after injections, birds were killed and samples saved for chromatographic resolution of vitamin A alcohol and esters. Blood sera of the 6 chicks in each lot were pooled to give

TABLE 2

Absorption and deposition of vitamin A in tissues at various time intervals following injection of vitamin A acetate into ligatured duodenal and posterior small intestinal (P.S.I.) sections

Time	Average vitamin A content					
	Duodenal tissue	Blood serum	Liver	P.S.I. tissue	Blood serum	Liver
hours	*μg/tissue*	*μg/100 ml*	*μg/liver*	*μg/tissue*	*μg/100 ml*	*μg/liver*
0	2 (2)[1]	0[2] (2)	1 (2)	2 (6)	1 (5)	1 (6)
0.25	58 (2)	1 (2)	1 (2)	25 (2)	1 (1)	1 (2)
0.5	73 (3)	9 (3)	3 (3)	42 (5)	2 (4)	2 (5)
0.75	71 (2)	13 (2)	8 (2)	52 (2)	3 (2)	2 (2)
1	71 (3)	21 (3)	13 (3)	54 (5)	4 (4)	2 (5)
1.25	58 (2)	22 (2)	16 (2)	43 (4)	7 (3)	2 (4)
1.5	74 (3)	28 (3)	18 (3)	64 (6)	9 (5)	2 (6)
1.75	52 (2)	28 (2)	18 (2)	51 (4)	10 (3)	2 (4)
2	74 (1)	37 (1)	17 (1)	58 (4)	11 (3)	4 (4)
2.25				72 (2)	14 (2)	5 (2)
2.5	42 (1)	39 (1)	20 (1)	70 (3)	16 (2)	9 (3)
2.75				47 (1)	17 (1)	14 (1)
3	60 (1)	43 (1)	25 (1)	69 (2)	23 (1)	9 (2)
3.5	45 (1)	49 (1)	20 (1)	37 (1)		14 (1)
4	51 (1)	47 (1)	20 (1)			

[1] Number of birds from which samples were taken. In duodenal studies, each received 700 to 710 USP units vitamin A acetate in 1 ml 20% Tween 40, and in posterior small intestinal studies, 700 to 740 USP units.
[2] No vitamin A detected.

TABLE 3

Absorption of preformed vitamin A palmitate and acetate from ligatured duodenal sections [1]

No. of birds	Vitamin A form administered	Vitamin A levels		
		Duodenal tissue	Blood serum	Liver
		µg/tissue	*µg/100 ml*	*µg/liver*
4 [2]	None	1	3	1
6 [3]	Palmitate	25	18	4
6 [4]	Acetate	58	55	20

[1] Birds killed for samples 4 hours after vitamin A injections.
[2] Negative controls. Received carrier only.
[3] Received 680 or 700 USP units vitamin A palmitate in 1 ml 20% Tween 40.
[4] Received 680 or 700 USP units vitamin A acetate in 1 ml 20% Tween 40.

sufficient vitamin A for chromatography. Results (table 4) indicated that about 90% [10] of the vitamin A injected as acetate was recovered from the duodenal tissue as ester. Both acetate and higher fatty acid esters of vitamin A were detected by chromatography, indicating at least partial hydrolysis and re-esterification. Blood serum vitamin A was about 65% alcohol and 35% ester; only higher molecular weight fatty acid esters were detected.

Although even more vitamin A palmitate than acetate was injected, there was less vitamin A in duodenal tissue (table 4), indicating that the palmitate was not so readily absorbed. About 85% of the duodenal vitamin A was the ester and represented by higher molecular weight types, similar to trials with acetate. Vitamin A alcohol, but no ester, was detected in the blood serum.

Results with injections of vitamin A alcohol generally agreed with those obtained by injecting acetate (table 4); however, total duodenal vitamin A content was lower. Most of the vitamin A ester in both tissue and serum was recovered as higher molecular weight types.

Regardless of the form of vitamin A injected, 85 to 90% of the vitamin A recovered in the doudenal mucosa and walls was the ester. In blood serum, some 30 to 35% of the vitamin A recovered after injecting vitamin A acetate or alcohol was the ester (mostly of higher fatty acid types), whereas only vitamin A alcohol was detected in serum after injecting vitamin A palmitate.

Different quantities of vitamin A acetate were injected into ligatured duodenal sections to study the relation of blood serum vitamin A levels to liver storage. Blood serum and liver samples were analyzed 4 hours after the injections. When birds were injected with 355 USP units vitamin

[10] Vitamin A ester and alcohol content was based on quantities recovered by chromatography. Total recoveries of vitamin A by column chromatography ranged from 83 to 108% of that determined prior to chromatography. Percentage recoveries are in footnotes to table 4 when recoveries were not practically 100%.

TABLE 4

Tissue vitamin A ester and alcohol levels 4 to 4.5 hours after injection of various forms vitamin A into ligatured duodenal sections

Form vitamin A injected	No. birds injected	Duodenal tissue				Blood serum [1]			
		Alcohol	Ester	Total	% ester	Alcohol	Ester	Total	% ester
		µg/tissue				*µg/100 ml*			
Vitamin A acetate [2]	6	5	49	54	91	24	14	38	37
Vitamin A palmitate [3]	6	2	12	14 [4]	86	12	0 [5]	12 [6]	0
Vitamin A alcohol [7]	6	3	21	24 [8]	88	26	10	36 [9]	28

[1] Pooled serum sample.
[2] Birds received 760 USP units vitamin A in 1 ml 20% Tween 40.
[3] Birds received 1025 USP units vitamin A in 1 ml 20% Tween 40.
[4] Recovery of 108%, based on values before chromatography.
[5] No apparent vitamin A ester recovered.
[6] Recovery of 83%, based on values before chromatography.
[7] Birds received 700 IU vitamin A in 1 ml 20% Tween 40.
[8] Recovery of 103%, based on values before chromatography.
[9] Recovery of 95%, based on values before chromatography.

A, serum vitamin A content was 11 μg/100 ml and liver vitamin A was 5 μg/liver. Substantial liver vitamin A storage (11 μg) was observed in birds injected with 670 USP units vitamin A, where a blood serum vitamin A content of 20 μg/100 ml was attained.[11]

In similar studies of injections into ligatured posterior small intestine sections, birds given 412 USP units vitamin A averaged 11 μg/vitamin A/100 ml of blood serum, but liver vitamin A content was not substantially higher than that of controls. With 615 USP units vitamins A, serum vitamin A levels averaged 14 μg/100 ml and liver, 10 μg/liver.

Data on relation of blood serum vitamin A levels and liver vitamin A storage also are shown in table 2. Birds had a serum vitamin A content of 9, 13 and 21 μg/100 ml at 0.5, 0.75, and 1 hour, respectively, after vitamin A was injected into ligatured duodenal sections, at which times the liver vitamin A content was 3, 8 and 13 μg/liver, respectively. In general, moderate liver vitamin A storage occurred only after serum vitamin A levels of some 12 to 15 μg/100 ml were attained.

Effects of low concentrations of dietary antioxidants on vitamin A and carotene utilization and on growth have been reported (11–14), but these effects probably are complicated by degree of protection given vitamin A during feed storage and while in the upper gastrointestinal tract after ingestion.

In a series of trials 335 or 715 USP units vitamin A acetate dispersed in 20% Tween 40, with or without 0.03% ethoxyquin,[12] was injected into ligatured duodenal sections to determine the effect of the antioxidant, ethoxyquin, on absorption. Duodenal tissue, serum, and liver were analyzed for vitamin A 1.25 or 4 hours later. Although there was an indication of decreased absorption at 1.25 hours, definite effects could not be discovered because of variability within treatments.

DISCUSSION

Vitamin A acetate injected into duodenal sections appeared in the blood earlier, was deposited in the liver faster, and reached higher levels than when injected into posterior small intestine sections. This difference is explainable, in part, in terms of absorptive surfaces in the 2 sections. Although the posterior small intestinal section was almost twice as long as the duodenal section, villi are longer and more numerous per area in the duodenum (15).

The vitamin A content of duodenal tissue was 2 to 4 times higher after injection of vitamin A acetate than after injection of the palmitate (tables 3 and 4). Most of the vitamin A recovered was in the higher molecular ester form; the remainder was largely vitamin A alcohol. Eden and Sellers (16) maintained that vitamin A esters were hydrolyzed and re-esterified during absorption instead of being absorbed in the unaltered form. Similar conclusions were reached by Murthy et al. (17). Krishnamurthy et al. (18) reported some hydrolysis of natural vitamin A esters by small intestinal tissue. Mahadevan et al. (19) observed that in vitro hydrolysis of vitamin A palmitate was activated by sodium taurocholate but inhibited by the presence of large amounts of Tween 20 and Tween 80. This effect alone, if also true in vivo, could account for the decreased absorption noted with the vitamin A palmitate. Hydrolysis of vitamin A esters by liver homogenates, however, varies inversely with chain length of the fatty acid portion (20, 21);[13] this may be true also in the intestine.

Although during active absorption a proportion of the free vitamin is present in mucosae and muscles of the small intestine and in mesenteric lymph fluid (22), it is transported from the intestine principally as long-chain fatty acid esters and by the lymphatics. Moore (2) concluded that the lymphatics are more important than the portal vein for absorption of vitamin A. However, Imbesi (4) suggested that vitamin A acetate was absorbed through both lymph and portal systems, and Murray and Grice (23) observed that blocking the lymphatic system of rats at the thoracic duct did not interfere with vitamin A absorption and suggested the portal system as an important alternative route for absorption.

---

[11] See footnote 2.

[12] Santoquin, Monsanto Chemical Company, St. Louis.

[13] High, E. G., J. R. Powell and H. B. Bright 1957 *In vivo* and *in vitro* hydrolysis of vitamin A esters and esterification of vitamin A alcohol. Federation Proc., *16:* 388 (abstract).

Normally, as confirmed in this study with chicks, most of the vitamin A in serum is the alcohol (2, 22, 24). The alcohol of serum may arise from 2 possible sources: absorption of vitamin A hydrolyzed in the intestinal lumen, or hydrolysis of serum vitamin A esters by tissues other than the small intestine. A small quantity (10 to 15%) of intestinal vitamin A was found as free alcohol; this may be significant, since it could be the source of blood serum vitamin A alcohol, via the portal system at a rate preventing build-up in intestinal tissue. Another possible source of serum vitamin A alcohol is hydrolysis of blood vitamin A esters by other tissues. Krishnamurthy et al. (18) reported that pancreatic tissue actively hydrolyzed natural vitamin A esters, liver tissue was inactive, whereas splenic tissue hydrolyzed a small quantity. Slow hydrolysis of the palmitate in intestinal lumen and thus lower concentrations of vitamin A in duodenal tissue, plus other tissue hydrolysis may account for lack of vitamin A esters in post-absorptive blood serum after the palmitate was injected. Further work is needed to define the mechanism controlling vitamin A alcohol levels of blood.

Transport of vitamin A across the intestinal wall apparently is energy-dependent (22), for it was accelerated by extra glucose or ATP, or both, whereas glycolytic inhibitors and uncoupling agents inhibited or reduced absorption. If ethoxyquin, an antioxidant, decreases absorption, as our study indicated, it might be through interference in energy dependent processes.

LITERATURE CITED

1. Jacobson, N. L., R. S. Allen, J. T. Blake and P. G. Homeyer 1954 The effect of method of administration on the absorption and storage of vitamin A by dairy calves. J. Nutrition, *54:* 143.
2. Moore, T. 1957 Vitamin A. Elsevier Publishing Company, New York.
3. Wise, G. W., N. L. Jacobson, R. S. Allen and S. P. Yang 1958 Rate of absorption of carotene and of vitamin A from the alimentary tract of dairy calves. II. Effects of methods of dispersion and of administration. J. Dairy Sci., *41:* 143.
4. Imbesi, A. 1958 Vitamin A absorption through the blood and lymph. Atti. Accad. Peloritana Pericolanti, Classe Sci. Med.-Biol., *56:* 203 (cited in Chem. Abs., *53:* 20350d).
5. Week, E. F., and F. J. Sevigne 1949 Vitamin A utilization studies. I. The utilization of vitamin A alcohol, vitamin A acetate, and vitamin A natural esters by the chick. J. Nutrition, *39:* 233.
6. Neff, A. W., D. B. Parrish, J. S. Hughes and L. F. Payne 1949 The state of vitamin A in eggs. Arch. Biochem., *21:* 315.
7. Association of Official Agricultural Chemists 1960 Methods of Analysis, ed. 9. Washington, D. C., p. 652.
8. Kimble, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med., *24:* 1055.
9. Parrish, D. B., G. H. Wise and J. S. Hughes 1947 The state of vitamin A in colostrum and in milk. J. Biol. Chem., *167:* 673.
10. Kaiser, E., and B. M. Kagan 1956 Separation of vitamin A alcohol and vitamin A esters by paper chromatography. Arch. Biochem. Biophys., *63:* 118.
11. Matterson, L. D., R. H. Bunnell, L. D. Stinson, E. P. Singsen and L. M. Potter 1955 The effect of incorporating diphenyl-*p*-phenylenediamine (DPPD) into a ration containing various levels of vitamin A from fish oils. Poult. Sci., *34:* 1080.
12. Shellenberger, T. E., D. B. Parrish and P. E. Sanford 1957 Effects of antioxidants, DPPD and BHT, on health, production and reproduction of laying hens. Poult. Sci., *36:* 1313.
13. Tiews, J. 1957 The influence of antioxidants and antibiotics on the utilization of vitamin A by chicken and rat. Landwirtsch. Forsch., Sonderh., no. *9:* 148.
14. Ascarelli, I. 1957 The use of vitamin A protected by DPPD (diphenyl-*p*-phenylenediamine) for the growth of chickens. Poult. Sci., *36:* 549.
15. Calhoun, M. L. 1954 Microscopic Anatomy of the Digestive Systems of the Chicken. Iowa State College Press, Ames, Iowa.
16. Eden, E., and K. C. Sellers 1950 Hydrolysis and esterification of vitamin A during absorption. Biochem. J., *46:* 261.
17. Murthy, S. K., S. Mahadevan, P. S. Sastry and J. Ganguly 1961 Fatty acid specificity for the esterification of vitamin A and cholesterol by intestinal and pancreatic enzymes in rats. Nature, *189:* 482.
18. Krishnamurthy, S., P. S. Sastry and J. Ganguly 1957 Studies on vitamin A esterase. II. The hydrolysis of natural vitamin A ester within the living animal. Indian J. Med. Res., *45:* 391.
19. Mahadevan, S., S. K. Murthy, S. Krishnamurthy and J. Ganguly 1961 Studies on vitamin A esterase. 4. The hydrolysis and synthesis of vitamin A esters by rat intestinal mucosae. Biochem. J., *79:* 416.

20. Krause, R. F., and L. T. Powell 1953 The *in vitro* hydrolysis and esterification of vitamin A. Arch. Biochem. Biophys., *44:* 57.
21. Laughland, D. H. 1958 The hydrolysis of vitamin A esters by rat liver homogenates. Arch. Biochem. Biophys., *73:* 95.
22. Ganguly, J. 1960 Absorption, transport, and storage of vitamin A. Vitamins and Hormones, *18:* 387.
23. Murray, T. K., and H. C. Grice 1961 Absorption of vitamin A after thoracic duct ligation in the rat. Canad. J. Biochem. Physiol., *39:* 1103.
24. Parrish, D. B., G. H. Wise and J. S. Hughes 1948 Effect of vitamin A supplements upon the state of vitamin A in blood serum of the dairy cow and in blood serum and liver of its neonatal calf. J. Biol. Chem., *172:* 355.

Vanadium Toxicity and Distribution in Chicks and Rats[1,2]

JOHN N. HATHCOCK, CHARLES H. HILL AND GENNARD MATRONE
North Carolina State College, of the University of North Carolina, Raleigh, North Carolina

ABSTRACT Studies of the effect of dietary vanadium revealed that 25 ppm vanadium added to a dry skim milk-glucose monohydrate diet was toxic to chicks as indicated by both depression of growth and mortality. Ammonium metavanadate and vanadyl sulfate were equally toxic. The addition of the essential trace elements, iron, copper, molybdenum, cobalt, zinc, and manganese to the basal diet did not affect the toxicity of vanadium. Scandium, titanium, and niobium, elements which resemble vanadium in many of their chemical and physical parameters, were not toxic when fed at 200 ppm nor did they influence the toxicity of vanadium. Radioisotope studies revealed that scandium and niobium were poorly absorbed or poorly retained compared to vanadium. EDTA completely prevented the toxicity of vanadium apparently by preventing its absorption from the intestinal tract. Ingested V^{48} was found to be mainly concentrated in the hone and kidney of young chicks and in the kidney of adult rats.

Dietary vanadium in low concentration has been found to be toxic to rats and chicks (1–4). Although considerable work has been carried out, the nature of vanadium toxicity remains unknown.

The work presented in this report was undertaken to: 1) further study vanadium toxicity in chicks and rats; 2) determine target organs by use of radioactive vanadium; 3) compare effects of vanadium with effects of elements similar to it; and 4) determine the effects of a dietary chelating agent on vanadium toxicity.

MATERIALS AND METHODS

The chicks used in the non-radioisotope studies, straight-run White Plymouth Rocks from a commercial hatchery, were weighed individually and distributed at random into experimental lots at one day of age. They were housed in electrically heated battery brooders with raised wire floors. Water and feed were supplied ad libitum. The basal diet used in all these studies is presented in table 1. Individual body weights of the chicks were recorded weekly and mortality was recorded daily.

In the first experiment, ammonium metavanadate was fed to chicks at levels of 10 and 25 ppm vanadium in the diet, and in the second experiment, both ammonium metavanadate and vanadyl sulfate were fed at a level of 25 ppm vanadium. Two lots of 20 chicks each were fed each diet.

In the third experiment, 100 ppm iron, 25 ppm copper, 70 ppm manganese, 100 ppm zinc, 50 ppm cobalt, and 50 ppm molybdenum were added to the basal diet in both the presence and absence of 25

TABLE 1
Basal diet

	%
Dried skim milk	60.0
Glucose	32.5
Cottonseed oil [1]	5.0
DL-Methionine	0.3
L-Arginine	0.5
Glycine	0.5
NaCl	0.5
$MgSO_4 \cdot 7H_2O$	0.5
	g/kg
$MnSO_4 \cdot H_2O$	0.22
$FeSO_4 \cdot 7H_2O$	0.53
$CuSO_4 \cdot 5H_2O$	0.10
Vitamin mix [2]	1.98

[1] Wesson Oil, The Wesson Oil Company, New Orleans, La.

[2] Supplies per kilogram of diet: Vitamin A, 11,000 USP units; vitamin D, 1980 IC units; thiamine, 3.52 mg; riboflavin, 5.72 mg; folic acid, 1.10 mg; α-tocopheryl acetate, 22.0 mg; menadione sodium bisulfite, 0.792 mg; biotin, 0.176 mg; pantothenic acid, 18.3 mg; niacin, 52.3 mg; pyridoxine, 5.7 mg; and cyanocobalamin, 8.8 μg.

Received for publication August 5, 1963.

[1] Published with the approval of the Director of the Experiment Station as Paper no. 1678 of the Journal Series.

[2] Supported in part by grants from the National Institutes of Health (AM05651-02), the Herman Frasch Foundation, and the Moorman Manufacturing Company.

ppm vanadium as ammonium metavanadate. Each of these diets was fed to 2 lots of 20 chicks each.

In the fourth study, scandium, titanium, and niobium were each fed at 200 ppm alone and in combination with 50 ppm vanadium to 2 lots of 10 chicks each. The compounds used were scandium trichloride, titanium tetrachloride, potassium niobate, and ammonium metavanadate.

In the fifth experiment, vanadium at 25 ppm was fed in the absence and presence of disodium ethylenediaminetetraacetate (EDTA) at 0.93 and 2.78 mmole/kg. These levels of EDTA are 2 and 6 times the molar concentration of vanadium. Two lots of 20 chicks each were fed each diet.

In the radioisotope experiments, 6-week-old chicks or mature rats were each orally administered a quantity of the radioisotope in 0.5 ml of solution. After allowing time for the adsorption and assimilation of the radioisotopes, the animals were killed and samples of the tissues and excreta were taken for both counting and weighing. The radioactivities of the counting samples were determined in a deep-well scintillation counter and compared on the basis of net counts per minute per gram of dry weight of the tissue.

In the first of these experiments, 5.6 μc of vanadium-48, in the form of vanadyl dichloride, were administered orally to each of 6 chicks. Forty-eight hours later, the radioactive vanadium content of various organs was determined.

In the second radioisotope experiment, 3 chicks fed the basal diet and 3 others fed that diet supplemented with EDTA at 0.93 mmole/kg were each given 5.1 μc of vanadium-48. Forty-eight hours later, the radioactive vanadium contents of liver, kidney, bone, and bone marrow were determined.

For the third experiment, 3 rats were fed the basal diet and 3 others were fed that diet with EDTA added at 0.93 mmole/kg. Three days after beginning to feed the animals these diets, they were orally given 10 μc of vanadium-48 each. The urine and feces were collected separately. Forty-eight hours later, the radioactive vanadium content of the liver, kidney, and bone was determined as well as that of the urine and feces.

In the fourth study, 4 rats were each given 5 μc of scandium-46, as scandium trichloride, and 4 others were each given 5 μc of niobium-95, as niobium oxalate. After 4 days, certain organs and the excreta were analyzed for content of these radioactive elements.

The results were analyzed for statistical significance by Duncan's multiple range test (5).

RESULTS AND DISCUSSION

The first study was conducted to determine the level of vanadium which would have toxic effects when included in the basal diet. The results, presented in table 2, experiment 1, show that the addition of 10 ppm vanadium to the basal diet failed to affect either the growth or mortality of the chicks at 2 weeks of age. However, 25 ppm vanadium significantly depressed growth and increased mortality. The control and 10 ppm vanadium groups were carried to 6 weeks of age and no significant differences were observed in either weight gains or mortality.

TABLE 2

Effects of dietary vanadium on chicks

Vanadium	2-Week mortality [1,2]	2-Week gain [1,2]
	%	g
Experiment 1		
0 ppm	15.0 [a]	75.7 [a]
10 ppm [3]	15.0 [a]	69.9 [a]
25 ppm [3]	95.0 [b]	10.0 [b]
Experiment 2		
None	5.0 [a]	77.1 [a]
NH_4VO_3 [4]	82.5 [b]	16.9 [b]
$VOSO_4$ [4]	85.0 [b]	23.0 [b]

[1] Mean of 2 replicates of 20 chicks each.
[2] Numbers with same superscripts are not significantly different ($P \leq 0.05$).
[3] As NH_4VO_3.
[4] At 25 ppm vanadium.

The second study was conducted to determine whether 2 different vanadium compounds with vanadium of different valences would have different effects on chicks when included in the diet. The results, shown in table 2, experiment 2, indicate that, in the diet, vanadyl sulfate with the vanadium in 4^+ valence, and ammonium metavanadate with the vanadium in 5^+ valence are equally toxic.

This toxicity manifested with a low dietary concentration indicates some specificity in the action of vanadium. That is, with this level of vanadium the deleterious effects observed could hardly be due to such a nonspecific property of metals as the binding of sulfhydryl groups of proteins in general.

It was possible that vanadium was antagonistic to an essential trace metal analogous to the antagonism of zinc for copper (6) or tungsten for molybdenum (7). To determine whether this was so, in the third experiment, vanadium was fed at a concentration of 25 ppm in the basal diet and in that diet supplemented with essential trace minerals. The results, presented in table 3, show that the additional amounts of the trace elements had no effect on the toxicity of vanadium as measured by either growth inhibition or mortality.

TABLE 3

Effect of vanadium on basal and fortified diets

Supplement [1]	2-Week mortality [2,3]	2-Week gain [2,3]
	%	g
None	0.0 [a]	82.2 [a]
Vanadium	65.0 [b]	21.3 [b]
Trace minerals	2.5 [a]	77.6 [a]
V + trace minerals	60.0 [b]	26.4 [b]

[1] Vanadium at 25 ppm as NH_4VO_3; trace mineral supplement includes 100 ppm iron as $FeSO_4 \cdot 7H_2O$, 100 ppm zinc as $ZnCl_2$, 70 ppm manganese as $MnSO_4 \cdot H_2O$, 50 ppm cobalt as $CoCl_2 \cdot 6H_2O$, 50 ppm molybdenum as $Na_2MoO_4 \cdot 2H_2O$, and 25 ppm copper as $CuSO_4 \cdot 5H_2O$.
[2] Mean of 2 replicates of 20 chicks each.
[3] Numbers with same superscripts are not significantly different ($P \leq 0.05$).

The 3 elements, scandium, titanium, and niobium, are very similar to vanadium: the ions of these elements, Sc^{3+}, Ti^{4+}, and Nb^{5+} are isoelectronic in their valence shells with the V^{5+} ion and all have a favored coordination number of six. The similarities of these elements to vanadium suggested that they, too, might be toxic or might influence the toxicity of vanadium. However, the results of the fourth experiment, presented in table 4, indicate that, at 200 ppm in the diet, scandium, titanium, and niobium are not toxic as measured by growth or mortality, nor do they affect the toxicity of vanadium.

TABLE 4

Effects of elements similar to vanadium

Treatment [1]	2-Week mortality [2,3]	2-Week gain [2,3]
	%	g
None	0.0 [a]	100.1 [a]
V	90.0 [b]	23.5 [b]
Sc	0.0 [a]	104.2 [a]
Ti	5.0 [a]	101.1 [a]
Nb	5.0 [a]	101.2 [a]
V + Sc	100.0 [b]	—
V + Ti	100.0 [b]	—
V + Nb	100.0 [b]	—

[1] Vanadium at 50 ppm as NH_4VO_3, scandium at 200 ppm as $ScCl_3$, titanium at 200 ppm as $TiCl_4$, and niobium at 200 ppm as $(K_2O)_4 \cdot (Nb_2O_5)_3 \cdot 16H_2O$.
[2] Mean of 2 replicates of 10 chicks each.
[3] Numbers with same superscripts are not significantly different ($P \leq 0.05$).

Studies with other elements have indicated that chelating agents can reverse metal toxicity by chelation and subsequent excretion (8) or by prevention of absorption (9). In the fifth experiment, EDTA was added at 2 and 6 times the molar concentration of vanadium in the diet to determine whether this chelating agent would affect the toxicity produced by 25 ppm dietary vanadium. The results, presented in table 5, show that both levels of EDTA protected the chicks against the vanadium toxicity.

TABLE 5

Effects of EDTA[1] on vanadium toxicity

Dietary supplement	2-Week mortality [2,3]	3-Week gain [2,3]
	%	g
None	5.0 [a]	90.4 [a]
Vanadium [4]	62.5 [b]	30.5 [b]
V [4] + 0.93 mmole/kg EDTA	12.5 [a]	78.3 [a]
V [4] + 2.78 mmole/kg EDTA	5.0 [a]	89.4 [a]

[1] Added as disodium ethylenediaminetetraacetate.
[2] Mean of 2 replicates of 20 chicks each.
[3] Numbers with same superscripts are not significantly different ($P \leq 0.05$).
[4] Vanadium at 25 ppm which equals 0.46 mmole/kg, as NH_4VO_3.

The results of the studies thus far reported raised several questions: 1) What are the target organs of dietary vanadium? 2) How does EDTA protect the chick from vanadium toxicity? 3) Are scandium, titanium, and niobium absorbed in appreciable amounts from the intestinal tract? The radioisotope studies presented in this report were undertaken to answer these questions.

The results of the first of these experiments, presented in table 6, show the distribution of the radioactivity in the tissues of the chicks 48 hours after administration of the radioactive vanadium. The concentrations were found to be greatest in the bone and kidney.

TABLE 6

Radiovanadium distribution in chick tissues

Tissue	
	mean net count/ min/g dry wt
Bone [1]	13,346
Kidney	10,931
Liver	2,292
Spleen	1,693
Gonads [2]	1,491
Duodenum	1,200
Lung	981
Heart	882
Proventriculus	861
Skin	700
Brain	527
Muscle [3]	505
Pancreas	231
	mean net count/ min/ml
Whole blood	275
Serum	201
Blood cells [4]	444

[1] From the central section of the right femur; includes marrow.
[2] Mean includes 3 males and 3 females; mean for ovaries was 4% less than for testes.
[3] From the right thigh.
[4] Calculated from the activities of the whole blood and serum.

The next experiment was conducted to determine the distribution of radioactive vanadium in certain tissues of the chick both in the presence and absence of dietary EDTA. The results, presented in table 7, experiment 1, show that dietary EDTA decreased the concentration of radioactive vanadium in all the tissues studied. Whether this decrease was due to increased excretion or decreased absorption could not be determined from this study with chicks.

To obtain some knowledge of the excretion and absorption patterns of vanadium in the presence and absence of dietary EDTA, rats were used in the next experiment. The distributions of radioactive vanadium in the tissues and excreta of rats fed the basal diet and rats fed the EDTA-containing diet are presented in table 7, experiment 2. Again in

TABLE 7

Effect of EDTA [1] on radiovanadium distribution

Tissue	Control	EDTA [1]
	mean net count/ min/g dry wt	
Experiment 1 [2]		
Liver	4,843	1,028
Kidney	21,210	1,760
Bone [3]	69,843	19,888
Marrow [4]	5,075	375
Experiment 2 [5]		
Liver	1,805	333
Kidney	8,942	2,127
Bone [6]	155	131
Urine	25,879	9,130
Feces	168,998	737,139

[1] Concentration in the diet was 0.93 mmole/kg as disodium ethylenediaminetetraacetate.
[2] Each value is the mean of 3 chicks.
[3] From the central section of the right femur with the marrow removed.
[4] Marrow from the femur section mentioned above.
[5] Each value is the mean of 3 rats.
[6] From the central section of the right femur including marrow.

the rats, as it had in the chicks, the presence of EDTA in the diet produced lower concentrations of vanadium-48 in the tissues. The urine of the EDTA-fed rats, also, contained less radioactive vanadium than that of the control rats. The feces of the EDTA-fed rats, however, contained more radioactivity than that of the control rats. These results indicate that in the presence of dietary EDTA less vanadium is absorbed from the intestinal tract. Thus, EDTA probably prevented vanadium toxicity by preventing its absorption from the intestine.

The low activity of the bone of all the rats may be explained by the fact that they were mature adults, whereas the chicks in the previous experiments were young and growing rapidly.

There are no data in the literature which provide an estimate of the absorption of scandium, titanium, and niobium. It was possible that these elements, unlike vanadium, were not absorbed to any appreciable extent from the intestinal tract. To estimate the absorption of these elements from the diet, radioisotopes of scandium and niobium were used to determine the absorption and distribution patterns of these elements when orally administered. None of the known radioisotopes of titanium have a half-life long enough for use in this type of experiment. The

TABLE 8

Distribution of scandium-46 and niobium-95 [1]

Tissue	Scandium-46 [2]		Niobium-95 [3]	
	mean net count/ min/g dry wt	*% of fecal counts* [4]	*mean net count/ min/g dry wt*	*% of fecal counts* [4]
Liver	69.0	0.0025	207.2	0.0121
Spleen	178.0	0.00045	414.5	0.0024
Kidney	78.8	0.00048	943.8	0.0085
Femur [5]	10.2	0.00028	356.5	0.0016
Muscle [5]	142.2	0.0034	152.2	0.0016
Urine	320.0	0.0071	5,609.5	0.2300

[1] All numbers given are means for 4 rats.
[2] As $ScCl_3$.
[3] As $Nb(HC_2O_4)5$.
[4] Calculated as: $\frac{\text{Count/min whole organ}}{\text{Count/min total feces}} \times 100$.
[5] Values for these tissues given on wet weight basis.

results of this experiment, presented in table 8, are shown as mean net counts per minute per gram of dry weight, and as a percentage of fecal counts.[3] The results indicate that scandium and niobium are poorly absorbed from the intestinal tract.

LITERATURE CITED

1. Berg, L. R. 1963 Evidence of vanadium toxicity resulting from the use of certain commercial phosphorus supplements in chick rations. Poult. Sci., *42:* 766.
2. Franke, K. W., and A. L. Moxon 1937 The toxicity of orally ingested arsenic, selenium, tellurium, vanadium, and molybdenum. J. Pharmacol. Exp. Therap., *61:* 89.
3. Nelson, T. S., M. B. Gillis and H. T. Peeler 1962 Studies of the effect of vanadium on chick growth. Poult. Sci., *41:* 519.
4. Romoser, G. L., W. A. Dudley, L. J. Machlin and L. Loveless 1961 Toxicity of vanadium and chromium for the growing chick. Poult. Sci., *40:* 1171.
5. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
6. Smith, S. E., and E. J. Larson 1946 Zinc toxicity in rats. J. Biol. Chem., *163:* 29.
7. Leach, R. M., and L. C. Norris 1957 Studies on factors affecting the response of chicks to molybdenum. Poult. Sci., *36:* 1131.
8. Sivjakov, K. I., and H. A. Braun, Jr. 1959 The treatment of acute selenium, cadmium, and tungsten intoxication in rats with calcium disodium ethylenediaminetetraacetate. Toxicol. Appl. Pharmacol., *1:* 602.
9. Longcope, W. T., and J. A. Luetscher, Jr. 1946 Treatment of acute mercury poisoning by BAL. J. Clin. Invest., *25:* 557.

[3] Calculated as: $\frac{\text{Count/min whole organ}}{\text{Count/min total feces}} \times 100$.

Mineral Balance Studies with the Baby Pig: Effects of Dietary Phosphorus Level upon Calcium and Phosphorus Balance[1,2]

E. R. MILLER, D. E. ULLREY, C. L. ZUTAUT, J. A. HOEFER AND R. W. LUECKE

Departments of Animal Husbandry and Biochemistry, Michigan State University, East Lansing, Michigan

ABSTRACT Calcium and phosphorus balance studies were conducted on 29 baby pigs receiving a synthetic milk diet containing phosphorus levels of 0.2, 0.4, 0.5, 0.6, 0.7 and 0.8% with 0.8% calcium. Growth rate, food intake and mineral retention were greatly depressed in pigs receiving 0.2% of phosphorus. Increasing dietary phosphorus levels to 0.5% resulted in increased total phosphorus retention (g) and percentage phosphorus retention. Increasing dietary phosphorus levels beyond 0.5% did not increase phosphorus retention (g) but decreased percentage phosphorus retention. Dietary phosphorus levels below 0.5% resulted in reduced calcium retention (g) whereas increasing dietary phosphorus levels above 0.5% did not affect calcium balance. The dietary phosphorus requirement for optimal utilization of calcium and phosphorus is therefore about 0.5%.

Mineral balance studies with animals provide information concerning mineral absorption and retention and offer a means of assessing the effects of dietary nutrient levels and other factors upon mineral utilization. Few mineral balance studies with baby pigs have been conducted. Danish (1), German (2, 3), Canadian (4) and American (5, 6) balance trials concerned with calcium and phosphorus utilization of young pigs receiving sow's milk, sow's milk substitutes or synthetic milk diets have been reported.

The present study was undertaken to determine the effects of dietary phosphorus level upon calcium and phosphorus utilization by the baby pig as determined by mineral balance trials and to provide supplementary information toward a more accurate determination of the dietary phosphorus requirement of the baby pig (7).

MATERIALS AND METHODS

Baby pigs used in the study were from 2 trials conducted to determine their phosphorus requirement (7). Calcium and phosphorus balance studies were conducted with pigs receiving 0.2, 0.4 or 0.6% of dietary phosphorus in the first trial, and 0.4, 0.5, 0.6, 0.7 or 0.8% of dietary phosphorus in the second trial. Casein furnished 0.2% of phosphorus in each of the diets with USP grade $CaHPO_4 \cdot 2H_2O$ supplying the additional phosphorus for the higher dietary levels. Dietary calcium level for all pigs was maintained at 0.8% by appropriate reduction of $CaCO_3$ when $CaHPO_4 \cdot 2H_2O$ was increased. Thus the sources of dietary calcium were $CaCO_3$ and $CaHPO_4 \cdot 2H_2O$ and the sources of dietary phosphorus were casein and $CaHPO_4 \cdot 2H_2O$. All diets contained 1800 IU of vitamin D_3/kg of solids.

Pigs used in the balance studies were placed in metal metabolism cages[3] for a 3- to 5-day adjustment period prior to the collection period. Pigs were removed from the metabolism cage to other cages to be fed (4 times daily while fed the liquid diet or 3 times daily with dry diet) an amount of food and water which they would consume within a 5- to 10-minute period. Their mouths were wiped clean and then they were returned to the collection cages. Constant daily feed intakes were maintained throughout the remainder of the adjustment period and the 3-day collection

Received for publication August 5, 1963.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal article no. 3179.

[2] Presented in part before the meeting of the American Institute of Nutrition, April, 1962 (Federation Proc., *21:* 308, 1962, abstract).

[3] LC-108/A, Geo. H. Wahman Manufacturing Company, Baltimore, Maryland.

period. Feces were collected separately from urine by means of a fine screen placed above the collection tray. Urine contamination was minimal because of the firm nature of the feces. In the first trial, collections were made at 2, 4 and 6 weeks of age with pigs receiving the synthetic diet as a liquid during the first 2 collections and as a dry meal in the final collection. In the second trial collections were made during the final 2 weeks (5 and 6 weeks of age) with all pigs receiving the dry meal diet. Rate of dietary consumption was depressed only in pigs receiving 0.2% of dietary phosphorus. Total dietary intakes of pigs fed other levels of dietary phosphorus were equated during simultaneous collection periods.

Total fecal collections were dried in a low temperature (70°C) convection oven, weighed, finely ground and stored in air-tight containers. Total urine collections were acidified with 6 M HCl to a pH of between 1 and 2, the volume accurately measured and 100-ml samples stored in air-tight bottles. Analyses of fecal and urine calcium and phosphorus concentrations were by modifications of the methods of Varley (8). Statistical analyses of data were performed using the multiple range test of Duncan (9).

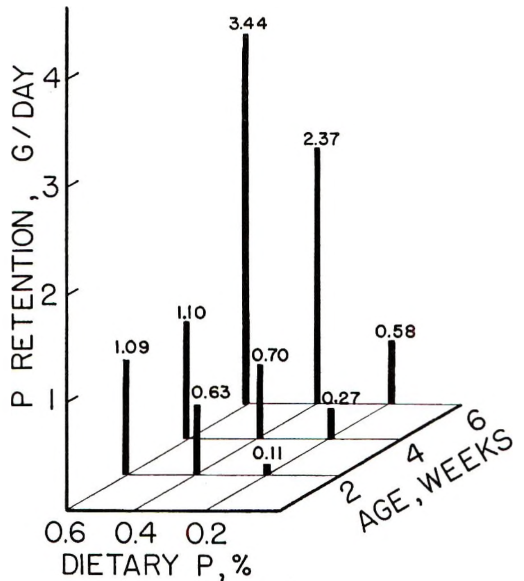


Fig. 1 Phosphorus retention of baby pigs as affected by age and phosphorus level (synthetic milk diet).

RESULTS AND DISCUSSION

Results of balance studies conducted during the first trial are presented graphically in figures 1 and 2. Dietary intake was depressed very early in the trial in pigs receiving 0.2% of dietary phosphorus. Animals receiving the 2 higher levels of dietary phosphorus received equal food intakes. Level of dietary phosphorus had a positive effect upon calcium and phosphorus retention at each age. Daily calcium and phosphorus retention increased with age at each level of dietary phosphorus. A great increase in calcium and phosphorus retention occurred from 4 to 6 weeks of age in pigs receiving 0.4 or 0.6% of dietary phosphorus. This increased retention was primarily due to the greater intakes of calcium and phosphorus that occurred when the dietary form was changed from a liquid (20% of solids) to a dry meal.

In the second trial littermates fed the different levels of dietary phosphorus received equal food intakes during simultaneous collections. The effects of dietary phosphorus level upon calcium and phosphorus utilization are shown in data presented in table 1. Pigs receiving 0.4 or 0.5% of dietary phosphorus excreted similar amounts of fecal and urinary phosphorus. Animals receiving the higher concentrations of dietary phosphorus excreted proportionately greater amounts of phosphorus, particularly in the urine, than animals receiving 0.5% of phosphorus. This resulted in similar phosphorus retention by pigs receiving dietary phosphorus levels of 0.5 to 0.8% which was significantly greater than that of pigs receiving 0.4% of phosphorus. Pigs receiving the lower levels of dietary phosphorus retained a higher percentage of the phosphorus intake than animals receiving 0.7 or 0.8% of phosphorus.

Fecal calcium excretion was significantly greater by pigs receiving 0.4% of phosphorus than by animals receiving the higher levels of dietary phosphorus. This appears to be an effect of the low level of dietary phosphorus per se, but it is also possible that this reduced apparent calcium

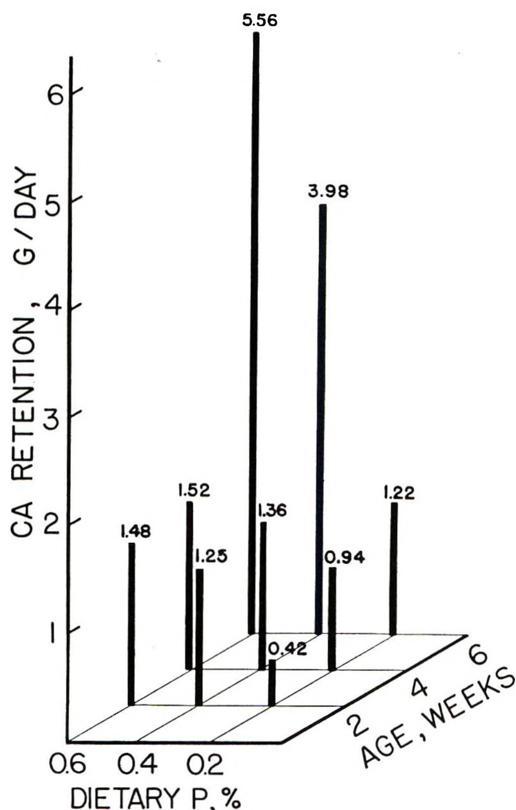


Fig. 2 Calcium retention of baby pigs as affected by age and phosphorus level (synthetic milk diet).

absorption may be due to dietary source of calcium since more of the calcium in the low phosphorus diet is being supplied in the less soluble carbonate form. The latter possibility does not, however, appear to be operative since no stepwise decrease in fecal calcium excretion occurs with other alterations of $CaCO_3$ and $CaHPO_4 \cdot 2H_2O$ as dietary phosphorus level is increased beyond 0.5%. Furthermore, calcium digestibility studies with rats (10) and with cattle (11) have shown no significant differences in calcium utilization when added to the diet in the carbonate or phosphate forms. Urinary calcium excretion was unaffected by dietary phosphorus level, contributing 5 to 10% of the total calcium excreted. This relationship between urinary and fecal calcium excretion has been shown to be quite consistent (1, 5, 6). Increased fecal calcium excretion by pigs receiving 0.4% of phosphorus resulted in a significantly reduced calcium retention. Since average dietary calcium intakes were equated the percentage calcium retention of pigs receiving 0.4% phosphorus was also significantly less than that of pigs receiving higher levels of dietary phosphorus. The effects of dietary phosphorus level upon calcium and phosphorus utilization by the baby pig are best illustrated by the graphs in figures 3 and 4. These indicate

TABLE 1

Calcium and phosphorus excretion and retention as affected by dietary phosphorus level

	Dietary phosphorus level, %				
	0.4	0.5	0.6	0.7	0.8
No. of collections	4	4	4	4	4
Daily food intake, g [1]	700	700	700	700	700
P balance					
Daily P intake, g [1]	2.8	3.5	4.2	4.9	5.6
Daily fecal P, g	0.3 ± 0.03 [2]	0.3 ± 0.03	0.5 ± 0.04	0.8 ± 0.03 [bb]	0.8 ± 0.14 [bb]
Daily urinary P, g	0.1 ± 0.07	0.1 ± 0.03	0.5 ± 0.03	1.0 ± 0.15 [bb]	1.5 ± 0.13 [bb]
Daily P retention, g	2.4 ± 0.2	3.1 ± 0.3 [a]	3.2 ± 0.4 [a]	3.1 ± 0.2 [a]	3.3 ± 0.3 [a]
P retention, %	84 ± 2.5 [bb]	88 ± 2.3 [bb]	75 ± 2.8 [b]	63 ± 1.5	59 ± 5.6
Ca balance					
Daily Ca intake, g [1]	5.6	5.6	5.6	5.6	5.6
Daily fecal Ca, g	2.0 ± 0.2	1.1 ± 0.1 [aa]	1.2 ± 0.1 [aa]	1.3 ± 0.1 [aa]	1.2 ± 0.2 [aa]
Daily urinary Ca, g	0.1 ± 0.08	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.02
Daily Ca retention, g	3.4 ± 0.3	4.4 ± 0.5 [a]	4.3 ± 0.6 [a]	4.2 ± 0.4 [a]	4.3 ± 0.6 [a]
Ca retention, %	60 ± 2.9	78 ± 3.3 [a]	78 ± 4.3 [a]	75 ± 0.8 [a]	77 ± 5.4 [a]

[1] Coefficient of variation (standard deviation ÷ mean) of all intake means is 0.14.

[2] SE.

[a] Significantly different from 0.4% P mean value ($P < 0.05$); [aa] $P < 0.01$.

[b] Significantly greater than least 2 values ($P < 0.05$); [bb] $P < 0.01$.

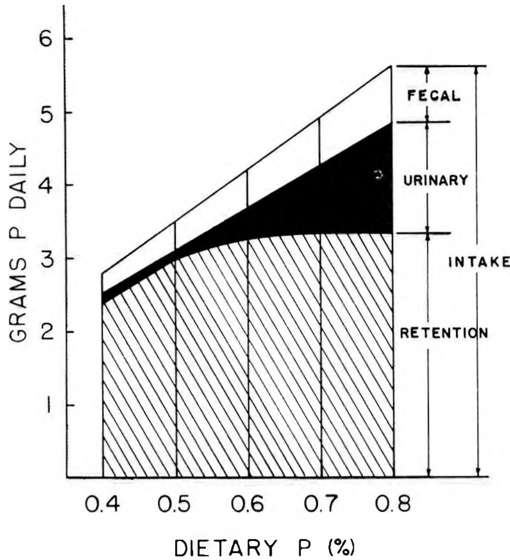


Fig. 3 Phosphorus balance as affected by dietary phosphorus level.

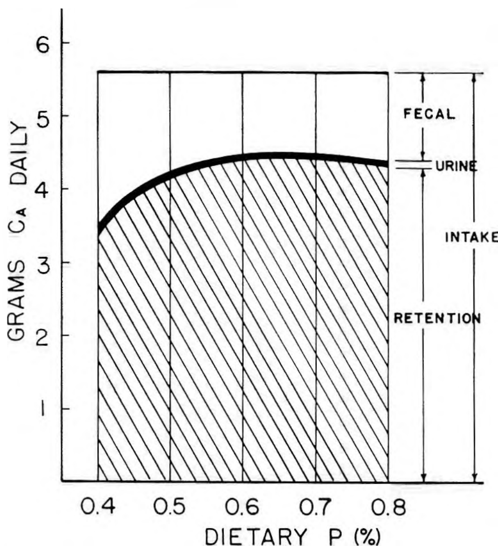


Fig. 4 Calcium balance as affected by dietary phosphorus level.

that the dietary phosphorus level for optimal utilization of calcium and phosphorus is about 0.5%.

LITERATURE CITED

1. Ludvigsen, J., and G. Thorbek 1960 Studier over pattegrises raekst of ernaering. II. Tilraekst og foderforbrug samt kvaelstof-, fosfor- og kalciumomsaetningen hos kunsfigt ernaerda pattergrise i aideren fra 5. til 35. dag. Beretning fra Forsøgslaboratoriet 320, København.
2. Freese, H. H. 1958 Undersuchungen über den Calcium-, Phosphor-, Magnesium- und Stickstoff-umsatz des Ferkels in Beziehung zum Wachstum bei Muttermilch und Muttermilchersatz. Arch. Tierernährung, *8:* 330.
3. Lenkeit, W., and H. H. Freese 1958 Zum Wachstum bei Muttermilch und Muttermilchersatz. Ztschr. Tierernährung u. Futtermittelk, *13:* 243.
4. Whiting, F., and L. M. Bezeau 1958 The calcium, phosphorus and zinc balance in pigs as influenced by the weight of pig and the level of calcium, zinc and vitamin D in the ration. Canad. J. Animal Sci., *38:* 109.
5. Hansard, S. L., W. A. Lyke and H. M. Crowder 1961 Absorption, excretion and utilization of calcium by swine. J. Animal Sci., *20:* 292.
6. Miller, E. R., D. E. Ullrey, C. L. Zutaut, B. V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1962 Calcium requirement of the baby pig. J. Nutrition, *77:* 7.
7. Miller, E. R., D. E. Ullrey, C. L. Zutaut, B. V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1963 Phosphorus requirement of the baby pig. J. Nutrition, *82:* 34.
8. Varley, H. 1960 Practical Clinical Chemistry. Interscience Publishers, Inc., New York.
9. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
10. Hayami, H., Y. Matsuno, K. Mori, M. Suzki and S. Saito 1955 The utilization of calcium from various sources. VIII. A comparison of calcium sulfate, carbonate and phosphate. National Institute of Nutrition Annual Report (Tokyo), *1955:* 24. (Cited in Chem. Abstr., *51:* 16753.)
11. Hansard, S. L., H. M. Crowder and W. A. Lyke 1957 Biological availability of calcium in feeds for cattle. J. Animal Sci., *16:* 437.

Adaptation of the Chick to Dietary Energy Source[1]

W. E. DONALDSON

Department of Poultry Science, North Carolina Agricultural Experiment Station, Raleigh, North Carolina

ABSTRACT One-day-old chicks were maintained from 0 to 14 days with high-carbohydrate (CHO) or high-fat (fat) diets. The chicks were then given fatty acid or glucose labeled with C^{14} and placed in metabolism cages. Excreta and respiratory CO_2 were collected for 24 hours to determine C^{14} dose retention and $C^{14}O_2$ production. Production of $C^{14}O_2$ was used as an index of fatty acid or glucose oxidation. Fat-fed chicks oxidized fatty acid at a maximal rate regardless of age, dietary choline level or dietary fatty acid level. The CHO-fed chicks oxidized fatty acid maximally at one day of age, but the rate decreased with age. Diet had no effect on the oxidation of glucose-C^{14}. Feeding the fat diet to CHO-adapted chicks at the time of fatty acid-C^{14} administration resulted in an increase of fatty acid oxidation to levels comparable to fat adapted chicks. When glucose-C^{14} was administered, tissues of chicks fed the CHO diet contained approximately 3 times as much radioactivity in the fat-soluble fraction as did the tissues of chicks fed fat. The results are discussed in terms of growth response to dietary fat.

Rand et al. (1) obtained consistent growth responses with chicks by the eqi-caloric substitution of corn oil for glucose. The growth responses to corn oil were obtained under both ad libitum and equalized feeding techniques. Carcasses of chicks fed either corn oil or glucose had a similar protein content despite the differences in body weight. These workers suggested that the corn oil effect could have been exerted by increased efficiency of metabolizable energy utilization or protein utilization.

Tepperman et al.[2] observed that liver slices from fat-fed rats oxidize palmitic-1-C^{14} acid more rapidly than do slices from carbohydrate-fed rats. This result coupled with other observations (2) suggested a metabolic adaptation dependent upon dietary energy source. Shifts in fatty acid-oxidizing enzymes were postulated as a possible explanation of the observed differences.

Boell (3) stated that the avian embryo derives over 90% of its total caloric requirement from fatty acid oxidation.

The above results are compatible with the hypothesis that at hatching, the chick is adapted to a fatty acid-oxidizing type metabolism and that inclusion of large amounts of carbohydrates in the diet results in adaptation to a carbohydrate-oxidizing type metabolism and loss of fatty acid oxidizing ability. Such a hypothesis becomes attractive as a partial explanation of the consistent growth responses obtained by the addition of fat to chick diets when the ratio of metabolizable energy to other nutrients is held constant.

The experiments reported here were designed to test the above hypothesis.

PROCEDURE

Rearing procedures. Female White Rock chicks ranging in age from one-day-old to 14 days were used. The chicks from each experiment were maintained in electrically heated batteries with raised-wire floors. One-half of the chicks were fed a high-carbohydrate diet and the remaining half were fed a high-fat diet (table 1). In experiment 2, choline was inadvertently omitted from both diets.

At appropriate ages, 3 chicks fed each diet, selected according to body weight,[3] were administered a single oral dose of palmitic-1-C^{14} acid, linoleic-1-C^{14} acid, or uniformly labeled glucose-C^{14} (size, specific activity, and type of dose are listed under

Received for publication July 1, 1963.

[1] Contribution from the Poultry Science Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as paper no. 1653 of the Journal Series.

[2] Tepperman, J., H. M. Tepperman and M. P. Schulman 1955 Oxidation of palmitic acid-1-C^{14} by liver slices of fat and carbohydrate diet-adapted rats. Federation Proc., 14: 152 (abstract).

[3] The corn oil diet consistently gave approximately a 10% growth response over the glucose diet. Chicks of similar body weight were chosen from each dietary treatment so that differences in metabolic rate due to differences in body weight would not result.

TABLE 1

Composition of test diets [1]

	High-carbohydrate diet	High-fat diet
	%	%
Glucose [2]	51.70	—
Corn oil [3]	—	28.05
Soybean oil meal, 50% protein	37.94	47.24
Cellulose [4]	4.75	17.72
Trace mineralized salt	0.47	0.59
Dicalcium phosphate	3.70	4.61
Ground limestone	0.95	1.18
Manganese sulfate	0.02	0.03
DL-Methionine	0.28	0.35
Vitamin mixture (glucose carrier) [5]	0.19	0.23

[1] The calculated ratios of metabolizable energy to protein were constant for both diets.
[2] Cerelose, Corn Products Company, New York.
[3] Fatty acid composition was determined by gas-liquid chromatography as follows: (%) myristic, 1.08; palmitic, 9.18; stearic, 2.56; oleic, 29.38; linoleic, 55.90; and linolenic, 1.90. Mazola, Corn Products Company, New York.
[4] Solka Floc, Brown Company, Boston.
[5] Each 100 g of the vitamin mixture supplied: (milligrams) thiamine·HCl, 176; Ca pantothenate, 924; niacin, 2640; riboflavin, 286; pyridoxine·HCl, 286; biotin, 8.8; folic acid, 55; vitamin K, 39.6; vitamin E acetate, 1100; choline chloride, 6400; and vitamin B_{12}, 440 μg; vitamin A 550,000 IU; and vitamin D_3, 99,000 ICU.

results). The proper doses were placed in gelatin capsules and inserted into the crops of the chicks. Glucose was in water solution. Fatty acids were dissolved in benzene solution and were pipetted into capsules and the benzene removed under dry nitrogen. These chicks were then placed in glass metabolism cages. Respiratory CO_2 was collected for 24 hours by means of drawing air through the cages with a vacuum pump and bubbling it through 200 ml of 2.5 N NaOH for 1- and 7-day-old chicks and 200 ml of 5.0 N NaOH for 14-day-old chicks. Excreta were collected for 24 hours. Feed and water were available ad libitum during the experiments. The chicks were fed the same diets while in the metabolism cages as previously except that in experiment 4 the chicks were starved for 2 hours before being given the C^{14} dose after which the diets were reversed.

Chemical procedures. All radioassays were made using a liquid scintillation spectrometer.[4] All samples were assayed sufficiently long to reduce counting error to $< 1.25\%$. Background ranged between 55 and 65 count/min.

Total $C^{14}O_2$ activity of samples collected from each chick was assayed by the method of Harlan.[5]

Excreta samples from each age group in each experiment were pooled according to diet. The excreta were diluted to 250 ml with distilled water and mixed in a Waring Blendor for 5 minutes. Duplicate 0.2-ml samples were withdrawn and placed on 20-ml counting vials containing scintillator. The vials were prepared by filling with thixotropic gel powder[6] and adding 15 ml of scintillator solution. Scintillator solution was prepared by mixing 378 ml of absolute ethanol with 600 ml of toluene containing 0.4% PPO (2,5-diphenyloxazole) and 0.0015% dimethyl POPOP (1, 4-bis - (2,4-methyl-5-phenyloxazolyl) - benzene). The samples were counted and recounted after addition of toluene-C^{14} internal standard. This method gave similar results to a procedure in which the excreta were extracted with diethyl ether-ethanol-HCl solvent and the extract counted in toluene containing 0.4% PPO and 0.01% POPOP.

In experiment 3, the 14-day old chicks were killed after CO_2 collection by dislocating the neck. The carcasses from the 3 chicks on each treatment were pooled and passed through a meat grinder. The ground tissues were thoroughly mixed and 10-g aliquots were blended with 10 ml distilled water for 5 minutes in a Servall Blendor. Duplicate 500-mg samples were made up to 30 ml with distilled water and were extracted by the addition of 1 ml concentrated HCl and 20 ml diethyl ether. The samples were shaken for 5 minutes, 20 ml of 95% ethanol were added and shaking continued for another 5 minutes. The ether layer was withdrawn by pipette and the samples were washed with three 10-ml portions of ether. Twenty milliliters of ether were added and the samples again were shaken for 5 minutes. The ether was withdrawn and the samples washed as previously. The ether extracts and washings were pooled and evaporated under

[4] Tri-Carb, Packard Instrument Company, La Grange, Illinois.
[5] Harlan, J. W. 1961 Liquid scintillation counting of aqueous carbonate solutions. Atomlight, no. 18: 8–11. Published by New England Nuclear Corporation, Boston.
[6] Cab-O-Sil, Packard Instrument Company, La Grange, Illinois.

dry nitrogen. The residues were weighed and taken up in 15 ml of scintillator solution and radioassayed by the same method as the excreta samples. The water-ethanol layer was sampled and reduced to < 0.5 ml under dry nitrogen. These samples were radioassayed as above. Moisture determinations were made on the tissues by drying for 24 hours at 90°C.

RESULTS AND DISCUSSION

The first experiment was designed to measure the influence of dietary energy source on fatty acid oxidation. From the working hypothesis, a relatively high rate of fatty acid oxidation was assumed in newly hatched chicks. Furthermore, the oxidation rate presumably would be maintained with fat feeding and decrease with carbohydrate feeding. The results, shown in table 2, support the hypothesis. Although the standard errors of the mean $C^{14}O_2$ production were high, at 14 days of age, the differences in oxidation rate between carbohydrate- and fat-fed chicks was statistically significant ($P = 0.01$).

Table 3 shows the recovery of $C^{14}O_2$ from one-day-old chicks in experiment 1 as a function of time. These data indicate that the differences in $C^{14}O_2$ recovery are a reflection of differing oxidation rates and not of a time lag in absorption of the labeled fatty acid.

The second experiment was designed to determine whether the oxidation rate of a fatty acid is related to the level of that fatty acid in the diet. The results are shown in table 4. The pattern of $C^{14}O_2$ recovery was similar when either labeled palmitic or linoleic acids were fed. The total amount of linoleic acid oxidized was greater than the total palmitic acid oxidation in the fat-fed chicks (assuming that the proportions of labeled to unlabeled fatty acids oxidized were constant) since the corn oil contained more linoleic than palmitic (55.9% vs. 9.2%). The data suggest that the chick oxidizes fat per se and that the proportion of a fatty acid that is oxidized is not affected by the level of that fatty acid in the dietary fat. The omission of added choline chloride from both diets in experiment 2 did not result in markedly decreased $C^{14}O_2$ production from the labeled fatty acid as compared to experiment 1. The calculated levels of choline (1100 and 1320 mg/kg in the carbohydrate and fat diets, respectively) appeared to be adequate for normal fatty acid oxidation.

Experiment 3 was designed to determine whether dietary energy source affected glucose oxidation. The working hypothesis suggested that glucose oxidation would be minimal in a newly hatched chick and that the minimal oxidation level would be maintained relatively constant with age when fat was fed. Conversely, carbohydrate feeding would result in increased glucose oxidation with age. The results are shown in table 5. In contrast to expectation, $C^{14}O_2$ recovery was relatively constant regardless of diet.

The calculated percentage of the total calories contributed by protein, carbohydrate and fat to the experimental diets are shown in table 6. Comparison of these calculations with the observed oxidation rates of labeled fatty acid or glucose shows that the total quantities of fatty acid oxidized were greater with the fat diet, and

TABLE 2

Effect of diet on palmitic-1-C^{14} acid oxidation by chicks (exp. 1)

Diet	Age [1]	C^{14} dosage [2]	Avg body wt	Apparent C^{14} retention	$C^{14}O_2$ produced [3]
	days	*μc/chick*	*g*	%	%
CHO	1	4	43	93.6	42.2 ± 6.60
Fat	1	4	43	92.9	51.7 ± 8.32
CHO	7	3	72	95.0	32.8 ± 9.05
Fat	7	3	72	96.7	48.8 ± 4.80
CHO	14	4	135	83.1	24.3 ± 5.93
Fat	14	4	135	97.5	54.5 ± 1.58

[1] Three chicks per treatment.
[2] Palmitic-1-C^{14} acid, specific activity 10 mc/mmole.
[3] Mean values of % absorbed dose recovered ± SE.

conversely the total quantities of glucose oxidized were greater on the carbohydrate diet. Total calories oxidized were calculated by applying oxidation rates for 14-day-old chicks from experiments 1 and 3 to the calorie values for the diets. These calculations are also shown in table 6. It was assumed that the protein calories oxidized were similar for both diets. Rand et al. (1) observed a 5% difference in nitrogen retention between glucose- and corn oil-fed chicks. This 5% difference would result in a negligible difference between diets in protein calories oxidized. These calculations suggested that if carbohydrate-fed chicks were suddenly shifted to the fat diet, the level of fatty acid oxidation would have to increase or else metabolic rate would decrease.

TABLE 3

Effect of diet on palmitic-1-C^{14} acid oxidation by chicks at various time intervals after C^{14} administration (exp. 1)

Diet	Hours after C^{14} administration [1]	$C^{14}O_2$ produced [2]
CHO	2	12.6
	4	22.0
	6	27.2
	24	42.2
Fat	2	12.1
	4	26.9
	6	35.8
	24	51.7

[1] Three day-old chicks per treatment. See table 2 for details of treatment.

[2] Cumulative mean values of % absorbed dose recovered.

In experiment 4 chicks maintained with a high-carbohydrate diet were shifted to a high-fat diet immediately after labeled fatty acid administration at 14 days of age. The data in table 7 show clearly that diet reversal results in reversal of $C^{14}O_2$ production (viz., level of $C^{14}O_2$ production from labeled fatty acid is a function of diet during CO_2 collection and not prior dietary experience). It is concluded that 1) the chick is able to adjust its metabolism to accommodate radical changes in dietary energy source in relatively short periods of time, and 2) any shift in glucose or fatty acid oxidizing enzyme levels due to feeding is not great enough to appreciably affect oxidation rate.

TABLE 4

Effect of diet on palmitic-1-C^{14} acid and linoleic-1-C^{14} acid oxidation by chicks (exp. 2)

Diet	Label	Age [1]	C^{14} dosage [2]	Avg body wt	Apparent C^{14} retention	$C^{14}O_2$ produced [3]
		days	*μc/chick*	*g*	%	%
CHO	palmitic	13	4	127	87.5	24.4 ± 4.70
Fat	palmitic	13	4	127	90.7	50.0 ± 5.76
CHO	linoleic	14	4	129	90.9	17.6 ± 3.22
Fat	linoleic	14	4	133	93.9	44.2 ± 0.57

[1] Three chicks per treatment.

[2] Palmitic-1-C^{14} acid and linoleic-1-C^{14} acid, specific activities 5 mc/mmole.

[3] Mean values of % absorbed dose recovered ± SE.

TABLE 5

Effect of diet on uniformly labeled D-glucose-C^{14} oxidation by chicks (exp. 3)

Diet	Age [1]	C^{14} dosage [2]	Avg body wt	Apparent C^{14} retention	$C^{14}O_2$ produced [3]
	days	*μc/chick*	*g*	%	%
CHO	1	1	39	95.4	46.9 ± 0.57
Fat	1	1	39	94.3	45.6 ± 5.89
CHO	7	2	73	97.3	45.7 ± 1.47
Fat	7	2	73	94.1	49.5 ± 1.78
CHO	14	2	138	93.5	54.1 ± 1.73
Fat	14	2	137	87.5	56.9 ± 3.22

[1] Three chicks per treatment.

[2] Uniformly labeled D-glucose-C^{14}, specific activity 3.2 mc/mmole.

[3] Mean values of % absorbed dose recovered ± SE.

The data on glucose and fatty acid oxidation do not establish a metabolic basis for growth response of chicks to dietary fat. A possible metabolic basis is provided by carcass analysis data from experiment 3 (glucose-C^{14} administration). No statistical analysis was possible since pooled samples were used. Carcass moisture and fat content were slightly lower in the fat-fed chicks. The data show that the carbohydrate-fed chicks converted considerably more carbohydrate to fat than did the fat-fed birds. Based on calculated diet analysis, total carbohydrate-to-fat conversion was approximately 12 times higher per 100 g of feed consumed with the carbohydrate diet. Total energy available for tissue synthesis should be comparable for chicks fed both diets if we assume that energy requirements for maintenance and body activity are similar. This implies that since carbohydrate-fed chicks require a measureable amount of energy to synthesize tissue fat (whereas fat-fed chicks deposit dietary fatty acids per se), energy available for tissue protein synthesis is reduced, and growth is reduced. If body weight gain decreases in comparison with fat-fed chicks, total energy requirements also decrease. But, at any given body weight, total energy available for tissue protein synthesis could be reduced because of the requirement for tissue fat synthesis. For the above hypothesis to be true, nitrogen retention must be poorer with carbohydrate feeding. Biely and March (4) observed that dietary fat had no consistent effect on nitrogen retention in the chick. These workers used a balance technique in which they measured feed intake and collected feces and urine during successive 24-hour periods, but no marker was incor-

TABLE 6

Calculated caloric composition of and energy production from the experimental diets

	CHO diet	Fat diet
% of total calories from:		
Protein	21.9	22.1
CHO	77.6	18.2
Fat	0.5	59.7
Kilocalories oxidized/100 kcal consumed: [1]		
Protein	Constant (K)[2]	Constant (K)[2]
CHO	41.9	10.3
Fat	0.1	32.5
Total	42.0+K	42.8+K

[1] Based on $C^{14}O_2$ recovery from 14-day-old chicks from experiments 1 and 3.

[2] It was assumed that amino acid oxidation was similar on both diets.

TABLE 7

Effect of diet reversal during $C^{14}O_2$ collection on palmitic-1-C^{14} acid oxidation by chicks (exp. 4)[1]

Diet	Diet during $C^{14}O_2$ collection	Age [2]	C^{14} dosage [3]	Avg body wt	Apparent C^{14} retention	$C^{14}O_2$ produced [4]
		days	*μc/chick*	*g*	%	%
CHO	Fat	14	2	139	75.1	52.8 ± 1.82
Fat	CHO	14	2	140	90.6	40.0 ± 1.00

[1] Chicks were starved for 2 hours, administered the C^{14} dose and fed the opposite diet during CO_2 collection.

[2] Three chicks per treatment.

[3] Palmitic-1-C^{14} acid, specific activity 10 mc/mmole.

[4] Mean values of % absorbed dose recovered ± SE.

TABLE 8

Effect of diet on carcass composition and deposition of uniformly labeled D-glucose-C^{14} in chicks (exp. 3)[1]

Diet	Age	Avg body wt	Carcass moisture	Carcass fat	Absorbed dose in tissues	
					H_2O-soluble	Fat-soluble
	days	*g*	%	%	%	%
CHO	14	138	71.3	11.5	19.6	14.4
Fat	14	137	70.0	9.7	31.0	5.0

[1] All values based on pooled samples of 3 chicks.

porated in the feed. Rand et al. (1) showed that dietary fat gave consistent improvements in nitrogen retention. In these studies, carcass protein analyses were used to calculate nitrogen retention. The experiments cited are typical of numerous conflicting reports in the literature concerning dietary fat and nitrogen retention.

Tepperman and Tepperman (5) reviewed extensive data on carbohydrate oxidation and lipogenesis. The data suggest that when the liver is exposed to increased concentrations of glucose (such as with carbohydrate feeding) hexose monophosphate shunt (HMP) activity is increased. Increased HMP activity results in an increased supply of reduced triphosphopyridine nucleotide (TPNH). Since TPNH is required for reductive fatty acid synthesis, it is postulated that control of lipogenesis is regulated by TPNH availability. Glucose oxidation under conditions of fat feeding follows the gycolytic pathway, and HMP activity is reduced.

More recently, Bortz et al. (6) presented convincing evidence that the block in lipogenesis resulting from feeding fat is caused by a failure of the reaction acetyl coenzyme A to malonyl coenzyme A and not by reduced TPNH supply or availability.

Although there is question as to how feeding fat inhibits lipogenesis, there is no question that inhibition occurs. The results presented herein support this concept.

LITERATURE CITED

1. Rand, N. T., H. M. Scott and F. A. Kummerow 1958 Dietary fat in the nutrition of the growing chick. Poultry Sci., *37:* 1075.
2. Tepperman, H. M., and J. Tepperman 1955 Ketogenesis in rats on high carbohydrate and high fat diets. Am. J. Physiol., *180:* 511.
3. Boell, E. J. 1955 Energy exchange and enzyme development during embryogenesis. In Analysis of Development, eds., B. H. Willier, P.A. Weiss and V. Hamburger. W. B. Saunders Company, Philadelphia.
4 Biely, J., and B. March 1957 Fat studies in poultry. 7. Fat and nitrogen retention in chicks fed diets containing different levels of fat and protein. Poultry Sci., *36:* 1235.
5. Tepperman, J., and H. M. Tepperman 1960 Some effects of hormones on cells and cell constituents. Pharmacol. Rev., *12:* 301.
6. Bortz, W., S. Abraham and I. L. Chaikoff 1963 Localization of the block in lipogenesis resulting from feeding fat. J. Biol. Chem., *238:* 1266.

Effect of Calcium, Phosphorus and Manganese on the Intestinal Synthesis of Thiamine in Rats

S. K. MEGHAL AND M. C. NATH
University Department of Biochemistry, Amravati Road, Nagpur, India

ABSTRACT When rats were fed thiamine-inadequate diets containing different levels of calcium and phosphorus with or without manganese, the urinary and fecal excretion of thiamine increased in rats receiving a low level of calcium and phosphorus and supplementation of manganese to this diet resulted in more urinary and fecal thiamine excretion. Higher levels of calcium and phosphorus in the diets, with or without manganese, and diets without an added amount of these minerals decreased the urinary and fecal excretion of thiamine. The presence of manganese in the diets reduced the lactobacilli count in the cecum and increased the coliform count. Rats receiving low calcium and phosphorus diets with or without manganese stored more thiamine in the liver and cecum than did other groups, whereas no increase was noted in the thiamine content of the heart. This observation indicates that low dietary calcium and phosphorus, with or without manganese, enhanced thiamine synthesis by the intestinal flora. This thiamine might be ultimately available to rats after coprophagy.

A great deal of research has been carried out on the effect of carbohydrates, fats, proteins and antibiotics, etc., on the intestinal synthesis of thiamine, but little attention has been directed toward the influence of salts and minerals. The intestinal bacteria present in animals are coliforms, anaerobes and lactics. Of these 3 microbes, the coliform organisms have been shown to synthesize thiamine to the greatest extent (1). Recently we have reported that the intestinal flora and thiamine synthesis depend upon the dietary constituents (2, 3). Friedman (4) reported that when rats became rachitic, the number of acid-producing organisms diminished, whereas non-acid-producing organisms increased, and also that rachitic rats had alkaline feces. Eppright et al. (5) studied the influence of mineral salts on the intestinal flora and postulated from their experiment that a diet low in inorganic salts favored coliform flora.

The essentiality of dietary manganese for rats was demonstrated by Orent and McCollum (6) and Kemmerer et al. (7). Hill and Holtkamp (8) have recently reported that storage of thiamine in liver increased with supplementation of manganese and thiamine. Balakrishnan and De (9) have reported that a diet low in phosphorus and high in calcium enhanced thiamine synthesis in rats. The present paper presents the results of feeding different amounts of calcium and phosphorus with or without manganese on the intestinal flora and biosynthesis of thiamine.

EXPERIMENTAL

Weanling albino rats were used in this study. They were grouped uniformly with respect to sex, weight and litter, and were caged individually, with food and water given ad libitum. They were fed the thiamine-inadequate diet for 5 days. The thiamine-inadequate basal diet contained: (in per cent) vitamin-free casein (acid alcohol-extracted), 25; dextrin, 20; sucrose, 37; groundnut oil, 15; and salt mixture (10), 1.5. The following vitamins were added per kilogram of diet: (in milligrams) thiamine·HCl, 0.4; riboflavin, 7; pyridoxine·HCl, 5; Ca pantothenate, 40; niacin, 40; inositol, 200; biotin, 0.2; folic acid, 2; vitamin B_{12}, 0.1; 2-methyl naphthoquinone, 10; vitamin A acetate, 3; vitamin D, 0.04; and α-tocopheryl acetate, 50.

The salt mixture recommended by Krieger and Steenbock (10) was used because it is free from calcium and phosphorus. For this experiment manganese sulfate was also removed from this salt mixture. The required amount of calcium was added to each diet as calcium carbonate, and phosphorus as an equimolar mixture of monobasic and dibasic phos-

Received for publication September 9, 1963.

phates of potassium (11). The details of the amounts of calcium and phosphorus in the diets are given in table 1. When manganese was incorporated in the diet it was fed at a level of 0.014% manganese as manganese chloride.

Rats were divided into 10 groups, with 6 rats per group. The first 5 groups received diets A, B, C, D, and E without manganese, respectively, and the remaining groups received the same diets as described above, respectively, but supplemented with manganese. Since rats were kept in wire-bottom cages, they consumed their feces along with the diets and no care was taken to prevent coprophagy during the experiment. The experiment was carried out for 4 weeks. Urine and feces were collected the last 3 days of the week. Urinary thiamine was determined by the thiochrome procedure as modified by Mawson and Thompson (12), and fecal thiamine as described by the Association of Vitamin Chemists (13).

During the 4-week experiment, the weekly fecal coliform count was taken of 3 rats from each group. The feces of each rat were collected aseptically the last 3 days of the week on sterile filter paper sheets and the sheets were pressed together to remove as much moisture as possible and then the feces were transferred to weighed sterile test tubes. A uniform suspension of about 0.1 g of feces with 10 ml of saline (0.9% NaCl solution) was made under aseptic conditions. Further dilutions were made with the saline, and the coliform colonies in the feces were counted by the plate method, using Mackonkey's agar medium (14).

At the end of the fourth week, all animals were killed by a sharp blow on the head and the livers and hearts excised. Each liver and heart was weighed and frozen and the thiamine content of each sample was determined by the method of Kratzing and Slater (15). The ceca of 3 rats from each group were removed and their contents were collected immediately under aseptic conditions. The cecal contents of each rat were refrigerated at 4° C before the next rat was killed. The cecal contents obtained from 3 rats in each group were pooled, mixed and 0.1 g of the mixed sample was weighed aseptically. The weighed samples were suspended in 10 ml of sterile saline (0.9%) and serial dilutions were made in sterile saline. Plate counts of the numbers of organisms in the cecal contents were made with Mackonkey's agar media for coliform bacteria, with nutrient agar media for "total number of organisms," and the lactobacillus selection medium (16) for lactobacilli. All plates were incubated at 37° C for 72 hours before counts were made of the colonies.

The cecal contents of the remaining rats from each group were weighed and digested in a flask with a sufficient amount of 0.1 N HCl; samples were then prepared in the recommended manner and total thiamine was estimated by the method of Hennessey and Cerecedo (17).

RESULTS

The effect of different levels of calcium and phosphorus with or without manganese on the urinary and fecal excretion of thiamine is presented in table 2. The daily urinary and fecal excretion of thiamine increased in the group receiving low calcium and low phosphorus (diet D) without manganese. Manganese supplementation of the above diet also increased daily urinary and fecal excretion of thiamine

TABLE 1

Calcium and phosphorus composition of diets [1]

	Diets				
	A	B	C	D	E
Ca ($CaCO_3$), % of diet	0.42	0.10	0.42	0.10	—
P (KH_2PO_4)	0.22	0.22	0.05	0.05	—
P (K_2HPO_4)	0.10	0.10	0.025	0.025	—
P from casein [2]	0.22	0.22	0.22	0.22	0.22
Total P, % of diet	0.54	0.54	0.295	0.295	0.22

[1] These minerals were given along with the basal diet.
[2] About 0.86% P.

TABLE 2

Effect of different levels of calcium and phosphorus with and without supplementation of manganese on the urinary and fecal excretion of thiamine

Diet [1]	Urinary excretion in weeks				Fecal excretion in weeks			
	1	2	3	4	1	2	3	4
	µg/24 hr/rat		µg/24 hr/rat		µg/24 hr/rat		µg/24 hr/rat	
	Without Mn							
A	0.48 ± 0.03 [2]	0.42 ± 0.02	0.38 ± 0.03	0.29 ± 0.01	0.58 ± 0.05	0.56 ± 0.02	0.55 ± 0.02	0.54 ± 0.02
B	0.32 ± 0.03	0.32 ± 0.04	0.30 ± 0.02	0.33 ± 0.02	0.53 ± 0.03	0.52 ± 0.05	0.40 ± 0.04	0.40 ± 0.05
C	0.53 ± 0.07	0.58 ± 0.03	0.75 ± 0.06	1.02 ± 0.05	0.92 ± 0.05	1.08 ± 0.07	1.16 ± 0.07	1.18 ± 0.06
D	0.59 ± 0.02	0.82 ± 0.03	1.23 ± 0.04	1.48 ± 0.05	1.17 ± 0.05	1.32 ± 0.05	1.88 ± 0.07	1.82 ± 0.08
E	0.28 ± 0.02	0.25 ± 0.02	0.22 ± 0.01	0.22 ± 0.03	0.36 ± 0.02	0.35 ± 0.05	0.28 ± 0.04	0.30 ± 0.05
	With Mn							
A	0.48 ± 0.03	0.48 ± 0.02	0.46 ± 0.04	0.39 ± 0.05	0.58 ± 0.04	0.62 ± 0.06	0.78 ± 0.04	0.78 ± 0.03
B	0.41 ± 0.03	0.47 ± 0.05	0.41 ± 0.07	0.40 ± 0.04	0.62 ± 0.04	0.62 ± 0.08	0.64 ± 0.08	0.68 ± 0.06
C	0.82 ± 0.03	0.87 ± 0.04	0.96 ± 0.05	1.08 ± 0.06	1.06 ± 0.03	1.22 ± 0.04	1.38 ± 0.03	1.42 ± 0.05
D	0.92 ± 0.04	1.07 ± 0.03	1.32 ± 0.06	1.42 ± 0.05	1.40 ± 0.03	1.66 ± 0.08	1.88 ± 0.10	2.08 ± 0.09
E	0.28 ± 0.02	0.28 ± 0.02	0.23 ± 0.04	0.25 ± 0.03	0.48 ± 0.05	0.40 ± 0.05	0.40 ± 0.06	0.38 ± 0.04

[1] Diet A, high Ca + high P; diet B, low Ca + high P; diet C, high Ca + low P; diet D, low Ca + low P; and diet E, very low P without Ca.
[2] Mean ± SD.

throughout the experiment. The diet without calcium and manganese (diet E) had an inhibitory effect on the urinary and fecal excretion. This indicates that low calcium and low phosphorus in the thiamine-inadequate diet had an enhancing effect on the intestinal synthesis of thiamine and this effect was more pronounced when manganese was added to the above diet. However, higher levels of calcium and phosphorus (diet A) decreased the urinary and fecal excretion of thiamine throughout the experiment and even supplementation of manganese to this diet did not have any favorable effect.

In table 3 data are presented showing the effect of different levels of calcium and phosphorus in the thiamine-inadequate diet either deficient or sufficient in manganese on the weekly fecal coliform count of rats. The analysis of variance shows that these data of fecal coliform count are statistically significant at 5% level (18). The low levels of calcium and phosphorus with or without manganese appear to have a favorable effect on the fecal coliform organisms of the rats. The coliform organisms were reduced in rats receiving the thiamine-inadequate diet without calcium even though addition of manganese to this diet showed a slight increase. This definitely leads to the conclusion that manganese had some favorable effect on the intestinal flora. Diet A (optimal calcium and phosphorus) and diet B (low calcium and optimal phosphorus) with or without manganese had no effect on the fecal coliform organisms; but a slight effect was shown with diet C (optimal calcium and low phosphorus).

Diet D (low Ca + low P) increased the tissue thiamine storage (table 4); supplementation of this diet with manganese improved the thiamine content of liver and cecum but had no effect on heart thiamine which was the same in all groups. The hepatic storage of the rats receiving low calcium and low phosphorus with or without manganese was greater ($P < 0.01$) than that of those receiving diet A and it was significantly reduced in those receiving diet E ($P < 0.01$).

The cecal contents and the coliform count are increased in those rats receiving diet D with or without manganese. Our

TABLE 3

Effect of different levels of calcium and phosphorus with or without manganese on the weekly fecal coliform count

Diet [1]	Coliform counts in weeks							
	Without Mn				With Mn			
	1	2	3	4	1	2	3	4
A	4.91 [2]	4.94	4.93	4.85	4.94	5.01	5.03	5.03
B	4.89	4.88	4.87	4.94	4.99	4.91	5.03	4.97
C	5.00	5.07	5.08	5.10	5.12	5.14	5.17	5.16
D	5.00	5.11	5.19	5.23	5.25	5.26	5.26	5.27
E	4.68	4.50	4.44	4.44	4.66	4.57	4.51	4.44

[1] Diet A, high Ca + high P; diet B, low Ca + high P; diet C, high Ca + low P; diet D, low Ca + low P; and diet E, very low P without Ca.
[2] Average $\log_{10}$ number of bacteria per gram of wet feces.

TABLE 4

Liver, heart and cecal thiamine and cecal flora as influenced by calcium, phosphorus and manganese

Diet [1]	Liver thiamine [2]	Heart thiamine [2]	Cecal thiamine [2]	Total cecal contents	Log_{10} number of bacteria/g wet cecal contents		
					Coliform	Total count	Lacto-bacilli
	$\mu g/g$	$\mu g/g$	$\mu g/g$	g			
			Without supplementation of Mn				
A	1.32 ± 0.08 [3]	1.20 ± 0.02	1.25 ± 0.04	2.02	4.65	5.07	4.91
B	1.08 ± 0.03	1.08 ± 0.04	1.01 ± 0.03	1.66	4.34	4.90	4.78
C	1.60 ± 0.06	1.09 ± 0.07	1.32 ± 0.04	2.10	4.92	5.23	4.91
D	2.28 ± 0.08 [4]	1.22 ± 0.06	1.89 ± 0.06	3.12	5.14	5.39	4.76
E	0.88 ± 0.02 [5]	0.98 ± 0.03	0.98 ± 0.02	1.40	4.07	4.69	5.45
			With supplementation of Mn				
A	1.65 ± 0.05	1.21 ± 0.06	1.58 ± 0.03	2.16	4.71	5.14	3.93
B	1.05 ± 0.03	1.12 ± 0.03	1.51 ± 0.02	1.74	4.36	4.95	4.04
C	2.07 ± 0.07	1.18 ± 0.02	1.52 ± 0.03	2.80	4.96	5.30	4.50
D	3.29 ± 0.06 [4]	1.28 ± 0.03	2.37 ± 0.09	3.95	5.44	5.44	4.50
E	0.92 ± 0.03 [5]	1.19 ± 0.05	0.93 ± 0.03	1.52	4.07	4.71	4.35

[1] Diet A, high Ca + high P; diet B, low Ca + high P; diet C, high Ca + low P; diet D, low Ca + low P; diet E, very low P without Ca.
[2] On wet-weight basis.
[3] SE of mean.
[4] Significantly increased from diet A at $P < 0.01$.
[5] Significantly reduced from diet A at $P < 0.01$.

results also indicate that those fed diets without manganese showed higher lactobacillus counts than those receiving manganese. This indicates that manganese might have an inhibitory effect on lactobacilli and might be favorable for coliform organisms.

The growth data (figs. 1, 2) show that diet D without manganese increased the body weight of rats and supplementation of manganese to this diet had more pronounced effect on the body weight of rats. However, the diet with low phosphorus resulted in a decrease in body weight. The statistical analysis shows that these growth data are significant at 5% level.

DISCUSSION

Inclusion of the Osborne and Mendel salt mixture by Eppright et al. (5) in the diet of rats produced an intestinal flora predominating lactobacilli and it has been further reported that, when calcium and phosphorus were added to a low salt diet, the flora was high in lactobacilli. Our results confirm that low calcium and low phosphorus increased the coliform count and decreased the lactobacilli. Recent work of Balakrishnan and De (9) shows that high calcium and low phosphorus increased the urinary and fecal excretion of thiamine in the presence of dietary manganese. Our results, however, indicate

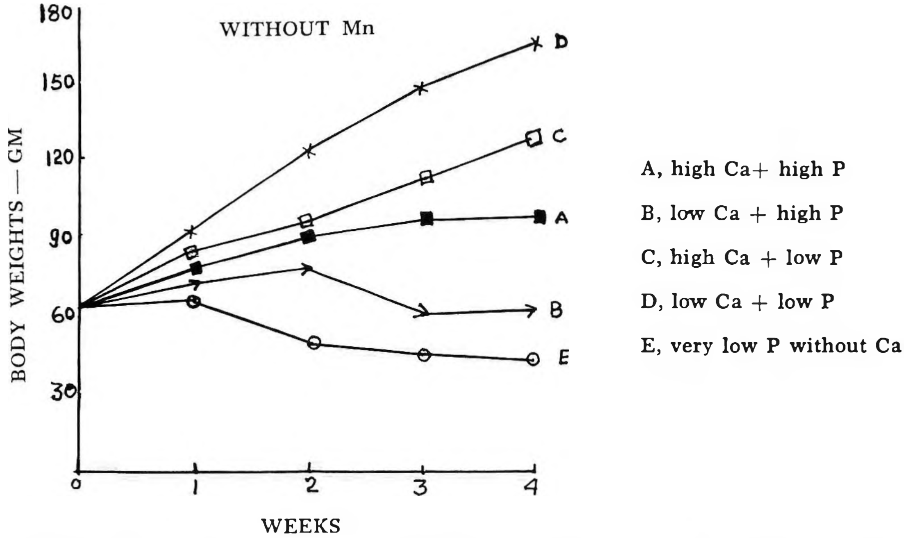


Fig. 1 Effect of different levels of calcium and phosphorus without manganese in thiamine-inadequate diet on weekly growth of rats.

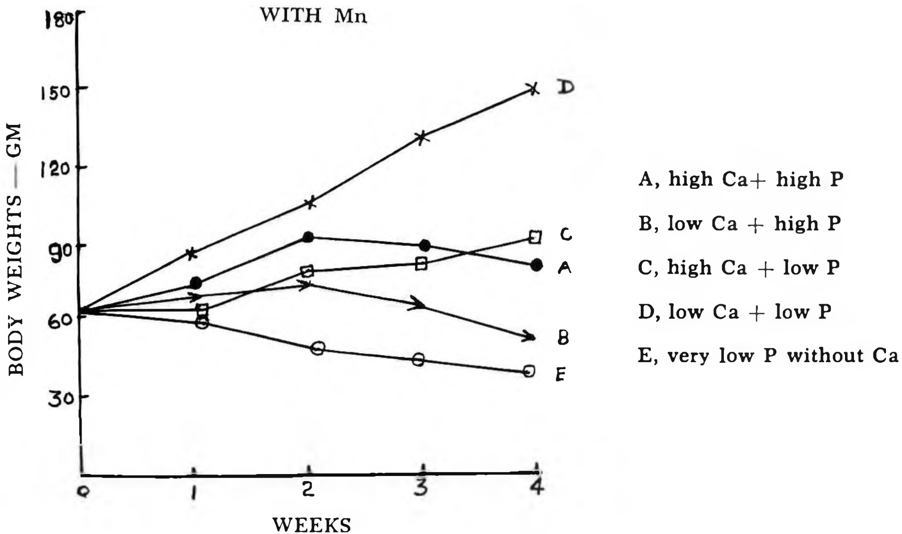


Fig. 2 Effect of different levels of calcium and phosphorus with manganese in thiamine-inadequate diet on weekly growth of rats.

that more thiamine was excreted in urine and feces with a diet low in calcium and phosphorus even though manganese was absent from the diet. Supplementation of manganese to the above diet increased the rate of thiamine excretion in urine and feces. Reen and Pearson (19) reported that during manganese deficiency there was no significant alteration in the vitamin content (niacin, riboflavin and pantothenic acid) of livers of duck. Our results, with rats, however, indicate that the manganese increases the thiamine storage in the liver of rats and reduces cecal lactobacilli. Hill and Holtkamp (8) have shown that supplementation of the diet to an intake of 0.17 mg of manganese/day, increased the average thiamine content of

liver and, furthermore, an increase of the manganese intake did not affect the thiamine storage. The metabolic interrelationship between thiamine and manganese was first reported by Perla et al. (20), but from our results it is possible that this effect of manganese may be attributed to the change in the intestinal flora brought about by its presence in the diet. The same reasoning applies to the effect of calcium and phosphorus which have been reported to affect the intestinal flora. It is also well established that the intestinal flora is known to synthesize thiamine.

The results of our present investigations indicate that low calcium and low phosphorus with or without manganese in the thiamine-inadequate diet enhances more thiamine synthesis than high calcium and low phosphorus in the diet as reported by Balakrishnan and De (9). It has also been observed in this laboratory that coprophagy is essential in order to make available flora-synthesized thiamine and to promote synthesis of thiamine in rats (21).

LITERATURE CITED

1. Najjar, V. A., and R. Barrett 1945 The synthesis of B vitamins by intestinal bacteria. Vitamins and Hormones, *3:* 23.
2. Nath, M. C., and S. K. Meghal 1961 Effect of carbohydrates on the intestinal synthesis of thiamine in rats. Biochem. J., *81:* 220.
3. Meghal, S. K., and M. C. Nath 1962 Inhibitory effect of sulfaguanidine on the biosynthesis of thiamine in rats and the beneficial role of penicillin and hydrolyzed glucose cycloacetoacetate. J. Nutrition, *76:* 265.
4. Friedman, H. 1936 Study of the fecal flora and lime test of normal rats, rachitic rats and healing rachitic rats. J. Nutrition, *12:* 165.
5. Eppright, E. S., G. Valley and A. H. Smith 1937 Influence of the salt in the diet on the intestinal flora of the albino rat. J. Bact., *34:* 81.
6. Orent, E., and E. V. McCollum 1931 Effect of deprivation of manganese in the rat. J. Biol. Chem., *92:* 651.
7. Kemmerer, A. R., C. A. Elvehjem and E. B. Hart 1931 Studies on the relation of manganese to the nutrition of mouse. J. Biol. Chem., *92:* 623.
8. Hill, R. M., and D. E. Holtkamp 1954 Storage of dietary manganese and thiamine in the rat. J. Nutrition, *53:* 73.
9. Balakrishnan, S., and S. S. De 1951 Effect of calcium and phosphorus on the intestinal synthesis of vitamin B_1. Indian J. Physiol. Allied Sci., *5:* 123.
10. Krieger, C. H., and H. Steenbock 1940 Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. J. Nutrition, *20:* 125.
11. Fuhr, I., and H. Steenbock 1943 Effect of dietary calcium, phosphorus and vitamin D on the utilization of iron. II. Effect of vitamin D on body iron and hemoglobin production. J. Biol. Chem., *147:* 65.
12. Mawson, E. H., and S. Y. Thompson 1948 A note on the estimation of vitamin B_1 in urine. Biochem. J., *43:* 2.
13. Association of Vitamin Chemists 1951 Methods of Vitamin Assay, ed. 2. Interscience Publishers, Inc., New York.
14. Balakrishnan, S., and R. Rajagopalan 1952 Studies on the influence of feeding milk and curd on the intestinal synthesis of vitamin B_1. Indian J. Physiol. Allied Sci., *6:* 143.
15. Kratzing, C. C., and E. C. Slater 1950 Effect of sulfonamides on the aneurine economy in animals. Biochem. J., *47:* 24.
16. Rogosa, M., A. J. Mitchell and R. F. Wiseman 1951 A selective medium for the isolation and enumeration of oral and fecal lactobacilli. J. Bact., *62:* 132.
17. Hennessey, D. J., and L. R. Cerecedo 1939 The determination of free and phosphorylated thiamine by a modified thiochrome assay. J. Am. Chem. Soc., *61:* 179.
18. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames.
19. Reen, R. V., and B. Pearson 1955 Manganese deficiency in the duck. J. Nutrition, *55:* 225.
20. Perla, D., M. Sandberg and O. M. Holly 1939 Interdependence of vitamin B_1 and manganese. III. Manganese, copper and iron metabolism in normal rats. Proc. Soc. Exp. Biol. Med., *42:* 371.
21. Meghal, S. K., and M. C. Nath 1963 Effect of prevention of coprophagy on the thiamine sparing action of mustard. Nature (Lond.), *198:* 89.

Effect of the Quality of Dietary Protein on Nitrogen Compounds in the Urine of Rats

SHUHACHI KIRIYAMA[1] AND KIYOSHI ASHIDA
Laboratory of Food and Nutrition, Department of Agricultural Chemistry, Nagoya University, Anjo, Aichi, Japan

ABSTRACT To learn in more detail the effect of dietary protein quality on the ratio of urinary N compounds of rats, the excretion rate of urea, allantoin, creatinine and their partition were determined when the rats of 4 ages (21-, 27-, 61- and 158-day-old) were fed each diet containing casein and wheat gluten at the 14% level. The nutritive value of both proteins was simultaneously compared using the usual methods for biological value, net protein utilization, net protein ratio and the ratio of creatinine N to total urinary N. In general, urea excretion increased with the decrease of protein quality and increased with protein intake or age, or both. Although allantoin excretion was more complex and less variable than urea excretion, that of the casein groups was consistently higher than that of gluten groups except in the case of the 158-day-old rats. In using the ratios between urinary N compounds to describe nutritive value of dietary protein, the values for (allantoin/urea) $\times$ protein intake were comparable to that of other criteria: biological value or net protein ratio, although the effect of age and dietary protein quality on allantoin excretion needs further investigation.

It is well known that Folin (1) investigated indirectly the metabolism of body protein by analyzing the urinary N compounds. Relatively few studies on the relationship between urinary N constituents and dietary protein quality have been reported. Two of these are the pioneering investigations of Murlin et al. (2, 3) in which protein quality was expressed by the percentage of creatinine N in total urinary N.

The change in either the quantity or quality, or of both, of the ingested protein causes alteration of body protein metabolism which influences the relative excretion rates of catabolic end products of protein and of other materials related to protein metabolism (mainly N compounds). If some regularity is found in the interrelationship between protein quality and urinary N compounds, it will become possible by the analysis of urinary N compounds to determine the quality of dietary protein. Murlin's investigations were also along this line.

In a preceding report (4), the authors determined urinary urea, allantoin, and creatinine, etc., when rats were fed a diet containing at various levels, egg albumin which is one of the proteins with the "highest" biological value, and gelatin which is one of the "lowest" as protein source. This study showed great variations in urea and allantoin excretion.

The present paper deals with a survey of the change of N compounds in urine of rats fed casein and gluten as the protein source at 4 defined stages of age, and with a comparison between prevailing methods to evaluate protein quality biologically.

METHODS AND MATERIALS

Sixty Wistar-strain male rats were used throughout this study. Four separate experiments were carried out using rats of 4 ages, 21, 27, 61, and 158 days old, respectively.

In each experiment, 15 rats were selected from standardized animals that had been fed a "standard" 25% casein diet for one week. They were then divided into 3 subgroups, and started to be fed the test diets A, B, and C as shown in table 1. All diets were fed ad libitum. Immediately before daily feeding, the synthetic test diets were mixed with water and made into a soft ball. The animals older than 27 days of age had been maintained be-

Received for publication August 1, 1963.

[1] Present address: The National Institute of Nutrition, 1 Toyamacho, Shinjukuku, Tokyo, Japan.

TABLE 1

Composition of "standard" and test diets

Constituents	"Standard" diet	Test diets [1]		
		A	B	C
	%	%	%	%
α-Starch [2]	65	89	75	75
Casein	25	—	14	—
Gluten	—	—	—	14
Sesame oil	5	5	5	5
Salts-B [3]	4	4	4	4
Vitamins [4]	1	1	1	1
Feces' marker (Cr_2O_3 or SiO_2)	—	1	1	1
Total	100	100	100	100
Average protein content (N × 6.25)	21.0	0	11.5	11.9

[1] The test diets for 21- and 158-day-old rats contained one per cent more of α-starch, substituting feces' marker.
[2] This is an instant starch or pregelatinized starch made from potato-β-starch.
[3] Harper (15).
[4] This mixture was identical with Harper's (15) except for omission of ascorbic acid and α-tocopherol.

fore standardization with the stock diet used in our laboratory.

Urine was collected from the animals while they were maintained in individual metabolic cages. The first urine collection period was for 3 days after 3 days' feeding of test diets, and the second collection period, for another 3 days after one day's interval. As a preservative for the urine, 10 ml of N sulfuric acid were placed in each collection flask at the beginning of the collection period.

At the end of both urine collection periods the rats were removed from metabolic cages. The cages, screens, and the funnels which were fitted with filter paper, were washed down with hot distilled water, the washings being combined with the collected urine.

The pooled 3-day urine samples obtained from each rat were diluted up to 250 ml with distilled water and a portion of this diluted sample was preserved with a small amount of chloroform and stored in deep-freeze ready for analysis. Chromium oxide, Cr_2O_3 was used to mark feces. The urine samples were analyzed in duplicate for allantoin, urea and creatinine by colorimetry using Kotaki's electrophotometer at 500, 430, and 530 mμ, and the methods described by Young and Conway (5), Engel and Engel (6) and Clark and Thompson (7), respectively.

Nitrogen determination was carried out by the Kjeldahl method.

These experiments were performed during the period extending from October, 1959, to March, 1960.

Environmental temperature ranged from 20 to 25°C under air conditioning.

The biological value was determined by the method of Njaa (8), and the net protein utilization (NPU) was calculated by multiplying digestibility by the biological value.

Net protein ratio (NPR), which is a modification of protein efficiency ratio proposed by Bender and Doell (9), was calculated from the difference between the gains in weight of the test and non-protein groups divided by the weight of ingested protein during 10 days of feeding.

RESULTS AND DISCUSSION

The daily N intake of the 61-day-old rats was the highest and was approximately 1.5 times higher than that of the 21- and 27-day-old rats. In each experiment, nitrogen intakes of the casein and gluten groups were about the same except at the second period for the 21- and 158-day-old rats, as shown in figure 1.

The digestibility of the test proteins did not differ significantly among ages and individuals (table 2).

Each group showed its own characteristic growth curves (figs. 2–5). As expected, the casein groups grew more rapidly than the gluten groups, although in the case of the 158-day-old rats the

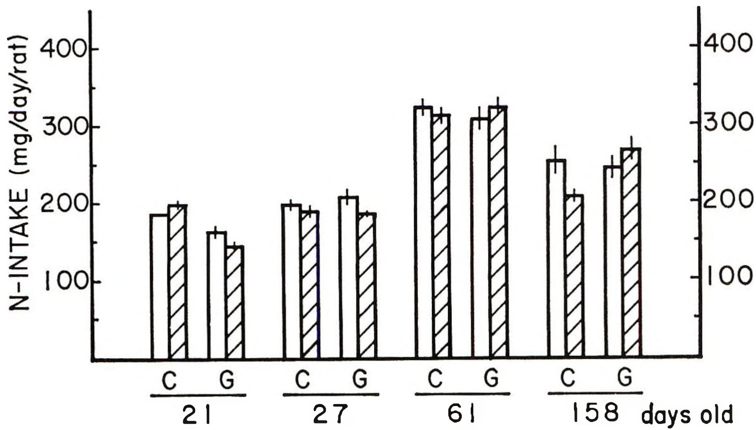


Fig. 1 Average nitrogen intake per day per rat at each stage of age, when rats were fed the casein (C) and wheat gluten (G) diet. White and shaded columns represent the value at the first and second periods. Vertical bars represent standard error of the mean.

TABLE 2

True digestibility[1] of casein and wheat gluten of rats at the various stages of age in each period

Diet	Period	Age in days			
		21	27	61	158
Casein	P-I	98.6 ± 0.37[2]	93.1 ± 1.59	94.1 ± 0.49	96.5 ± 0.93
	P-II	97.1 ± 0.38	93.4 ± 0.21	98.0 ± 0.87	95.5 ± 0.67
Gluten	P-I	95.1 ± 0.86	94.1 ± 0.91	97.2 ± 0.61	98.5 ± 0.54
	P-II	96.1 ± 1.14	92.9 ± 0.78	96.1 ± 1.09	92.7 ± 0.86

[1] $\frac{\text{N intake} - (\text{Fecal N in protein-fed rat} - \text{fecal N in nonprotein-fed rat})}{\text{N intake}} \times 100.$

[2] SE of mean.

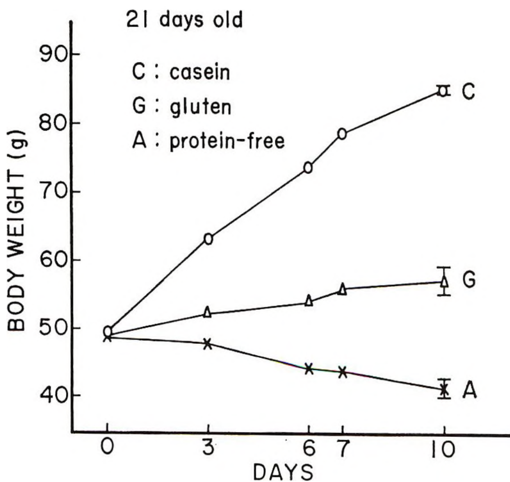


Fig. 2 Growth rate of 21-day-old rats fed test diets. ○——○, casein; △——△, wheat gluten; ×——×, protein-free groups. Vertical bars represent standard error of the mean.

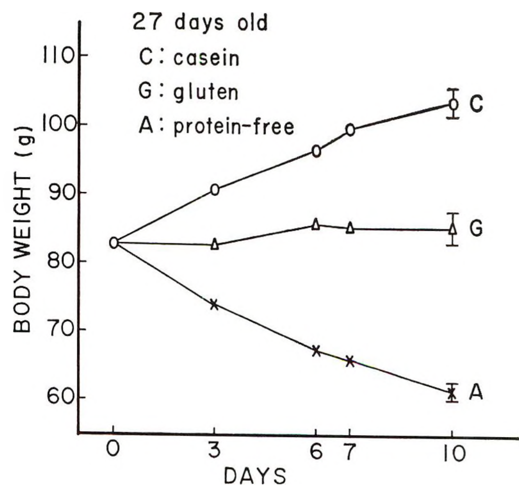


Fig. 3 Growth rate of 27-day-old rats fed test diets. ○——○, casein; △——△, wheat gluten; ×——×, protein-free groups. Vertical bars represent standard error of the mean.

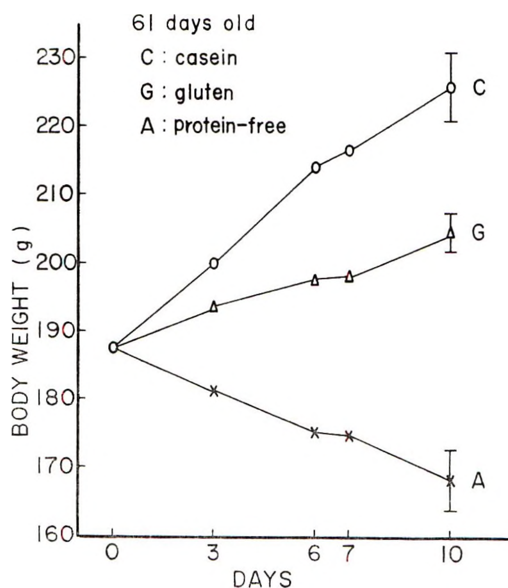


Fig. 4 Growth rate of 61-day-old rats fed test diets. ○——○, casein; △——△, wheat gluten; ×——×, protein-free groups. Vertical bars represent standard error of the mean.

growth curve of the former became stationary approaching the latter. These results are reasonable considering that the amino acid composition of casein is better balanced with respect to the requirement pattern of rats than that of gluten.

Urea excretion of the gluten groups was consistently higher than that of the casein groups (table 3), even though N intakes were almost the same (fig. 1). In growing animals, urea excretion increased in the second period except for that of the 21-day-old rats fed the gluten diet, whereas no difference was noted in the 158-day-old animals. Rats fed gluten consistently excreted a greater percentage of the urinary

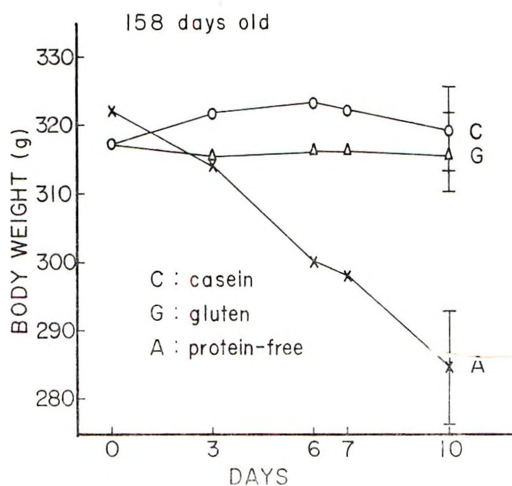


Fig. 5 Growth rate of 158-day-old rats fed test diets. ○——○, casein; △——△, wheat gluten; ×——×, protein-free groups. Vertical bars represent standard error of the mean.

TABLE 3

Average urea excretion and percentage of urea N in total urinary N at the various stages of age and periods when rats were fed casein, gluten and nonprotein diets

Age	Diet	Period P-I		Period P-II	
		Urea	Urea N in total urinary N	Urea	Urea N in total urinary N
days		*mg/rat/day*	%	*mg/rat/day*	%
21	Nonprotein	8.4 ± 1.3 [1]	33.8	7.1 ± 1.1	35.2
	Casein	93.4 ± 8.3	60.3	108 ± 3.2	62.6
	Gluten	180 ± 12.1	77.0	155 ± 8.9	76.3
27	Nonprotein	23.8 ± 3.7	53.9	16.4 ± 1.2	54.2
	Casein	118 ± 2.7	62.5	134 ± 5.4	73.0
	Gluten	150 ± 12.6	70.0	223 ± 3.4	77.6
61	Nonprotein	45.6 ± 4.0	47.7	29.5 ± 3.4	44.2
	Casein	202 ± 12.1	60.9	226 ± 13.8	68.2
	Gluten	342 ± 24.9	70.2	413 ± 17.9	77.9
158	Nonprotein	44.0 ± 7.0	45.3	46.7 ± 5.8	48.1
	Casein	217 ± 21.1	69.2	205 ± 21.6	68.3
	Gluten	313 ± 22.6	74.5	320 ± 24.0	75.7

[1] SE of mean.

N as urea than rats receiving casein irrespective of age or period of collection (table 3). In the nonprotein group, the percentage of urea N in total urinary N was significantly lower than that of both protein groups. This value was in a state of equilibrium in first period. This may have occurred because urea excretion ceases more rapidly than excretion of other N compounds immediately after the protein intake is suspended.

Allantoin excretion of the casein groups was higher than that of the gluten groups except in the 158-day-old rats (table 4), but the difference in allantoin excretion between the 2 groups was not as marked as the differences in urea excretion. Allantoin excretion of the casein groups increased along with growth up to the second period, but in the gluten groups it was almost the same in both periods except in the 27-day-old group. This might be considered to be due to the extremely slow growth rate of the gluten groups as compared with that of casein groups. The increments of allantoin excretion appear to be affected not only by absolute body weight, but also by growth rate of rats. The larger the animal the greater is, in general, its total allantoin excretion; but when allantoin excretion is computed per unit of body weight, the smaller the animal, the higher the value is (fig. 6).

TABLE 4

Average allantoin excretion of rats at the various stages of age and periods when fed casein, gluten and nonprotein diets

Period	Groups		
	Nonprotein	Casein	Gluten
	mg/day/rat	*mg/day/rat*	*mg/day/rat*
	21-day-old		
P-I	12.4 ± 0.5 [1]	20.0 ± 1.3	15.5 ± 0.6
P-II	7.9 ± 0.3	23.3 ± 1.3	13.5 ± 0.8
	27-day-old		
P-I	7.6 ± 1.5	14.7 ± 1.8	9.4 ± 1.4
P-II	8.4 ± 0.6	22.8 ± 1.9	19.9 ± 0.9
	61-day-old		
P-I	25.8 ± 1.4	37.9 ± 0.8	34.9 ± 0.7
P-II	20.8 ± 0.8	43.5 ± 1.2	33.0 ± 1.1
	158-day-old		
P-I	31.4 ± 1.7	32.2 ± 1.7	34.8 ± 1.2
P-II	28.7 ± 1.0	36.5 ± 1.9	34.4 ± 1.2

[1] SE of mean.

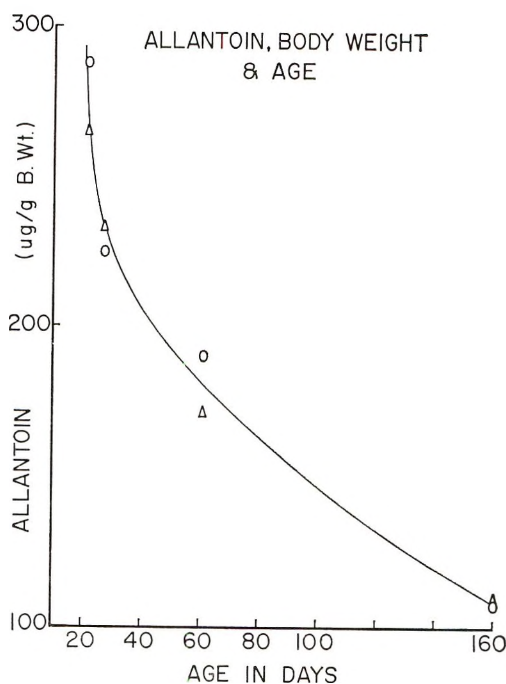


Fig. 6 The difference of allantoin excretion rate in various ages. The values are plotted in micrograms of allantoin per gram of body weight per day against each age. ○ and △: represent casein and gluten groups, respectively.

Actually, as reported in the previous studies (4), when rats were fed extremely low quality protein such as gelatin, allantoin excretion decreased. However, as shown above, allantoin excretion was affected rather by age than by dietary protein quality. Thus, allantoin excretion is complex, and the effect of age and dietary protein quality on allantoin excretion should be investigated further.

It was apparent that the changes of urinary N compounds obtained above were caused by the quality of the ingested protein; when the quality or quantity of dietary protein, or both, are unbalanced or in excess of the requirements of rats, it is considered that the portion of dietary protein utilized for body protein synthesis would be small, and urea excretion would become relatively high. On the other hand, in view of present knowledge of the intimate correlation of ribonucleic acid metabolism with protein synthesis, it would be expected that allantoin excretion would be increased with the increase of nutritive

value of ingested protein, because it appears probable that when protein synthesis actively increases, the increased excretion of allantoin as the catabolic end product of purine bases derived from nucleic acids might be attributed to the increased turnover rate of ribonucleic acids.

Indirect evidence for the above assumptions has been reported by Muramatsu and Ashida (10, 11), who showed that urea excretion increased almost linearly and allantoin excretion increased curvilinearly against the increment of dietary level or ingested amounts of casein. Also, Ashida and Harper (12), and Schimke (13) have reported a similar response of urea excretion in their studies on the biological regulation of protein metabolism.

We have attempted to determine the nutritive value of dietary protein by pursuing such variations as described above. Part of this research has been reported previously (4, 14). In these reports, the values of allantoin (A)/urea (U) ratio were greatly lowered with the increase of protein consumption, because urea excretion was primarily dominated by protein intake (I_p). Hence, to eliminate the effect due to variation of protein consumption within a group, (A/U) was multiplied by I_p. Subsequently, obtained values of $(A/U) \times I_p$ fell into a certain constant point within a group, and they were unbalanced only by protein quality. The values of $(A/U) \times I_p$ and also the values obtained by applying some of prevailing methods to estimate the nutritive value of protein are summarized in table 6.

At first glance, it appears that within a defined stage of age the $(A/U) \times I_p$ values of the casein group are constantly higher than that of the gluten group.

Only the biological value in the 27-day-old rats of the gluten group in the first period was significantly higher than the general trend. The biological value and net protein utilization are expressed on a percentage scale, whereas $(A/U) \times I_p$ and net protein ratio are expressed as a relative value. Therefore, direct comparison between these methods of measurement is impossible. To compare them, the relative expression (the value of gluten/the value of casein $\times$ 100) is given in the lower part of table 6. The relative values of $(A/U) \times I_p$ and NPR of 21-day-old rats were appreciably lower than those of older rats, but the relative values of biological value and NPU of 21-day-old rats were slightly higher than the values of both 27- and 61-day-old rats, especially in the second period. The values of NPR of both the casein and gluten groups successively approached each other as age progressed or physiological development proceeded.

The ratio of creatinine N to total N excreted is an expression developed by Murlin et al. (2, 3). Their studies are based on creatinine excretion not being affected by exogenous proteins or by other nutrients. The values obtained by this method are shown in table 5. The values for the casein are always higher than for gluten. And, there are general trends similar to biological value in the relative values. Because complete urine collection is not required in this method, C/N_t is simple and useful in the assessment of protein quality when the protein intake of various protein groups is equal because this value would greatly fluctuate by the change of I_p.

In case of the casein diet, the absolute $(A/U) \times I_p$ values for 27-day-old rats were lower than those of the 21-day-old group. This might be explained either by the rapidly decreased allantoin excretion with age (cf. fig. 6) or by the increased urea excretion per unit of I_p in 27-day-old rats. Decreased utilization of absorbed protein

TABLE 5

Ratios of creatinine excreted (mg) to total urinary nitrogen (mg), $(C/N_t) \times 100$, and relative values when rats were fed casein or wheat gluten diets

Age	Period	$(C/N_t) \times 100$		G/C × 100 [2]
		Casein	Gluten	
days				
21	P-I	2.89 ± 0.08 [1]	1.72 ± 0.08	59.5
	P-II	3.02 ± 0.06	2.02 ± 0.08	66.8
27	P-I	2.45 ± 0.08	2.00 ± 0.12	81.7
	P-II	2.59 ± 0.16	1.73 ± 0.05	66.8
61	P-I	4.02 ± 0.07	2.69 ± 0.20	66.9
	P-II	4.41 ± 0.16	2.56 ± 0.15	58.1
158	P-I	7.20 ± 0.48	5.71 ± 0.33	79.3
	P-II	7.91 ± 0.74	5.69 ± 0.39	71.9

[1] SE of mean.
[2] The ratio of the value for gluten to the value for casein (%).

TABLE 6

Comparison of methods evaluating protein quality when rats were fed casein and wheat gluten diets at each stage of age and period

Age	Diets	Biological value		NPU		$(A/U) \times I_p$		NPR (10 days)
		P-I[1]	P-II	P-I	P-II	P-I	P-II	
days								
				Absolute values				
21	Casein	68.1 ± 1.5[2]	63.1 ± 1.1	67.1 ± 1.5	61.3 ± 1.0	26.5 ± 2.8	26.8 ± 1.0	3.70 ± 0.06
	Gluten	38.4 ± 2.0	39.9 ± 1.6	36.5 ± 1.8	38.3 ± 1.3	8.9 ± 0.2	8.1 ± 0.4	1.48 ± 0.05
27	Casein	65.3 ± 1.5	59.5 ± 2.3	60.9 ± 2.5	55.6 ± 2.2	15.7 ± 2.2	20.0 ± 1.4	3.30 ± 0.12
	Gluten	58.4 ± 4.9	30.4 ± 0.7	55.0 ± 4.9	28.2 ± 0.7	8.1 ± 0.2	10.4 ± 0.5	1.90 ± 0.15
61	Casein	64.2 ± 0.9	60.1 ± 1.8	60.4 ± 0.9	58.9 ± 1.7	38.6 ± 1.5	38.2 ± 2.3	2.90 ± 0.09
	Gluten	38.8 ± 2.0	31.2 ± 1.7	37.8 ± 2.0	30.0 ± 1.5	19.7 ± 0.8	16.4 ± 0.5	1.80 ± 0.11
158	Casein	58.9 ± 1.8	52.2 ± 5.3	56.7 ± 2.2	50.0 ± 5.3	24.2 ± 3.1	23.4 ± 1.8	2.62 ± 0.22
	Gluten	38.3 ± 1.4	38.7 ± 2.6	37.8 ± 1.5	36.0 ± 2.7	17.2 ± 0.5	18.0 ± 0.7	2.34 ± 0.16
				Relative values				
21	G/C[3]	56.4	63.2	54.4	62.5	33.6	30.2	40.0
27	G/C	89.4	51.1	90.3	50.7	51.5	52.0	57.0
61	G/C	60.4	51.9	62.6	51.0	51.0	43.0	63.2
158	G/C	65.0	74.1	66.6	72.0	71.2	77.0	89.4

[1] Indicates period.
[2] SE of mean.
[3] The ratio of the value for gluten to the value for casein (%).

may be assumed from the growth curves. At this age, however, the relative values between the casein and gluten groups are comparable to the others. Higher absolute values in 61-day-old rats might be also explained by the higher growth rate of this group caused by higher food intake. In this case, both urea and allantoin excretion increased, but the increased urea excretion was not large enough to decrease the $(A/U) \times I_p$ value. The fact that relatively low absolute values of $(A/U) \times I_p$ were obtained in the 158-day-old rats (although they had the highest weight) might be due to extremely slow gain. Similar explanations also appear possible in the case of the gluten diet for 61-day-old rats although the situation is reversed.

We have not been able to decide whether a consideration of allantoin excretion was essential in the calculation of nutritive value. However, surveying the partition of nitrogenous constituents in urine would be pertinent and of great significance from the following point of view. All organisms take up their nutrients from their environments and excrete their wastes continually, and by these basal processes they can support life. As long as life is maintained by a balance of the above 2 basal processes, organisms acquire a fixed metabolic pattern corresponding to the nutrient intake. This "corresponding metabolic pattern" can be secured only by changing the ratios among the velocities of numerous reaction processes in the body. Therefore, the degree of inconsistence, in both quantity and quality of the nutrients ingested, between the nutrient pattern of food and requirement pattern of animals would be reflected in the velocity ratios of reaction processes in the body, and consequently, these fluctuations, especially of protein metabolism, would become detectable in urine with a certain ratio among various urinary nitrogenous end products.

Studies on the responses of urinary N partition to the graded incorporation of a single essential amino acid into amino acid mixtures are now in progress.

LITERATURE CITED

1. Folin, O. 1905 Laws governing the chemical composition of urine. Am. J. Physiol., *13:* 66.
2. Murlin, J. R., T. A. Szymanski and E. C. Nasset 1948 Creatinine nitrogen percentage as a check on the biological values of proteins. J. Nutrition, *36:* 171.
3. Murlin, J. R., A. D. Hayes and K. Johnson 1953 Correlation between the biological value of protein and the percentage of creatinine N in the urine. J. Nutrition, *51:* 149.
4. Kiriyama, S., and K. Ashida 1960 The relation between quantity and quality of dietary protein and nitrogen compounds in urine of rat. I. The ratio of the amount of allantoin to that of urea in urine of the rats fed egg albumin or gelatin as protein source. Seikagaku (J. Japanese Biochem. Soc.), *32:* 185.
5. Young, E. G., and C. F. Conway 1942 Estimation of allantoin by the Rimini-Schryver reaction. J. Biol. Chem., *142:* 839.
6. Engel, M. G., and F. L. Engel 1947 The colorimetric microdetermination of urea nitrogen by the xanthydrol method. J. Biol. Chem., *167:* 535.
7. Clark, L. C. Jr., and H. L. Thompson 1949 Determination of creatine and creatinine in urine. Anal. Chem., *21:* 1218.
8. Njaa, L. R. 1959 A shortened version of the Mitchell method for determination of the biological value of protein. Growth rate as a source of error. Brit. J. Nutrition, *13:* 137.
9. Bender, A. E., and B. H. Doell 1957 Biological evaluation of proteins: a new aspect. Brit. J. Nutrition, *11:* 140.
10. Muramatsu, K., and K. Ashida 1955 Composition of nutrients and metabolism of tissue protein. II. The effect of varying levels of dietary protein upon the enzyme activities and nitrogen metabolites. J. Agr. Chem. Soc. Japan, *29:* 725.
11. Muramatsu, K., and K. Ashida 1957 Composition of nutrients and metabolism of tissue protein. IV. Relation between rat liver xanthine oxidase, arginase activity and urinary nitrogen metabolites. J. Agr. Chem. Soc. Japan, *31:* 607.
12. Ashida, K., and A. E. Harper 1961 Metabolic adaptations in higher animals. VI. Liver arginase activity during adaptation to high protein diet. Proc. Soc. Exp. Biol. Med., *107:* 151.
13. Schimke, R. T. 1962 Adaptive characteristics of urea cycle enzymes in the rat. J. Biol. Chem., *237:* 459.
14. Kiriyama, S., and K. Ashida 1962 The relation between quantity and quality of dietary protein and nitrogen compounds in urine of rat. II. The values of (allantoin/urea) × protein intake of adult rats fed the diets incorporating egg albumin, casein or wheat gluten as protein source. Japanese J. Nutrition, *20:* 121.
15. Harper, A. E. 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, *68:* 405.

Pantothenic Acid Deficiency in Cats[1]

S. N. GERSHOFF AND L. S. GOTTLIEB
Department of Nutrition, Harvard School of Public Health, the Mallory Institute of Pathology, Boston City Hospital, and Tufts University School of Medicine, Boston, Massachusetts

ABSTRACT Cats were fed purified diets containing varying levels of calcium pantothenate. In pantothenic acid-deficient cats the most prominent changes were growth failure and histologic changes in the small intestines and the liver. The results of gross and histologic examinations, measurements of acetylation of urinary *p*-aminobenzoic and the ratio of urinary pantothenic acid to creatinine indicate that in this study, cats required approximately 5 mg of calcium pantothenate/kg of diet.

During the past 10 years, a program designed to determine the nutrient requirements of cats has been pursued in this laboratory. In the present paper, pantothenic acid deficiency in cats will be described and the minimal requirements for pantothenic acid of cats fed purified diets will be reported.

MATERIALS AND METHODS

In this work, kittens approximately 3 months old of mixed breed and sex were used. Before being admitted to the laboratory, the cats were dewormed, dusted against external parasites, and vaccinated against feline distemper. The animals were housed in individual cages and maintained with food and water ad libitum. The purified diet used consisted of: (in per cent) casein, 32.1; sucrose, 37.6; corn oil, 12.5; hydrogenated fat, 12.5; cod liver oil, 1.0; salts IV (1), 4; and choline, 0.3 and (in mg/kg diet) thiamine, 4; riboflavin, 8; pyridoxine, 4; niacin, 40; folic acid, 1; biotin, 0.2; menadione, 1; and vitamin B_{12}, 0.1. The levels of calcium pantothenate fed were 0, 1, 3, 5, 10 and 20 mg/kg of diet.

During the third experimental month, the cats were injected intraperitoneally with 6 mg of *p*-aminobenzoic acid/kg of body weight and acetylated and total urinary *p*-aminobenzoic acid were determined with the reagents and in the manner described for sulfanilamide by Bratton and Marshall (2). In the fourth experimental month, urinary creatinine (3) and pantothenic acid (4) estimates were made.

Throughout the study, hematological examinations were made and when the cats died or were killed, they were autopsied and their tissues fixed in 10% buffered formalin. Sections of heart, lung, trachea, gastrointestinal tract, liver, spleen, kidney, adrenal, lymph node, skin and bone marrow were stained routinely with hematoxylin and eosin and sections of gastrointestinal tract were stained by the periodic acid-Schiff reaction.

RESULTS

Eight cats were fed diets without added calcium pantothenate. The terminal stages of acute deficiency were observed in 2 of these after 2 months, and after 4 to 4.5 months in the other six. The deficiency state was characterized chiefly by emaciation. Loss or greying of hair and blood dyscrasias were not observed. Four cats receiving 1 mg of calcium pantothenate/kg of diet died after being fed the diet for approximately 3.5 to 4.5 months. Of the 4 cats fed 3 mg of calcium pantothenate/kg of diet, three died after 6 months, two of massive respiratory infections and one lived 9.5 months. Five milligrams of calcium pantothenate per kilogram of diet appeared to be sufficient to support good growth and health in 4 cats. Two groups of 4 cats were fed the diets containing 5

Received for publication July 11, 1963.

[1] Supported in part by grants-in-aid from the Nutrition Foundation and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts, and by Public Health Service Research Grant AM-04165 (National Institute of Arthritis and Metabolic Diseases).

TABLE 1

Weight changes of cats fed diets with varying levels of calcium pantothenate[1]

Ca pantothenate	No. of cats	Month 0	1	2	3	4
mg/kg of diet		*g*	*g*	*g*	*g*	*g*
0	8	1019	1141	1351	1363(6)[2]	1162(6)
1	4	1272	1452	1520	1412	1495(3)
3	4	1273	1460	1788	1808	1628
5	4	1108	1564	2128	2348	2555
10	4	873	988	1493	1802	2073
20	4	966	1419	1781	2041	2285

[1] Each group contained equal numbers of male and female kittens. Mean weights of each group are presented in grams.
[2] Figures in parentheses represent number of cats in group alive.

and 10 mg of calcium pantothenate/kg for almost 2 years and cats have been maintained with diets containing 20 mg of calcium pantothenate/kg for up to 3 years before being killed.

Table 1 presents the growth records of the 6 groups of cats during the first 4 months of this study. These data indicate that following an initial growth response, the cats receiving 0, 1 or 3 mg of calcium pantothenate/kg of diet started to lose weight during the third or fourth month, whereas the mean weights of the cats in the other groups continued to increase.

Lesions attributable to pantothenic acid deficiency were observed in cats receiving 0, 1 and 3 mg of calcium pantothenate/kg of diet. The livers and intestinal tracts of these animals were particularly affected. The livers showed moderate to marked fatty metamorphosis and both fine and coarse vacuolar formation. Lipid was evenly deposited and showed no zonal preference. There was no increase in portal fibrous tissue and no evidence of cirrhosis. A few of the livers showed moderate amounts of lipofuschin pigment in hepatic cells and in portal areas. Lesions of the gastrointestinal tract were confined primarily to the small bowel. Sections of the esophagus and stomach were normal except for evidence of superficial acute erosions in 2 stomachs. In the small intestines, some animals showed giant blunted villi in the areas of the jejunum and upper ileum as well as a brown-yellow lipofuschin present at the tips in fibrocytes and macrophages. In some, there was an increase in the number of round cells in the tunica propria. Some animals showed a loss of polarity of the nuclei of the columnar cells. The glands were occasionally dilated and contained protein material and degenerating polymorphonuclear lymphocytes. In some animals, the epithelial columnar cells were enlarged in size with vesicular nuclei at the bases of the crypts with many gland-like forms containing inspissated protein material. The tops of the villi in some animals appeared to show infarct necrosis and one animal showed focal areas of repair of a previous ulcer with loss of villi and increase of fibrous tissue in the tunica with tract glands showing inflammatory cells. Sections of the colon showed no changes or slight dilatation of the glands and inspissated protein and polymorphonuclear lymphocytes.

Histologic changes related to the pantothenic acid content of the diets were not observed in the other tissues examined. Histologic changes associated with pantothenic acid deficiency were not observed in cats receiving 5, 10 or 20 mg of calcium pantothenate/kg of diet except for a slight fatty metamorphosis observed in the liver of one of the animals fed 5 mg of calcium pantothenate/kg of diet.

In figure 1, the results of the studies of acetylation of *p*-aminobenzoic acid and urinary excretion of pantothenic acid are presented. These data show that as the content of the diets increased from 3 to 5 mg of pantothenic acid/kg, there were marked increases in the excretion of acetylated *p*-aminobenzoic acid and the pantothenic acid, creatinine ratio.

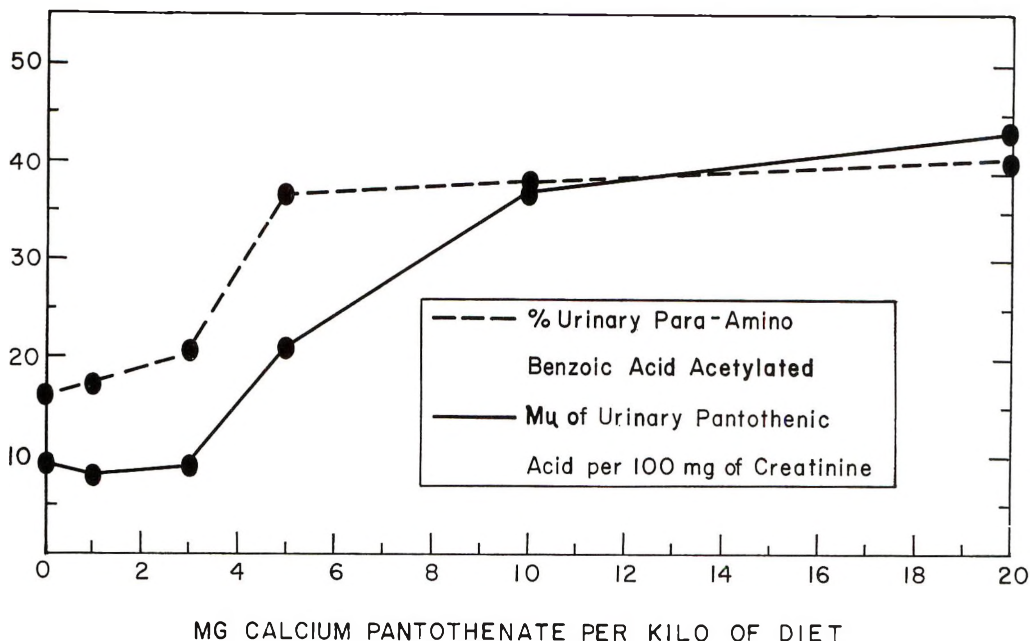


Fig. 1 Effect of dietary levels of calcium pantothenate on the urinary excretion of acetylated *p*-aminobenzoic acid and pantothenic acid.

DISCUSSION

The effects of pantothenic acid deficiency vary widely from species to species. In the cat, the most prominent changes were growth failure and histologic changes in the small intestines and the liver. Many of the deficient animals showed evidence of bronchopneumonia or lobar pneumonia. Dermatitis, achromotrichia, adrenal and kidney necrosis and blood dyscrasias, which have been often reported in deficient animals of other species, were not observed in this work. The histologic changes of the gastrointestinal tract are similar but not identical to those described in the pig (5). The spinal cord was not examined in this study, but ganglion cells, present in the periadrenal fat showed no evidence of chromatolysis. There was no evidence of necrosis of the adrenal cortex as has been previously noted in the rat (6) and no evidence of cortical hemorrhage. However, cats that died while being fed the experimental diet showed marked loss of cortical lipid.

A dietary source of pantothenic acid is required for all species of animals that have been studied. The role of this vitamin in intermediary metabolism, particularly acetylation reactions, is well established. In vitamin requirement studies, determinations of urinary levels of the vitamin being studied and metabolites whose excretion is related to dietary intake of the vitamin are often used as measures of the degree of tissue saturation with the vitamin. Riggs and Hegsted (7, 8) have reported that following the injection of 6 mg of *p*-aminobenzoic acid/kg in normal rats and rabbits, approximately 70% of the *p*-aminobenzoic acid excreted in the next 24 hours was acetylated. Pantothenic acid-deficient rats acetylated 50% of a 1-mg dose and 37% of a 2.5-mg dose. In this work cats receiving the highest level of pantothenic acid acetylated approximately 40% of a 6 mg/kg *p*-aminobenzoic acid load test and deficient cats about 17%. As the calcium pantothenate content of the diets increased from 3 to 5 mg/kg there was a sharp increase in acetylation of *p*-aminobenzoic acid from 20 to 37%. There was also a sharp increase in the urinary ratio of pantothenic acid to creatinine. However, this ratio did not reach a maximum until 10 mg of calcium pantothenate/kg of diet were fed. It appears from these data and the results of gross and histological examinations

that the minimal requirement of cats fed the purified diets used in this work was approximately 5 mg of calcium pantothenate/kg of diet. This is considerably less than the recommended minimal requirements of other laboratory animals (9–11), most of which require 8 mg or more of calcium pantothenate/kg of diet for growth when fed diets of less caloric density than those used in this study.

LITERATURE CITED

1. Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.
2. Bratton, A. C., and E. K. Marshall, Jr. 1939 A new coupling component for sulfanilamide determination. J. Biol. Chem., *128:* 537.
3. Clark, L. C., and H. L. Thompson 1949 Determination of creatine and creatinine in urine. Anal. Chem., *21:* 1218.
4. Hoag, E. H., H. P. Sarett and V. H. Cheldelin 1945 Use of Lactobacillus arabinosus 17–5 for microassay of pantothenic acid. Ind. Eng. Chem. (Anal. ed.), *17:* 60.
5. Wintrobe, M. M., R. H. Follis, Jr., R. Alcayaga, M. Paulson and S. Humphreys 1943 Pantothenic acid deficiency in swine with particular reference to the effects on growth and on the alimentary tract. Bull. Johns Hopkins Hosp., *73:* 313.
6. Deane, H. W., and J. M. McKibbin 1946 The chemical cytology of the rat adrenal cortex in pantothenic acid deficiency. Endocrinology, *38:* 385.
7. Riggs, T. R., and D. M. Hegsted 1948 The effect of pantothenic acid deficiency on acetylation in rats. J. Biol. Chem., *172:* 539.
8. Riggs, T. R., and D. M. Hegsted 1951 Size of dose and acetylation of aromatic amines in the rat. J. Biol. Chem., *193:* 669.
9. National Research Council, Committee on Animal Nutrition 1953 Nutrient Requirements for Dogs, pub. 300. National Academy of Sciences — National Research Council, Washington, D. C.
10. National Research Council, Comittee on Animal Nutrition 1960 Nutrient Requirements of Poultry, pub. 827. National Academy of Sciences — National Research Council, Washington, D. C.
11. National Research Council, Committe on Animal Nutrition 1962 Nutrient Requirements of Laboratory Animals, pub. 990. National Academy of Sciences — National Research Council, Washington, — D. C.

Influence of High Protein Diets on Vitamin A Metabolism and Adrenal Hypertrophy in the Chick

G. S. STOEWSAND AND M. L. SCOTT
Department of Poultry Husbandry and Graduate School of Nutrition, Cornell University, Ithaca, New York

ABSTRACT High dietary levels of isolated soybean protein, purified isolated soybean protein, casein-gelatin, vitamin-free casein, or amino acid mixtures formulated to simulate isolated soybean protein were administered to chicks. When adequate dietary intake of the high protein diets was obtained, a significantly decreased liver vitamin A storage was observed as compared with that of pair-fed chicks consuming the same protein at moderate dietary levels. Isolated soybean protein diets had the most marked effects in reducing vitamin A liver stores. The depletion of vitamin A ester in the liver was more complete than the depletion of vitamin A alcohol. Enlargement of the adrenal glands occurred in chicks consuming the high isolated soybean protein diets. This enlargement appeared to be due primarily to hypertrophy of the adrenal cortical tissue.

Studies with a number of species have shown that high protein diets reduce vitamin A in the liver and decrease survival time of animals fed diets low in vitamin A. In an earlier report from this laboratory (1) it was shown that chicks consuming high amounts of isolated soybean protein had a decreased amount of vitamin A in liver and blood serum as compared with vitamin A levels in pair-fed chicks receiving a normal amount of this protein. Olsen et al. (2) observed that vitamin A liver stores of chicks decreased when protein-deficient diets were made adequate by supplementation with additional protein. However, in this study the increased growth obtained from the higher protein diets may have been at least partially responsible for the reduced liver vitamin A stores, since the vitamin A requirement is directly related to body weight (3). Ferrando and Mainguy[1] observed decreased liver vitamin A in chicks when the protein content of the diet was gradually increased from 16 to 36%. Mayer and Krehl (4) observed that the average survival time of rats fed a 60% casein-vitamin A-deficient diet was markedly decreased compared with that of rats fed vitamin A-deficient diets containing normal protein levels. Bohman et al. (5) observed that when extra protein was fed to wintering beef calves on range, vitamin A and carotene levels in liver and blood were lowered. Erwin et al. (6) reported steers fed isocaloric diets showed decreased liver vitamin A reserves when higher amounts of protein were fed.

High levels of dietary protein also have been observed at times to produce adrenal gland hypertrophy in mammals. Tepperman et al. (7) and Leathem (8) reported that high dietary protein levels increased rat adrenal weights. Ingle et al. (9), however, did not observe significantly higher adrenal gland weights in rats fed high amounts of protein, and in one experiment by Benua and Howard (10) mice fed high levels of protein did not have hypertrophied adrenals.

The results presented in the present paper provide additional evidence that high levels of dietary protein from various sources influence the vitamin A status of the chick and that high dietary protein levels also cause hypertrophy of the adrenals in young chicks.

METHODS AND MATERIALS

Experiment 1. Twelve groups of 14 one-day-old White Plymouth Rock cockerel

Received for publication September 3, 1963.

[1] Ferrando, R., and P. Mainguy 1962 Influence du taux de protides et de lipides totaux de la ration sur la reserve hepatique de vitamine A chez le poulet. Twelfth World's Poultry Congress, Proceedings. Sydney, Australia, pp. 168–171.

chicks obtained from a commercial hatchery were randomly distributed into 12 pens within an electrically heated chick battery, and 6 treatments were randomly assigned to the pens, 2 lots per treatment. At the end of the first week, 10 chicks were selected on the basis of similar body weights to be maintained with the respective diets for 3 additional weeks. The composition of the diets is presented in table 1. Either isolated soybean protein or casein and gelatin were used as the source of protein in this experiment. Chicks eating the moderate (normal) protein diets (2 and 4) were either pair-fed to the chicks consuming the corresponding high protein diets (1 and 3) or were fed ad libitum (diets 5 and 6). After 4 weeks of age, 5 livers of chicks from each pen were collected, immersed immediately in liquid nitrogen, and stored at – 10°C until liver vitamin A analyses were made. The livers were analyzed individually for vitamin A according to the method of Ames et al. (11). Statistical significance of the differences in liver vitamin A content of pair-fed chicks was determined by Duncan's new multiple range test according to Steel and Torrie (12).

Experiment 2. The second experiment was conducted with chicks of the same breed and from the same source as those used in experiment 1. At one day of age, 11 chicks per lot were randomly allotted to each of 15 experimental pens. At the end of one week 8 chicks were selected to be maintained with the experimental treatments for 3 additional weeks. Each treatment was fed to triplicate lots of chicks. The diets were similar to the isolated soybean protein diets used in experiment 1 (diets 1 and 2, table 1). All chicks were fed the same amount of feed consumed by the chicks receiving the high protein diet which showed the lowest feed consumption.

To determine whether impurities in the crude, commercial isolated soybean protein exerted any effect upon vitamin A liver storage, 2 experimental groups received "purified" isolated soybean protein[2] substituted for "crude" soy protein in both diet

TABLE 1

Composition of experimental diets

Diet no.	1	2	3	4	5	6
	%	%	%	%	%	%
Isolated soybean protein [1]	84.35	28.00	—	—	—	—
Casein	—	—	63.15	21.00	—	—
Vitamin-free casein [2]	—	—	—	—	77.605	25.00
Gelatin	—	—	22.00	7.00	—	—
Glucose monohydrate	—	58.05	—	57.15	—	58.845
Cellulose	3.00	3.00	3.00	3.00	3.00	3.00
Stripped lard [3]	3.00	3.00	3.00	3.00	3.00	3.00
Mineral mixture	5.63 [4]	5.63 [4]	5.63 [4]	5.63 [4]	6.21 [5]	6.21 [5]
Vitamin mixture [6]	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine	1.20	0.70	0.80	0.80	0.90	0.30
L-Cystine	1.00	—	—	—	—	—
L-Arginine·HCl	—	—	0.80	0.80	4.50	1.50
Glycine	0.50	0.30	—	—	2.40	0.80
Choline chloride (70%)	0.22	0.22	0.22	0.22	0.22	0.22
$NaHCO_3$	—	—	—	—	1.00	—
Antioxidant [7]	0.0125	0.0125	0.0125	0.0125	0.0125	0.0125
% Protein (N × 6.25)	74.9	25.0	80.6	26.6	83.3	27.4

[1] Archer-Daniels-Midland, Minneapolis.
[2] Nutritional Biochemicals Corporation, Cleveland.
[3] Distillation Products Industries; Rochester, New York.
[4] Mineral mixture supplies per kg of diet: (in g) $CaHPO_4$, 20.70; $CaCO_3$, 14.80; KH_2PO_4, 10.00; KCl, 1.00; NaCl, 6.00; $NaHCO_3$, 0.80; $MgSO_4$, 3.00; $FeSO_4 \cdot 7H_2O$, 0.333; $MnSO_4 \cdot H_2O$, 0.333; KI, 0.0026; ZnO, 0.062; $CuSO_4 \cdot 5H_2O$, 0.0167; $CoCl_2 \cdot 6H_2O$, 0.0017; $NaMoO_4 \cdot 2H_2O$, 0.0093; Na_2SeO_3, 0.0001.
[5] Chloride low mineral mixture contains: (g/kg) $CaHPO_4$, 17.00; $CaCO_3$, 18.30; KH_2PO_4, 13.77; $NaC_2H_3O_2$, 8.42; $NaHCO_3$, 0.80; $MgSO_4$, 3.00; $FeSO_4 \cdot 7H_2O$, 0.333; $MnSO_4 \cdot H_2O$, 0.333; KI, 0.026; ZnO, 0.125; $CuSO_4 \cdot 5H_2O$, 0.0167; $CoCl_2 \cdot 6H_2O$, 0.0017; $NaMoO_4 \cdot 2H_2O$, 0.0083; Na_2SeO_3, 0.0001.
[6] Vitamin mixture supplies per kg of diet: (in IU) Vitamin A, 5,000; vitamin D_3, 975; (in mg) α-tocopheryl acetate, 600; niacin, 50; Ca pantothenate, 20; riboflavin, 10; thiamine·HCl, 10; pyridoxine·HCl, 10; folic acid, 4.0; menadione sodium bisulfite, 1.52; biotin, 0.5; (in μg) vitamin B_{12}, 20 (diets 2, 4, 6); 50 (diets 1, 3); 60 (diet 5); and 9.9 g of added glucose monohydrate.
[7] Santoquin, Monsanto Chemical Company, St. Louis.

TABLE 2

Effect of washing isolated soybean protein on body weight and liver vitamin A of chicks fed approximately the same amount of diet

Lot no.	Treatment	Protein in diet [1]	4-Week weights	Vitamin A in the liver [2]			
				Total	Liver retention [3]	Alcohol	Ester
		%	g	IU/liver	%		
1	High purified	80.5	236 [4]	78	4.7	+	0
2	High crude	77.6	220	0	0	0	0
3	Moderate purified	24.6	267	429	22.5	+	+
4	Moderate crude	23.5	255	354	18.6	+	+
5	Moderate crude + washing	27.0	241	471	26.3	+	+

[1] N × 6.25.
[2] Diet contained 5,000 IU vitamin A/kg of diet.
[3] $\frac{\text{Total liver vitamin A/chick}}{\text{Total amount of vitamin A consumed/chick}} \times 100.$
[4] All diets pair-fed equivalent to the feed consumed ad libitum by the chicks receiving the "high crude" protein diet.

TABLE 3

Amino acid diets that simulate isolated soybean protein diets in experiment 3

	High [1] protein	Moderate protein
	%	%
L-Arginine·HCl	6.71	2.02
L-Aspartic acid	1.29	0.40
L-Cystine	1.49	0.13
L-Glutamic acid	12.35	3.74
Glycine	4.21	1.30
L-Histidine·HCl	2.11	0.64
DL-Isoleucine	9.69	2.93
L-Leucine	6.07	1.82
L-Lysine·HCl	5.49	1.67
DL-Methionine	2.00	0.94
DL-Phenylalanine	8.07	2.44
L-Proline	2.02	0.60
DL-Serine	2.82	0.84
DL-Threonine	6.31	1.92
DL-Tryptophan	1.62	0.49
L-Tyrosine	2.76	0.82
DL-Valine	8.89	2.69
Glucose monohydrate	—	59.545
Stripped lard [2]	3.00	3.00
Cellulose	3.00	3.00
Mineral mixture [3]	6.21	6.21
Vitamin mixture [2]	1.00	1.00
Choline chloride (70%)	0.22	0.22
$NaHCO_3$	1.50	0.50
Antacid [4]	1.00	1.00
Antioxidant [2]	0.0125	0.0125
% Protein (N × 6.25)	64.2	20.5

[1] At the end of the first week of the experiment an additional 2.25% $NaHCO_3$ and 2.25% $KHCO_3$ was mixed into this diet in order to neutralize the HCl radical of the basic amino acids.

[2] See table 1; vitamin B_{12} (μg/kg of diet) high, 60; moderate, 20.

[3] See table 1, footnote 5.

[4] Gelusil (aluminum hydroxide — magnesium trisilicate), Warner-Chilcott Laboratories, Morris Plains, New Jersey.

1 and diet 2. A further treatment group (lot no. 5 of table 2) received diet 2 with the additional washings from the "purification" of the soy protein to make the total "soy protein impurities" equivalent to those in the high "crude" soy diet.

Livers were collected, pooled (4 per lot) and analyzed for vitamin A. In addition, vitamin A alcohol and vitamin A acetate were separated by thin layer chromatography[3] from aliquots of the ether solutions of liver vitamin A. These were determined qualitatively.

Experiment 3. The third experiment was conducted to determine whether the depressing effect of the high soybean protein upon liver vitamin A storage was a peculiarity of soybean protein or whether it also occurred when casein diets or pure amino acid diets were fed.

White Plymouth Rock × Vantress cockerels produced by a controlled flock of hens were used in this experiment. Ten chicks were randomly placed in each of 8

[2] The isolated soybean protein was washed with 41°C tap water at a pH of 4.6, pressed in a small cheese press, dried in a forced-air draft oven at 65°C and finely ground. Analysis for total sulfur by the sodium peroxide method (17) showed the sulfur was reduced from 0.55% in the "crude" protein to 0.15% in the "purified" isolated soybean protein.

[3] Glass plates, 20 × 20 cm, were spread with 27% silica gel G. Aliquots (50 to 100 μliters) of the diethyl ether solution from the total vitamin A analysis were pipetted onto the base line of the plate which was then placed into a covered glass jar containing a mixed solvent of 75% petroleum ether (bp 30° to 60°) and 25% diethyl ether. After the solvent had risen 10 cm above the base line, the plates were either quickly placed under a UV light and the separated vitamin A alcohol and vitamin A acetate spots outlined with a pencil before fading ensued, or a 25% solution (w/v) of antimony trichloride in chloroform was applied by dropper and the blue color of the Carr-Price reaction with vitamin A compounds was observed in the separated spots.

duplicated treatments; 6 chicks were selected at one week of age and continued to be fed the experimental diets for the subsequent 3 weeks as in experiments 1 and 2. Two treatments received the same "high" and "moderate" crude or purified isolated soybean protein diets used in experiment 2. The other treatments were high and moderate amino acid diets formulated to simulate isolated soybean protein (table 3) and the high and moderate vitamin-free casein diets (table 1, diets 5 and 6). Chicks consuming the moderate or normal protein diets were pair-fed to the chicks fed the respective high protein diets. Vitamin A was fed at 100,000 IU/kg of diet to measure fecal vitamin A which was done by the same method as the liver vitamin A analyses used in experiments 1 and 2. Vitamin A absorption was determined during the last week of the experiment. Feed intake and total excreta were carefully measured for 4 days, and averaged on an individual chick basis. The vitamin A liver retention values presented in table 4 were analyzed for statistical significance by the method described in experiment 1.

Both adrenal glands were dissected from 6 chicks per dietary treatment, trimmed of excess tissue and weighed. Since the body weights of the chicks varied widely, an adjustment of the paired adrenal weights was made by an analysis of covariance (12). The results are presented in table 4.[4]

RESULTS AND DISCUSSION

The results of experiment 1 are shown in table 5. Chicks consuming the high isolated soybean protein diet showed a significant decrease in total liver vitamin A as compared with chicks fed the other diets. Chicks fed the high casein-gelatin diet had significantly lower levels of total liver vitamin A than the chicks pair-fed the moderate casein-gelatin diet, but these were much higher than total liver vitamin A levels of the chicks fed the high isolated soybean

[4] The standard error of the difference between the adjusted adrenal pair means ($s\bar{d}$) of chicks consuming the same high and moderate protein diets were used in determining significance of the differences (i.e., $t = \bar{d}/s\bar{d}$ where $\bar{d}$ is the difference of the two means).

TABLE 4

Effect of high levels of dietary protein and amino acids on 4-week body weights, utilization of vitamin A, and chick adrenal pair weights

Treatment	Protein in diet [1]	4-Week wt	Liver vitamin A [2]			Mean adrenal pair wt [4]
			Apparent absorption	Total	Retained in liver [3]	
	%	g	%	IU/liver	%	mg
1 High crude isolated soy protein	76.2	209 (12/12) [5]	73	11,000	44 [6]	56.5 [7]
2 Moderate crude isolated soy protein	24.0	262 (12/12)	83	24,106	75	35.7
3 High purified isolated soy protein	80.5	220 (12/12)	73	15,325	61	50.9 [7]
4 Moderate purified isolated soy protein	24.8	254 (12/12)	74	21,312	74	35.2
5 High casein	83.3	99 (9/12)	93	11,544	53 [6]	35.8
6 Moderate casein	27.4	269 [8] (12/12)	83	27,018	76	27.4
7 High amino acids	64.2	110 (6/12)	82	16,167	72	39.9
8 Moderate amino acids	20.5	165 (12/12)	82	20,223	82	32.5

[1] N × 6.25.
[2] Diet contained 100,000 IU vitamin A/kg diet.
[3] $\frac{\text{Total liver vitamin A/chick}}{\text{Total vitamin A apparently absorbed/chick}} \times 100.$
[4] Adjusted to a common body weight by analysis of covariance.
[5] Number of chicks survived/number of chicks started.
[6] Significantly ($P < 0.05$) lower than the respective moderate protein pair-fed controls.
[7] Significantly higher ($P < 0.01$) than the respective moderate protein pair-fed controls.
[8] Pair-fed to high isolated soy protein diets.

TABLE 5

Effect of high levels of isolated soybean protein and casein-gelatin on 4-week weight, feed conversion, and vitamin A liver storage

	Treatment	Protein in diet	4-Week wt	G feed/ g gain	Total mean liver vitamin A [2]
		%	*g*		*IU/liver*
1	High isolated soy protein	74.9	270	2.04	60 [a 3]
2	Moderate isolated soy protein (pair-fed to lot no. 1)	24.9	278	1.88	807 [bc]
3	High casein-gelatin	80.5	269	1.72	646 [b]
4	Moderate casein-gelatin (pair-fed to lot no. 3)	26.3	263	1.74	916 [c]
5	Moderate isolated soy protein (ad libitum)	24.9	541	1.64	1387 [d]
6	Moderate isolated casein-gelatin (ad libitum)	26.3	468	1.60	1348 [d]

[1] N × 6.25.
[2] Diet contained 5,000 IU vitamin A/kg of diet.
[3] Values that are significantly different ($P < 0.05$) are followed by different letters.

protein diet. Analyses showed no vitamin A or carotene in the casein used in this experiment.

The results of experiment 2 are shown in table 2. In this experiment no vitamin A was detected in livers of chicks fed the high crude soybean protein diet. A very low level of liver vitamin A was observed in the chicks consuming the high purified protein diets. The results with the moderate protein diets were approximately equivalent and there was no decrease in liver retention of vitamin A when the washings from the "purification" of the crude isolated protein were added to the moderate soybean diet. The small amount of liver vitamin A in the chicks consuming the high purified protein diet was shown to be vitamin A alcohol; there was no detectable vitamin A ester in the livers of these chicks. Both vitamin A ester and alcohol were observed in the livers of all chicks receiving the moderate protein diets.

The results of experiment 3 are presented in table 4. Chicks receiving the high casein diet could not consume a normal amount of food because the casein became impacted in the beaks of the chicks, which interfered markedly with food consumption. Chicks consuming the high amino acid diet grew poorly and had 50% mortality at the end of the 4 week experimental period, perhaps because of certain excess amino acid toxicities (13).

Liver vitamin A, expressed as the amount of vitamin A retained as a percentage of the total amount of vitamin A apparently absorbed, again showed a significant decrease in the chicks consuming the high crude isolated soybean protein diet and in chicks consuming the high casein diet. However, the high casein-fed chicks had livers that were very small (average weight < 3 g) at 4 weeks of age, and the amount of vitamin A/g of liver was not decreased compared with that of the larger chicks fed the moderate protein diets. The chicks fed the high purified isolated soy protein diet had lower vitamin A liver stores than the pair-fed chicks consuming the moderate purified isolated soy protein diet, but this difference was not quite significant ($P < 0.05$).

The 2 high isolated soybean protein diets produced chicks with significantly heavier adrenal glands as compared with those of chicks receiving the moderate protein diets. Histological examination (hematoxylin and eosin stain) of the adrenals showed no changes in these enlarged glands except for some hypertrophy of the cortical tissue, especially of the periphery of the organ. Further studies in this laboratory (14) indicate that chicks fed high isolated soybean

protein diets have an increased production of corticosterone.

The results of these experiments show, therefore, that high protein diets, especially high soybean protein diets, cause a marked reduction in total vitamin A liver stores, and also that this reduction is accompanied by a complete depletion of vitamin A esters in the liver and at the same time a marked hypertrophy of the adrenal cortex. According to evidence presented in a review on vitamin A storage and transport by Ganguly (15) it appears that the animal maintains the vitamin A alcohol content of its tissues at the expense of the liver ester, whereas the liver vitamin A alcohol content is usually quite constant. Selye (16) and Constantinides[5] have shown that high protein diets augment various other stresses in animals. Since, in the present studies, the high crude isolated soybean protein diet has been shown to decrease vitamin A liver reserves and at the same time produces increases in adrenal cortical size and corticosterone production, it appears possible that perhaps vitamin A utilization is mediated either directly or indirectly via adreno-cortical mechanisms.

LITERATURE CITED

1. Stoewsand, G. S., and M. L. Scott 1961 Effect of protein on utilization of vitamin A in the chick. Proc. Soc. Exp. Biol. Med., *106:* 635.
2. Olsen, E. M., J. D. Harvey and H. D. Branion 1959 Effect of dietary protein and energy levels on the utilization of vitamin A and carotene. Poultry Sci., *38:* 942.
3. Guilbert, H. R., and G. H. Hart 1935 Minimum vitamin A requirements with particular reference to cattle. J. Nutrition, *10:* 409.
4. Mayer, J., and W. A. Krehl 1948 The relation of diet composition and vitamin C to vitamin A deficiency. J. Nutrition, *35:* 523.
5. Bohman, V. R., M. A. Wade and C. Torrell 1959 Effect on animal fat and protein supplement in range beef cattle. J. Animal Sci., *18:* 567.
6. Erwin, E. S., R. S. Gordon and J. W. Algeo 1963 Effect of antioxidant, protein and energy on vitamin A and feed utilization in steers. J. Animal Sci., *22:* 341.
7. Tepperman, J., F. L. Engel and C. N. H. Long 1943 Effect of high protein diets on size and activity of the adrenal cortex in the albino rat. Endocrinology, *32:* 403.
8. Leathem, J. H. 1951 Adrenal and thyroid weights in rats following high protein diets and testosterone propionate. Exp. Med. Surg., *9:* 138.
9. Ingle, D. J., G. B. Gunther and J. Nezamis 1943 Effect of diet in rats on adrenal weights and on survival following adrenalectomy. Endocrinology, *32:* 410.
10. Benua, R. S., and E. Howard 1945 The effect of dietary protein on adrenal weights and on growth after unilateral adrenalectomy. Endocrinology, *36:* 170.
11. Ames, S. R., H. A. Risley and P. L. Harris 1954 Simplified procedure for extraction and determination of vitamin A in liver. Anal. Chem., *26:* 1378.
12. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw Hill Book Company, New York.
13. Snetsinger, D. C., and H. M. Scott 1961 The relative toxicity of intraperitoneally injected amino acids and the effect of glycine and arginine thereon. Poultry Sci., *40:* 1681.
14. Stoewsand, G. S., and M. L. Scott 1964 Effect of stress from high protein diets on vitamin A metabolism in chicks. J. Nutrition, in press.
15. Ganguly, J. 1960 Absorption, transport and storage of vitamin A. Vitamins and Hormones, *18:* 387.
16. Selye, H. 1946 The general adaptation syndrome and the diseases of adaptation. J. Clin. Endocrinol., *6:* 117.
17. Association of Official Agricultural Chemists 1960 Methods of Analysis. Washington, D. C.

[5] Constantinides, P. 1950 Influence of diet composition on alarm reaction to acute stress. Federation Proc., *9:* 25 (abstract).

Heat of Combustion Values of the Protein and Fat in the Body and Wool of Sheep[1,2]

O. L. PALADINES,[3] J. T. REID, A. BENSADOUN AND
B. D. H. VAN NIEKERK[4]
Department of Animal Husbandry, Cornell University, Ithaca, New York

ABSTRACT The heat of combustion values of protein and fat were determined in the ash-free, dry matter of the bodies of 63 sheep, and of the wool of 45 sheep. As a consequence of the ranges in age at the time of slaughtering and the dietary treatments imposed, the animals represented a wide range in body composition. After each animal was shorn and slaughtered, the ingesta-free body was separated into 4 parts, consisting mainly of the blood, viscera, hide, and carcass. The mean calorific values (kcal/g) of the protein and fat, respectively, in the various body parts were as follows: blood, 5.854 ± 0.052 and 7.435 ± 0.052; viscera, 5.428 ± 0.057 and 9.312 ± 0.057; hide, 5.458 ± 0.086 and 9.305 ± 0.086; and carcass, 5.327 ± 0.068 and 9.424 ± 0.068. For the total, ingesta-free body, the calorific values were 5.379 ± 0.051 kcal/g of protein and 9.405 ± 0.051 kcal/g of fat. As the result of the manner by which they were derived, these values represent the calorific value of the composite body proteins and lipids. As a consequence, these values are more accurate for use in studies involving the metabolism and storage of energy than are the older values which are based chiefly on the analysis of body (generally muscle) proteins and body fats purified to various degrees. Although the calorific value of fat (9.405 kcal/g) representing the total body was only slightly lower than the commonly used value (9.5 kcal/g), the calorific value of protein representing the total body (5.379 kcal/g) was considerably lower than that (5.7 kcal/g) which is generally used. The calorific value of wool protein was found to be 5.609 ± 0.0023 kcal/g and that of wool fat was 9.741 ± 0.0035 kcal/g. Since wool protein contained 16.85% of nitrogen, the obligatory factor for converting the percentage of nitrogen to the percentage of protein is 5.933.

The use of body composition estimated by indirect methods as the basis of indirect calorimetry requires the use of calorific values of the composite proteins and fats of the animal body. Also, in the use of the slaughter-analysis method as an indirect calorimetric method, the application of calorific values for the directly determined body protein and fat would obviate the need to determine heat of combustion values on the body tissues.

Although the average values of 5.7 kcal/g of protein and 9.5 kcal/g of fat as recorded by Armsby (1) have been used extensively, their accuracy when applied to the composite proteins and fats of the body has been questioned by Blaxter and Rook (2) and Stroud.[5] Armsby's values, derived from the analytical data of chiefly late-nineteenth century German workers, represent the calorific values of protein and fat isolated principally from muscles and fat depots, respectively, of animals of various species. However, the values do not necessarily represent the body as a whole, and they do not take into account substances other than pure protein and triglycerides deposited in the ash-free, dry matter of the animal body. Since the chemical composition of the nitrogen-containing substances and lipids determines their energy value, the analysis of purified preparations isolated from a few tissues could yield erroneous values with respect to application to the total body.

Received for publication August 5, 1963.

[1] This investigation was supported by a research grant (A-2889) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

[2] The data presented here are a part of those presented in the Ph.D. degree thesis by O. L. Paladines to the Graduate School, Cornell University, 1963.

[3] Recipient of Organization of American States scholarship (1960-62) and Rockefeller Foundation fellowship (1962–63); present address: Inter-American Institute of Agricultural Sciences, Turrialba, Costa Rica.

[4] Present address: Grootfontein College of Agriculture, Middelburg, South Africa.

[5] Stroud, J. W. 1961 The development of indirect methods for the estimation of the chemical composition and energy value of living cattle. Ph.D. Thesis, Cornell University, Ithaca, New York.

The purpose of the study to be reported here was to determine the calorific values of the protein and fat representing the total body and the wool of sheep.

EXPERIMENTAL PROCEDURE

Animals, dietary history and slaughter and tissue-sampling procedures. The present study was conducted in connection with a nutritional experiment in which 63 sheep were slaughtered and analyzed. Just prior to the beginning of the experimental feeding period, 18 sheep, of which 9 were 8 months old and 9 were 20 months old, were slaughtered and analyzed. Each of the remaining 45 sheep was fed a diet composed of either chopped hay; pelleted, finely ground hay; or a pelleted mixture of 55% hay and 45% corn meal for 196 days (at which time their age was either 15 or 27 months) at one of 3 levels of intake: low (slightly above maintenance); medium (intermediate between low and high); and high (ad libitum). Just prior to slaughter, the sheep were shorn and the wool produced since the beginning of the feeding period was analyzed separately from the remainder of the body.

The body was separated into 4 analytical groups: 1) blood, obtained by severing the main vessels in the neck; 2) viscera, which included the empty digestive tract, mesenteric fat, lungs, heart, liver, pancreas, spleen, and urinary bladder; 3) hide, including remnants of wool not removed by a close shearing, ears and the hooves; and 4) one-half of the skinned, eviscerated carcass, with superficial fat, one kidney with its fat and including one-half of the skinned head. The carcass was divided into halves along the median line and the left side was used in the chemical analysis. Each analytical group, except blood which was freeze-dried in toto, was ground and mixed 5 or 6 times by a high-capacity grinder and samples weighing 1 to 2.3 kg were freeze-dried. The freeze-dried samples were mixed with crushed dry ice and ground in a Wiley mill equipped with a screen having holes 1.59 mm in diameter. During the grinding process the samples gained some moisture and in the final form for analysis contained about 95% of dry matter. Concurrently with each of the chemical analyses listed below, the dry matter content was determined by freeze-drying.

Analytical methods. Ash was determined by the common AOAC method (3) and nitrogen was determined by the macro-Kjeldahl method using the boric-acid modification proposed by Scales and Harrison (4). However, for the purposes of this study, the protein content of the body-tissue groups was computed as the difference between the total weight and the sum of the moisture, ether extract, and ash contents. This scheme was considered to be more accurate than multiplying the nitrogen content by an arbitrary protein factor since the nitrogen content of the protein is different in the various tissues. On the basis of the data obtained in the present studies, it is indicated that the nitrogen content of the composite proteins of the ingesta-free, wool-free body of sheep is of the order of 15.58%, and that the factor for converting nitrogen to protein is 6.42.

Fat was determined as the difference in weight between the freeze-dried sample and the freeze-dried residue remaining after extraction with ether. Four to 8 samples weighing approximately 10 g were extracted continuously for 7 days. Thus, the calorific values derived for so-called "fat" in the present study apply strictly to the ether-soluble fraction in the water-free body parts. A complete extraction of lipids would require the use of a mixture of polar and non-polar solvents (e.g., methanol and chloroform). However, for the purposes of indirect calorimetry in which the lipid fraction is determined most conveniently by ether extraction, the values obtained in the present work are strictly valid and applicable.

The heat of combustion value of the tissue samples was determined with an oxygen bomb calorimeter.

The wool samples obtained from the 45 sheep used in the feeding experiment were studied. The wool fiber and fat were separated by successive extractions of the wool with ether and washings with warm water. Moisture, ash, protein and gross energy in the clean wool fiber and moisture and gross energy in the ether extract of the wool, were determined directly.

The heat of combustion of the ash-free, dry matter of the 4 body-tissue groups and of the total, ingesta-free body was partitioned between the protein and fat. This was effected by regressing the calorific value on the percentage of protein or fat in the ash-free, dry matter of the tissue groups. The intercept values of the respective equations represent the calorific value of fat and protein. The calorific value of the protein and fat of wool was computed on the moisture-free, ash-free basis.

A more detailed description of animals, dietary treatments and the slaughter and analytical procedures used is given in the report of Van Niekerk et al. (5).

RESULTS AND DISCUSSION

Chemical composition and gross energy value of ash-free, dry matter. The composition of the ash-free, dry matter of the bodies of the 63 sheep studied is shown in table 1. The wide range in these data is the result of the range in the dietary treatments imposed and in the age of the animals.

Calorific value of body protein and fat. In table 2 are summarized the statistics concerned with the relationships between the heat of combustion value and the percentages of protein and fat in the various tissue groups and the reconstituted, ingesta-free body. Both the regression and correlation coefficients are highly significant. (Since protein and fat constitute 100% of the ash-free, dry matter, the correlation between the calorific value and fat concentration is identical to that between the calorific value and protein concentration.)

Although the correlation coefficient between the concentration of fat or protein and the calorific value of blood is highly significant, it is nevertheless too low for the relationship to have prediction value. The low correlation between these variables for blood is associated with the narrow range in the variables. As shown in table 1, the protein content of blood ranged only from 94.20 to 100.00%. The few values observed below 98% probably reflect dilution by small quantities of superficial body fat which, during the

TABLE 1

Chemical composition of the ash-free, dry bodies of 63 sheep

Body part	Protein		Fat		Calorific value	
	Mean	Range	Mean	Range	Mean	Range
	%	%	%	%	*kcal/g*	*kcal/g*
Blood	98.80	94.20–100.00	1.20	0.00– 5.80	5.873	5.723–5.991
Viscera	33.50	12.88– 73.38	66.50	26.62–87.12	8.011	6.535–8.877
Hide	74.05	61.28– 81.63	25.95	18.37–38.72	6.473	6.076–7.038
Carcass	40.18	18.31– 66.68	59.82	33.32–81.69	7.778	6.758–8.667
Ingesta-free body	43.33	20.57– 71.20	56.67	28.80–79.43	7.661	6.607–8.581

TABLE 2

Relation between the concentration of protein or fat and the calorific value of the ash-free, dry matter of the sheep body

Body part	Regression equations [1]		Correlation coefficient [2]	$S_{y \cdot x}$[3]	S_b[4]
	Protein	Fat			
Blood	$Y = 7.435 - 0.01583X$	$Y = 5.854 + 0.01583X$	0.341	0.052	0.00564
Viscera	$Y = 9.312 - 0.03881X$	$Y = 5.428 + 0.03881X$	0.995	0.057	0.00051
Hide	$Y = 9.305 - 0.03847X$	$Y = 5.458 + 0.03847X$	0.893	0.086	0.00248
Carcass	$Y = 9.424 - 0.04097X$	$Y = 5.327 + 0.04097X$	0.989	0.068	0.00079
Total, ingesta-free body	$Y = 9.405 - 0.04025X$	$Y = 5.379 + 0.04025X$	0.994	0.051	0.00057

[1] In equations, Y = calorific value (kcal/g), and X = protein or fat (% of ash-free, dry matter).
[2] Correlation coefficient between calorific value (kcal/g of ash-free, dry matter) and the concentration of protein or fat (% of ash-free, dry matter); all coefficients highly significant ($P < 0.01$).
[3] SE of estimate; highly significant ($P < 0.01$).
[4] SD of regression coefficient.

slaughtering process, inadvertently fell into the blood-drip pan and were analyzed as a normal component of the blood.

As the correlation coefficients, standard deviations from regression, and standard deviations of the regression coefficients recorded in table 2 indicate, the relationships between the concentration of fat or protein and the calorific value of the other tissue groups have a high degree of prediction value. Of these, the relationship for the hide was the lowest. This is probably the result of variable amounts of wool remaining on the hide even though the animals were shorn closely.

From the regression equations shown in table 2, the heat of combustion value of protein and of fat can be computed individually. Since the ash-free, dry matter is composed wholly of protein and fat, the regression equation for either protein or fat may be used. For example, substitution of the values, 0.0 and 100.0 for X in the protein regression will resolve the calorific value of fat and protein, respectively, for a given tissue group or the total body. Conversely, substitution of the same values in the fat regression will allow the calorific value of protein and fat, respectively, to be computed for a given tissue group or the whole body. Obviously, the intercept value of the protein-regression equation represents the calorific value of fat and the intercept value of the fat-regression equation is the calorific value of protein. The calorific values so computed and their 95% confidence intervals are presented in table 3. Statistical treatment of the mean calorific values of the protein and fat revealed no significant differences among the viscera, hide, carcass and whole, ingesta-free body. However, the calorific value of blood protein was significantly higher than that of the protein of the hide ($P < 0.05$), and the viscera, carcass, and the ingesta-free body ($P < 0.01$). The calorific value of the fat of blood was significantly lower than that of the fat of the other body parts at the same levels of probability as indicated above for protein.

The calorific value per gram of nitrogen or crude protein (nitrogen × 6.25) can be computed from the data in table 3. These values for the total, ingesta-free, wool-free body are 0.838 kcal/g of nitrogen and 5.238 kcal/g of nitrogen × 6.25.

Comparison of results of present study with those of older investigations. A comparison of the data in table 3 with those of other investigators reveals that the calorific values of protein determined in the present study for the carcass (5.327 kcal /g) and the whole, ingesta-free body (5.379 kcal/g) are considerably lower than the average values of 5.656 and 5.7 kcal/g proposed by Rubner (6) and Armsby (1), respectively. On the other hand, the values derived in the present study are in good agreement with that (5.322 kcal/g) of the tissue proteins (containing 16% nitrogen) of cattle determined by Blaxter and Rook (2) and the calorific value of 5.447 kcal/g of protein representing the total body of cattle as reported by Stroud.[6] The difference between the various values is traceable to the method by which the values were derived. The early workers were concerned with determining the calorific value of isolated muscle proteins in varying

[6] See footnote 5.

TABLE 3

Calorific value of the protein and fat in the sheep body

Body part	Calorific value			
	Protein		Fat	
	Mean	95% Confidence interval	Mean	95% Confidence interval
	kcal/g		*kcal/g*	
Blood	5.854	5.748–5.960	7.435	6.299–8.571
Viscera	5.428	5.296–5.560	9.312	9.190–9.434
Hide	5.458	5.242–5.674	9.305	8.899–9.711
Carcass	5.327	5.161–5.493	9.424	9.274–9.574
Total, ingesta-free body	5.379	5.257–5.501	9.405	9.290–9.520

states of purity and did not necessarily intend that their values be applied to the total proteins of the body. Nevertheless, their values have been used for this purpose, even though they do not correspond to the protein of the total body. The method used by Stroud[7] is the same as that used in the present study. The method employed by Blaxter and Rook (2), although similar to that of the present study, involved the regression of the calorific value of various tissues on their nitrogen concentration. It is noteworthy that the calorific value (5.322 kcal/g) of protein (% nitrogen × 6.25) in the body tissues of cattle as determined by Blaxter and Rook (2) is almost identical to the value (5.327 kcal/g) derived for the carcass of sheep in the present study. Although Stroud's value (5.447 kcal/g) for protein of the total body of cattle is slightly higher than that (5.379 kcal/g) obtained for sheep in the present study, the 2 values are essentially the same considering the errors attached to the estimates.

Although in better agreement with recent observations than was the case for the calorific value of protein, the average calorific value of fat (9.5 kcal/g) suggested by Armsby (1), is somewhat higher than recent studies have revealed. The following values (kcal/g) have been obtained: 9.367, for cattle tissues (2); 9.499, for cattle tissues (Stroud);[8] and 9.405 for sheep tissues (present study). It is probable that these values are not significantly different from 9.5 kcal/g.

Calorific value of wool protein and fat. The mean calorific value of the protein of wool was 5.609 ± 0.0023 kcal/g, and the 95% confidence interval was 5.607 to 5.611 kcal/g. The mean calorific value of wool fat was 9.741 ± 0.0035 kcal/g, with the 95% confidence interval of 9.738 and 9.744 kcal/g. Since the degree of variation about the means is so small, a high degree of accuracy is attached to the use of these values for the prediction of the calorific values of wool fat and protein. The use of these values in future experiments would obviate the need to determine the heat of combustion value of wool fiber and fat, which is difficult and laborious.

The calorific value of both the protein and fat of wool is strikingly higher than that of the corresponding components of the body proper. These differences are reflected in the great differences in the chemical composition between wool and other tissues. Block (7) reported that wool protein is a keratin containing 14.3% of cystine, 3.9% of sulfur, and 16.8% of nitrogen. Other tissues such as the hair, hoof, and horn also contain keratins. In the present study it was found that wool protein contains 16.85% of nitrogen, a value almost identical to that (16.8%) reported by Block (7). As a consequence, it is suggested that the factor, 5.933 (having the 95% confidence interval of 5.930 to 5.936), should be used to compute the protein concentration from the percentage of nitrogen in wool.

ACKNOWLEDGMENTS

The authors wish to thank L. D. Van Vleck for his suggestions concerning statistical analyses, Mrs. Thelma Zablan for help with chemical analyses, and R. E. Allyn, J. Mauboussin, and R. P. Gerbasi for help in the care and slaughtering of experimental animals.

LITERATURE CITED

1. Armsby, H. P. 1917 The Nutrition of Farm Animals. The Macmillan Company, New York.
2. Blaxter, K. L., and J. A. F. Rook 1953 The heat of combustion of the tissues of cattle in relation to their chemical composition. Brit. J. Nutrition, 7: 83.
3. Association of Official Agricultural Chemists 1955 Official Methods of Analysis, ed. 8. Washington, D. C.
4. Scales, F. M., and H. E. Harrison 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem., 12: 350.
5. Van Niekerk, B. D. H., J. T. Reid, A. Bensadoun and O. L. Paladines 1963 Urinary creatinine as an index of body composition. J. Nutrition, 79: 463.
6. Rubner, M. 1885 Calorimetrische untersuchungen. Ztschr. Biol., 21: 250.
7. Block, R. J., and D. Bolling 1948 The Determination of Amino Acids, rev. ed. Burgess Publishing Company, Minneapolis.

[7] See footnote 5.
[8] See footnote 5.

Some Biochemical Lesions Associated with Liver Fat Accumulation in Threonine-deficient Rats[1]

DOROTHY ARATA, CATHERINE CARROLL[2] AND DENA C. CEDERQUIST

Department of Foods and Nutrition, College of Home Economics, Michigan State University, East Lansing, Michigan

ABSTRACT The metabolism of 2 groups of co-factors, the pyridine nucleotides and the adenosine polyphosphates, was altered in fatty livers produced in weanling rats by feeding a low protein diet deficient in threonine. Both total pyridine nucleotide levels and the PN/PNH ratio were significantly decreased by the twenty-fifth day in livers from threonine-deficient animals. These values returned to control levels upon subsequent supplementation with threonine. The amount of labile phosphorus derived from ADP and ATP from the fatty livers was significantly less than that from livers of control rats. The activity of the DPN-cytochrome c reductase enzyme system was significantly lower in livers from threonine-deficient rats than in livers from threonine-supplemented rats throughout the experimental period. The activity of the fatty acid oxidase system declined sharply during the early stages of fat accumulation in the liver. The subsequent, complete recovery of this enzyme system was followed closely by a return of liver fat concentrations to near-control levels. The ultimate dependence of all the above functions on active electron transport is noted, and the suggestion made that a threonine deficiency might affect some phase of this system.

A threonine imbalance, characterized by accumulation of excess fat in the liver, can be produced in weanling rats by feeding a 9% casein diet supplemented with methionine and tryptophan.[3] This study was undertaken to investigate further the effects of a threonine deficiency on the metabolic functions of liver tissue.

Evidence has accumulated which suggests a faulty metabolism of the adenosine polyphosphates and pyridine nucleotides in livers infiltrated with fat under different experimental conditions. Dianzani (1) noted a disruption in the metabolism of both of these groups of co-factors in fatty livers resulting from carbon tetrachloride poisoning, white phosphorus posioning, or choline deficiency. Total pyridine nucleotide levels, as well as the ratio of the oxidized to the reduced forms (PN/PNH), were lower in the fatty livers than in those from control rats (1). Furthermore, ATP concentration in liver tissues was decreased by fatty infiltration induced by any of the above means (2). In fatty livers associated with threonine deficiency, Arata et al. (3) observed a similar reduction in pyridine nucleotides. An extension of the latter study is reported here.

The PN/PNH ratios[4] were determined for fatty livers and for livers from control animals. In addition, the activity of an enzyme system involved in reoxidation of DPNH during electron transport, DPN-cytochrome *c* reductase, was measured in livers from threonine-deficient and from control rats. To assess the extent of involvement of ATP in this fatty liver syndrome, determinations were made of labile phosphorus from the adenosine polyphosphates, and of endogenous inorganic phosphorus. Finally, livers were assayed for fatty acid oxidase activity, inasmuch as both DPN and ATP are obligatory cofactors in the series of reactions involved in the oxidation of fatty acids (4, 5).

EXPERIMENTAL

A total of 90 male, weanling rats of the Sprague-Dawley strain was used in this study. For each series, the animals were divided into 2 groups. The rats in group 1 were fed a basal ration consisting of the

Received for publication August 5, 1963.

[1] Supported in part by United States Air Force contract no. AF 49(657-276). Journal Article no. 3201 from the Michigan Agricultural Experiment Station.

[2] Present address: Department of Home Economics, University of Arkanasas, Fayetteville, Arkansas.

[3] Singal, S. A., V. P. Sydenstricker and J. M. Littlejohn 1949 The lipotropic action of threonine. Federation Proc., 8: 251 (abstract).

[4] The PN/PNH ratios were determined by one of the authors (D.A.) in the Department of Biochemistry, University of Wisconsin, under the direction of Dr. J. N. Williams, Jr. His helpful guidance is hereby gratefully acknowledged.

following: (in grams) casein, 9.0; choline chloride, 0.15; salts,[5] 4.0; vitamin mix,[6] 0.25; corn oil,[7] 5.0; DL-methionine, 0.3; DL-tryptophan, 0.1; and sucrose to 100. The diet fed to rats in group 2 was identical with that above, except that 0.36 g DL-threonine was added at the expense of sucrose.

Pyridine nucleotide content of liver tissues was determined after rats had been fed the experimental diets for 25 days. Livers were assayed for labile phosphorus (from ADP and ATP), endogenous inorganic phosphorus, fat and for activities of the DPN-cytochrome *c* reductase and fatty acid oxidase enzyme systems, after 2, 3, and 4 weeks of feeding. A 4-week experimental period was judged to be of insufficient length for assay of the fatty acid oxidase system. A second experiment was devised wherein fatty acid oxidase activity was measured over a period of 6 weeks. The data from the 2 experiments were combined.

Total pyridine nucleotide, oxidized pyridine nucleotide (PN), and reduced pyridine nucleotide (PNH) were determined according to a procedure developed by Feigelson et al. (6). Total labile phosphorus from ADP and ATP was determined by charcoal adsorption of the adenosine phosphates from aliquots of liver homogenates, followed by hydrolysis in HCl to release the labile phosphorus from the polyphosphates (7). Fatty acid oxidation was measured manometrically, using the Warburg apparatus. The method used was essentially that of Lehninger for mitochondrial preparations (8), except that 1 ml whole homogenate (33%) was used in place of 0.5 ml of mitochondrial suspension. The DPN-cytochrome c reductase activity was determined by the method of Potter (9). Crude malic dehydrogenase preparations required for this system were isolated from fresh beef hearts as described by Potter (9). Fat was determined in dried, ground liver homogenates by continuous ether extraction in a Goldfisch apparatus.

RESULTS

Results from the differential assay for pyridine nucleotide content of the livers are summarized in table 1. The data for total pyridine nucleotide values are in agreement with those reported previously (3). Livers from threonine-deficient rats (group 1) contained more than twice as much PNH as the livers from control rats, whereas the concentration of the oxidized form of these coenzymes (PN) was significantly decreased. The decrease in PN together with the increase in PNH in livers from threonine-deficient rats resulted in a greatly reduced PN/PNH ratio, equal to only about one-quarter of the ratio in livers from control rats. In view of these results, it was considered pertinent to determine whether a short interval

[5] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339. Obtained as Salt Mixture-W from Nutritional Biochemicals Corporation, Cleveland.

[6] The vitamin mix provided the following in mg/100 g ration: thiamine·HCl, 0.5; riboflavin, 0.5; niacin, 1.0; pyridoxine, 0.25; Ca pantothenate, 2.0; inositol, 10.0; folic acid, 0.02; vitamin B_{12}, 0.002; biotin, 0.01; vitamin A powder, 10.0 (200 USP units); and calciferol, 0.18 (150 USP units).

[7] Mazola, Corn Products Company, Argo, Illinois, containing 7.5 mg α-tocopheryl acetate and 0.375 mg menadione/5 g.

TABLE 1

Liver pyridine nucleotide [1] and DPN-cytochrome c reductase [2] data from rats fed 9% casein diets with and without supplementary threonine for 2 weeks

Group no.[3]	No. rats	Total pyridine nucleotide	PN [4]	PNH [5]	PN/PNH	DPN-cytochrome c reductase activity
1	9	773 ± 9 [6]	674 ± 21	99 ± 14	6.9	139 ± 11
2	9	1021 ± 10	986 ± 14	35 ± 8	28.2	182 ± 13

[1] Expressed as μg/g fresh tissue.
[2] Expressed as μliter O_2/hr/g fresh tissue.
[3] Group 1, 9% of casein, no supplementary threonine; group 2, 9% of casein + 0.36% of DL-threonine.
[4] Oxidized pyridine nucleotide.
[5] Reduced pyridine nucleotide.
[6] SE of mean.

of feeding the threonine-supplemented diet to the remaining rats in group 1 would cause the PN/PNH ratio to return to a normal level. Six rats from group 1 and 3 rats from group 2 were maintained with their respective diets for a period of 39 days. At that time, 3 of the 6 rats in group 1 were fed diet 2 (9% of casein + threonine). All animals were fed for an additional 5 days. At the close of this 5-day period, the rats were killed and their livers analyzed for PN and PNH. Rats that had been maintained with the threonine-deficient diet for a total of 44 days had an average liver PN/PNH ratio of 4.5. Those rats maintained with the control diet for 44 days had a ratio of 16.0. The rats fed the deficient diet for 39 days and the control diet for 5 days had an average PN/PNH ratio of 19.9, essentially identical with that of the control group.

The activity of the DPN-cytochrome c reductase system in liver was depressed throughout the experimental period in threonine-deficient rats as compared with threonine-supplemented rats (table 1). This difference was significant at the 5% level.

A progressive accumulation of fat in livers of threonine-deficient rats was again observed. In previous studies (3, 10) liver fat levels had reached maxima in 2 to 3 weeks. In this experiment the peak in liver fat deposition appeared more rapidly than in previous runs. As a consequence, the sampling procedures were improperly spaced and a clear cut peak was not observed. An additional experiment was devised as an adjunct to the one reported here. Data were collected during the period of fat accumulation in liver tissues and through the diminishing phase of the liver fat curve. Data from the 2 experiments were combined, and are presented in table 2. Liver fat levels in the control group (group 2) did not increase significantly above the levels at zero time.

Fatty acid oxidase activity per gram of liver from rats fed the threonine-deficient diet was significantly lower than the activity in livers from control rats during the phase of fat deposition in the liver. During fat mobilization out of the liver, activity of the fatty acid oxidase system in threonine-deficient rats was significantly greater than the activity of the same system during fat deposition (fig. 1).

When fatty acid oxidase activities were calculated on the basis of the total weight of liver tissues, a similar curve was obtained. In fact, the depression in the total activity of this enzyme system was more

TABLE 2

Liver fat and fatty acid oxidase data from rats fed 9% casein diets with and without supplementary threonine for 6 weeks [1]

	Group 1 [2]			Group 2 [3]	
No. rats	Liver fat	Fatty acid oxidase activity		Liver fat	Fatty acid oxidase activity
	% of dry wt	*μl O₂/hr/g liver*	*% of control*	*% of dry wt*	*μl O₂/hr/g liver*
12	8.2 ± 1.0 [4]	552 ± 43 [4]	100	8.2 ± 1.0 [4]	552 ± 43 [4]
8	15.4 ± 1.5	449 ± 40	84	7.0 ± 0.8	532 ± 39
4	20.2 ± 2.1	318 ± 24	41	8.4 ± 1.2	782 ± 83
4	23.2 ± 6.6	267 ± 76	43	10.3 ± 1.3	621 ± 60
5	31.7 ± 2.6	450 ± 51	108	13.5 ± 4.7	415 ± 45
5	17.4 ± 2.9	565 ± 45	128	7.8 ± 2.4	441 ± 66
5	11.2 ± 3.7	614 ± 47	146	6.4 ± 0.5	442 ± 54
4	17.1 ± 2.2	735 ± 13	137	8.3 ± 0.4	538 ± 35
5	11.9 ± 0.7	470 ± 80	81	5.6 ± 0.4	581 ± 72

[1] These figures represent a compilation of 2 separate experiments. The time factor varied somewhat between the experiments; therefore, time notations would be approximate and thus are omitted. The complete experiment ran for 6 weeks and the peak in liver fat deposition occurred at approximately 2 weeks.

[2] Fed 9% of casein, no supplementary threonine.

[3] Fed 9% of casein + 0.36% of DL-threonine.

[4] SE of mean.

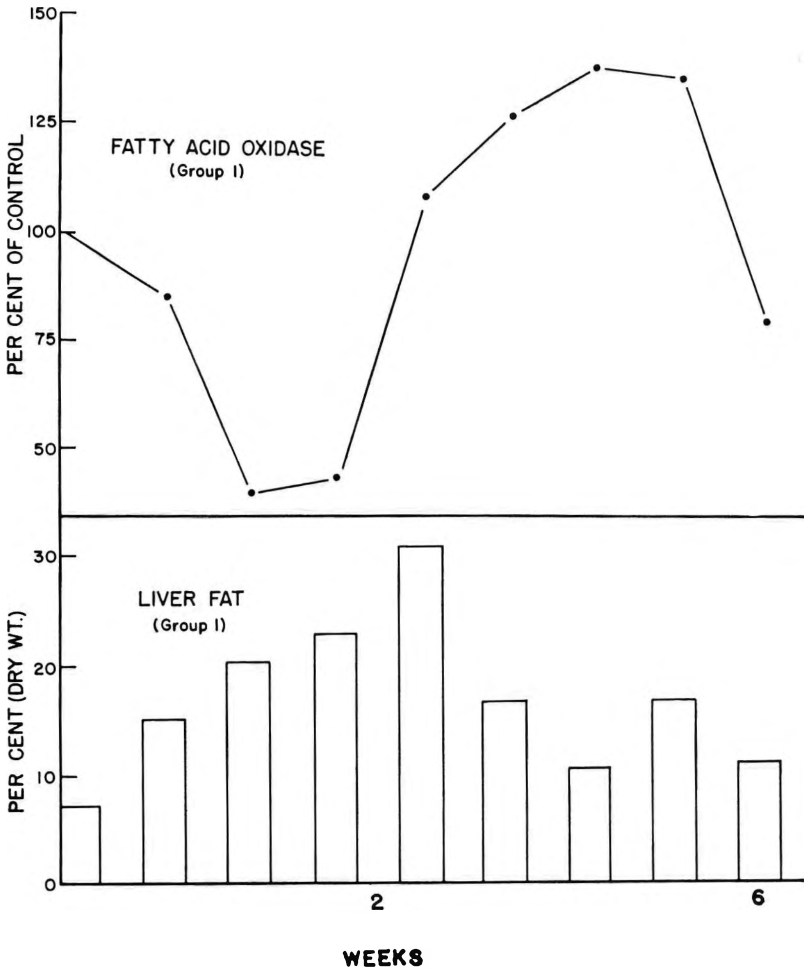


Fig. 1 Changes in fatty acid oxidase activity in livers from threonine-deficient rats during accumulation and removal of excess fat.

severe during the phase of fat deposition, falling to 26% of the control. These results were spot-checked in another group of rats, and the same relationship was observed.

The amount of labile phosphorus derived from ADP and ATP in livers from threonine-deficient rats (group 1) varied somewhat over the 4-week period, but showed no substantial fluctuations from the level established at zero time. In livers from the control group, on the other hand, labile phosphorus values increased sharply in the first 2 weeks, continued to increase during the third week, then leveled off during the fourth week (fig. 2). Amounts of labile phosphorus from the threonine-deficient group were significantly lower ($P < 0.01$) than those from the control group throughout the experimental period (table 3). The concentration of endogenous inorganic phosphorus in livers from both groups of rats increased at comparable rates for 3 weeks. However, during the fourth week, when labile phosphorus had leveled off in the control group, there was a much sharper increase in inorganic phosphorus in this group than in the deficient group (table 3). The significance of this observation is undetermined.

TABLE 3

Liver phosphorus data from rats fed 9% casein diets with and without supplementary threonine

Weeks fed diet	No. rats	Group 1 [1]		Group 2 [2]
		μg/ total liver	*% of control*	*μg/ total liver*
		Labile phosphorus from ADP and ATP		
0	4	307	100	307
2	8	343 ± 33 [3]	61	560 ± 53 [3]
3	4	289 ± 48	46	627 ± 15
4	4	359 ± 47	56	639 ± 66
		Endogenous inorganic phosphorus		
0	4	735	100	735
2	8	723 ± 44	82	880 ± 69
3	4	865 ± 62	86	1008 ± 91
4	4	1079 ± 145	59	1829 ± 183

[1] Fed 9% of casein, no supplementary threonine.
[2] Fed 9% of casein + 0.36% of DL-threonine.
[3] SE of mean.

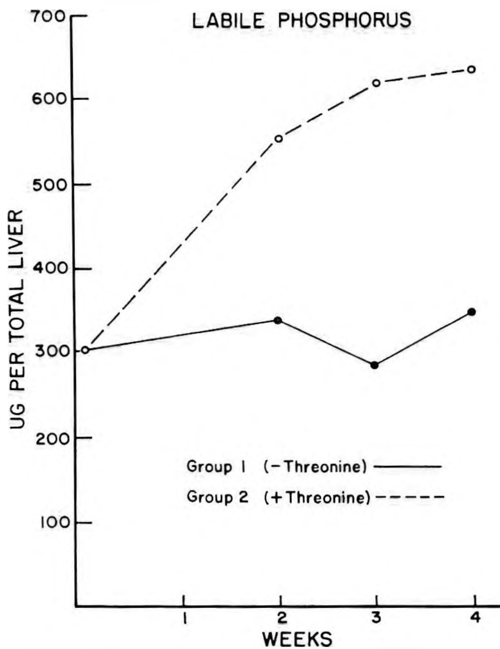


Fig. 2 Labile phosphorus from ADP and ATP in livers from threonine-deficient and threonine-supplemented rats.

DISCUSSION

Concurrent changes in activity of the fatty acid oxidase system and in concentration of fat in livers from threonine-deficient rats appeared to follow a pattern (fig. 1). As the activity of the enzyme system decreased, fat began to accumulate in the livers; then, after the enzyme system had fully recovered, fat concentration decreased rapidly to near-control levels. It was not a true inverse relationship, since enzyme activity changes preceded liver fat changes. This suggests that the fatty acid oxidase system is an important instrument in regulating liver fat levels.

The activity curve for fatty acid oxidase presented here is strikingly similar to activity curves for xanthine oxidase and malic dehydrogenase in threonine-deficient rats (10). These enzymes, too, began to recover *before* the accumulated fat was mobilized out of the liver. A similar observation was made by Arata et al. (3), namely, maximal stimulation of endogenous oxidation by added DPN in livers from threonine-deficient rats occurred *before* the peak in liver fat deposition. Using histological techniques for the determination of liver fat induced by carbon tetrachloride, Recknagle and Anthony (11) suggested that biochemical lesions and liver fat accumulation are not interdependent. However, the observations above strongly suggest a definite temporal relationship between changes in the activities of some enzyme systems and movement of fat into and out of the liver.

The temporal changes in the activities of the oxidative enzymes mentioned above appear to be nonspecific responses to the deficiency of threonine. It is unlikely that any of these systems reflects a primary biochemical lesion. The appearance of fatty livers in threonine-deficient animals may, in fact, be a compensatory mecha-

nism to allow recovery of the enzyme systems in a manner similar to the one suggested by Wilgram et al. (12) for choline deficiency.

The disturbance observed in fatty acid oxidation could have been secondary to changes in concentration of pyridine nucleotides, since DPN acts as a coenzyme in the fatty acid oxidase system (5, 13). The reduced quantity of total PN observed in the livers of threonine-deficient rats in this experiment would limit the supply of DPN available for fatty acid oxidation. At the same time, the pile-up of PNH (including DPNH and TPNH) would tend to favor fatty acid synthesis (14–16). In fact, Yoshida and Harper (17) demonstrated an increased rate of fatty acid synthesis from C^{14}-labeled acetate in threonine-deficient rats.

The major factor contributing to the depression in total pyridine nucleotide content of the fatty livers could well have been a defect in ATP metabolism. The decrease in labile phosphorus in livers from threonine-deficient rats probably indicates a restricted quantity of ATP. Dianzani (2) reported the concentration of ATP to be markedly reduced and that of ADP somewhat increased in fatty livers, resulting in a net decrease in total ADP plus ATP. When Stekol et al. (18) induced a decrease in liver ATP levels by feeding ethionine, they noted a parallel decrease in DPN synthesis. In the present study, the decrease in labile phosphorus values in threonine-deficient rats (expressed as percentage of control) tended to parallel the decrease in fatty acid oxidase activity in the same rats during the period of fat accumulation in the liver.

The metabolic lesions observed in this study (decreased supply of ATP, depressed activity of the DPN-cytochrome c reductase system, greatly reduced PN/PNH ratio, lower levels of total pyridine nucleotides, and depressed rate of fatty acid oxidation, with accumulation of fat in the liver) all appear to point to some portion of the electron transport system as the possible primary target of a threonine deficiency. The major mechanisms for ATP generation are coupled with electron transport (19); DPN-cytochrome c reductase is a component of the electron transport system, and is one of the chief sites of conversion of DPNH to DPN; ATP is required for synthesis of DPN (18, 20); the rate of fatty acid oxidation is dependent on availability of DPN; and the oxidation of fatty acids is a major route for removal of fat from the liver. Also, the activity of the cytochrome oxidase enzyme system, which accepts electrons derived from DPNH, is depressed in livers of threonine-deficient rats.[8] A primary attack on one of the links in electron transport would probably affect the normal functioning of other components of the system, through deficiency of substrates or pile-up of products, or both, and could conceivably lead to all the lesions noted above.

The fact that the activity of the fatty acid oxidase system returned to normal or above, whereas the PN/PNH ratio and the activity of the DPN-cyctochrome c reductase system remained at suboptimal levels, suggests that some biochemical adaptation took place. It is possible that the limited amount of DPN available was used preferentially for fatty acid oxidation, that adjustments occurred in PN/PNH transhydrogenase activities, that alternate pathways became more active, or that some other adaptive change occurred. The data presented here do not provide sufficient evidence to identify a specific mechanism by which fatty acid oxidation and liver fat levels approached control levels with time.

The temporal behavior of these systems has important implications with respect to the original lesion. The complete recovery of the PN/PNH ratio in depleted rats after only 5 days' supplementation with threonine, together with the failure to recover with time of those systems most closely associated with transfer of electrons from DPNH to cytochrome c, strongly suggest that a threonine imbalance directly influences a phase of electron transport essential for maximal reoxidation of DPNH.

LITERATURE CITED

1. Dianzani, M. U. 1955 Content and distribution of pyridine nucleotides in fatty livers. Biochem. Biophys. Acta, 17: 391.
2. Dianzani, M. U. 1957 The content of adenosine polyphosphates in fatty livers. Biochem. J., 65: 116.

[8] Unpublished observations.

3. Arata, D., G. Svenneby, J. N. Williams, Jr. and C. A. Elvehjem 1956 Metabolic factors and the development of fatty livers in partial threonine deficiency. J. Biol. Chem., *219:* 327.
4. Lehninger, A. L. 1945 The relationship of the adenosine polyphosphates to fatty acid oxidation in homogenized liver preparations. J. Biol. Chem., *157:* 363.
5. Lehninger, A. L. 1945 On the activation of fatty acid oxidation. J. Biol. Chem., *161:* 437.
6. Feigelson, P., J. N. Williams, Jr. and C. A. Elvehjem 1950 Spectrophotometric estimation of pyridine nucleotides in animal tissue. J. Biol. Chem., *185:* 741.
7. Seitz, I. F. 1956 Determination of adenosine di- and tri-phosphates. Bull. Exp. Biol. Med. (U.S.S.R.), *22:* 235.
8. Lehninger, A. L. 1955 Fatty acid oxidation in mitochondria. Methods in Enzymology, vol. 1, eds., S. P. Colowick and M. O. Kaplan. Academic Press, Inc., New York, p. 545.
9. Potter, V. R. 1946 The assay of animal tissues for respiratory enzymes. V. The malic dehydrogenase system. J. Biol. Chem., *165:* 311.
10. Carroll, C., D. Arata and D. C. Cederquist 1960 Effect of threonine deficiency on changes in enzyme activity and liver fat deposition with time. J. Nutrition, *70:* 502.
11. Recknagel, R. O., and D. D. Anthony 1959 Biochemical changes in carbon tetrachloride fatty liver: separation of fatty changes from mitochondrial degeneration. J. Biol. Chem., *234:* 1052.
12. Wilgram, G. F., C. F. Holloway and E. P. Kennedy 1960 The content of cytidine diphosphate choline in the livers of normal and choline-deficient rats. J. Biol. Chem., *235:* 37.
13. Green, D. E. 1954 Fatty acid oxidation in soluble systems of anmal tissues. Biol. Rev., Cambridge Phil. Soc., *29:* 330.
14. Van Baalan, J., and S. Gurin 1953 Cofactor requirements for lipogenesis. J. Biol. Chem., *205:* 303.
15. Langdon, R. G. 1957 The biosynthesis of fatty acids in rat liver. J. Biol. Chem., *226:* 615.
16. Wakil, S. J. 1961 Mechanisms of fatty acid synthesis. J. Lipid Res., *2:* 1.
17. Yoshida, A., and A. E. Harper 1960 Effect of threonine and choline deficiencies on the metabolism of C^{14}-labeled acetate and palmitate in the intact rat. J. Biol. Chem., *235:* 2586.
18. Stekol, J. A., E. Bedrak, U. Mody, N. Burnette and C. Sommerville 1963 Inhibition of the synthesis of diphosphopyridine nucleotide by ethionine administration in the liver of rats and mice. J. Biol. Chem., *238:* 469.
19. Lehninger, A. L., C. L. Wadkins, C. Cooper, T. M. Devlin and J. L. Gamble, Jr. 1958 Oxidative phosphorylation. Science, *128:* 450.
20. Preiss, J., and P. Handler 1958 Biosynthesis of diphosphopyridine nucleotide. II. Enzymatic aspect. J. Biol. Chem., *233:* 493.

Influence of Nicotinic, Picolinic and Pyridine-3-sulfonic Acids on Cholesterol Metabolism in the Rat[1]

DAVID KRITCHEVSKY AND SHIRLEY A. TEPPER
The Wistar Institute, Philadelphia, Pennsylvania

ABSTRACT The addition of 1% of nicotinic, picolinic or pyridine-3-sulfonic acid to a cholesterol-free rat diet for 3 weeks did not affect serum cholesterol levels. Liver cholesterol levels of the nicotinic acid-fed rats were lower than those of the controls. When these compounds were fed at 1% of the diet to rats also receiving 2% cholesterol and 0.5% cholic acid, all test groups displayed elevated serum cholesterol levels and the nicotinic acid group also had a higher liver cholesterol content than did the controls. Hepatic cholesterogenesis from sodium acetate-1-C^{14} in rats fed 1% nicotinic acid or pyridine-3-sulfonic acid was significantly reduced, whereas that of the group fed picolinic acid was below that of the control group but not significantly so.

The mechanism by which nicotinic acid exerts its hypocholesterolemic effect is not yet clear (1). Neither common derivatives, such as nicotinamide (2), nor metabolites, such as nicotinuric acid (3), display any hypocholesterolemic properties. The authors of the initial report on the action of nicotinic acid (4) suggested that it might act by increasing the in vivo oxidation of cholesterol. Studies of the influence of nicotinic acid on the oxidation of cholesterol by rat liver mitochondria (5) showed that mitochondrial preparations from livers of rats fed nicotinic acid oxidized more cholesterol-26-C^{14} to $C^{14}O_2$ than did similar preparations from untreated rats. It was further observed that addition of nicotinic acid (6 to 15 mg) to control mitochondrial preparations also enhanced cholesterol oxidation. A nicotinic acid homologue, 3-pyridylacetic acid has been shown to lower serum cholesterol levels in man (6) and to inhibit Triton[2]-induced hypercholesterolemia in rats (7). In experiments with a series of nicotinic acid homologues, only 3-pyridylacetic acid was active in vitro in enhancing cholesterol oxidation by rat liver mitochondria (8). In a subsequent series of experiments (9) it was observed that although neither nicotinamide nor its homologues affected mitochondrial oxidation of cholesterol, 2 compounds related to nicotinic acid (picolinic acid, pyridine-2-carboxylic acid, and pyridine-3-sulfonic acid) increased oxidation. The present report deals with the in vivo effects of these compounds upon cholesterol metabolism in the rat and their comparison with nicotinic acid.

METHODS

All studies were carried out in male Wistar rats weighing 150–160 g. The basal test diet contained, per 100 g: (in grams) cereal mixture, 70; wheat germ, 6.25; skim milk powder, 20; vitamin mixture, 2.75; and test compound, 1.0.[3] Sufficient water was added to make a thick dough. This diet provides 20% protein, 11% fat and 62% carbohydrate and was well received by the animals. The control diet contained 70 g of cereal mixture. In the hypercholesterolemic diet, cholesterol (2 g) and cholic acid (0.5 g) were added at the expense of the cereal mixture. Rats were maintained with the respective diets for 3 weeks at which time they were killed and the serum and liver analyzed for cholesterol by the Mann (10) modification of the method of Zlatkis et al. (11). Aliquots of the liver were dissolved in 15% alcoholic KOH and the color reaction carried

Received for publication August 19, 1963.
[1] This work was supported, in part, by USPHS Research Grant HE-03299 and Career Award 5-K6-HE-734 from the National Heart Institute.
[2] Rohm and Haas, Philadelphia.
[3] Cereal mixture, Pablum, Mead Johnson and Company, Evansville, Indiana; wheat germ, Kretschmer Corporation, Carrollton, Michigan; skim milk powder, Carnation Company, Los Angeles; vitamin mixture, Vionate, E. R. Squibb and Sons, New York; nicotinic acid, Eastman Organic Chemicals, Rochester, New York; picolinic acid-3-pyridinesulfonic acid, and cholic acid, Calbiochem, Inc., Los Angeles; cholesterol, Wilson and Company, Chicago; sodium acetate-1-C^{14}, New England Nuclear Corporation, Boston.

out on the petroleum ether extract of the saponification mixture. In the biosynthesis experiment each animal was given an intraperitonial injection of sodium acetate-1-C^{14} (1 μc/100 g) and killed 6 hours later. One-gram aliquots of the livers of these animals were taken for cholesterol determination. The remaining liver was also saponified and the nonsaponifiable material extracted with petroleum ether. The digitonide complex of the cholesterol present in the dried extract was prepared. The digitonides were dissolved in 1 ml of Hyamine[4] and assayed for radioactivity using a Packard Tri-Carb Liquid Scintillation Spectrometer (12).

RESULTS AND DISCUSSION

In our earlier experiments (5) we found that the addition of 0.75% nicotinic acid to a cholesterol-free diet over a 3-week period had no significant effect on the serum cholesterol levels of either male or female rats. Duncan and Best (13) made a similar observation in rats fed 1% nicotinic acid for either 8 or 42 days. After 8 days they noted no differences in the liver cholesterol levels of the fed or control rats, but at 42 days the liver cholesterol levels of the nicotinic acid-fed rats were somewhat higher than those of the control group. This observation was true in pair-fed rats as well as in rats fed ad libitum. Friedman and Byers (14) reported that the addition of 1% nicotinic acid to the diet lowered cholesterol levels in rats examined at either 12 or 14 days and attributed this to an anorexic effect of the drug since the nicotinic acid-fed groups gained less weight than did the controls. Duncan and Best (13) also observed smaller weight gains in nicotinic acid fed rats. Gaylor et al. (15) compared the effects of 1% of nicotinic acid, nicotinamide and isonicotinic acid (pyridine-4-carboxylic acid) upon the serum and liver cholesterol levels of rats fed a diet containing 10% lard for 4 weeks. The nicotinic acid group showed somewhat higher serum cholesterol levels and lower liver cholesterol levels than did the controls. The picolinic acid group showed lower serum and liver cholesterol levels than the control group and nicotinamide had no effect. Our data are presented in table 1. In 3 weeks none of the test compounds (nicotinic acid, picolinic acid, pyridine-3-sulfonic acid) had a signficant effect upon serum cholesterol levels and the liver cholesterol levels of the nicotinic acid-fed group were lower than those of the 3 other groups. No anorexia (as evidenced by reduced weight gain) was observed in any group.

Since the clinical use of nicotinic acid involves its administration to hypercholesterolemic subjects, of greater interest was the influence of nicotinic acid and the other test compounds upon the serum and liver cholesterol levels of rats being tested with a hypercholesterolemic regimen. Nath et al. (16) observed that when 1% nicotinic acid was fed to rats in a diet containing 1% cholesterol and 0.5% cholic acid there was an increase in both serum and liver cholesterol levels compared with those of controls. Gaylor et al. (15) added 1% nicotinic acid to the diets of rats pre-fed cholesterol (1%) and cholic acid (0.5%) and reported a retardation of cholesterolemia. Friedman and Byers (14) carried out a study in which 1% nicotinic acid was fed to rats with a modified Hartroft (17) hypolipotropic diet and observed a retardation of hypercholesterolemia in

[4] Packard Instrument Company, La Grange, Illinois.

TABLE 1

Autopsy data — rats fed normal diets plus 1% test compound for 21 days

Group	Survival	Wt gain	Liver wt	Liver cholesterol	Serum cholesterol
		g	*g*	*mg/g*	*mg/100 ml*
Nicotinic acid	4/5	106 ± 3.9 [1]	8.9 ± 0.8 [1]	1.71 ± 0.09 [1]	78.4 ± 4.5 [1]
Picolinic acid	4/5	89 ± 6.8	9.8 ± 0.9	1.86 ± 0.11	89.3 ± 11.6
Pyridine-3-sulfonic acid	5/5	87 ± 12.4	9.3 ± 0.7	2.23 ± 0.22 [2]	84.9 ± 11.2
Control	5/5	94 ± 13.3	9.7 ± 1.2	1.96 ± 0.05 [2]	75.2 ± 7.1

[1] SE of mean.
[2] Test for significance: nicotinic acid vs. pyridine-3-sulfonic acid or control $0.05 > P > 0.01$.

ad libitum fed rats but none in pair-fed groups. Schön (18) reported that 1% nicotinic acid had only a slight effect on the liver cholesterol levels of rats fed a special hypolipotropic diet. Our own observations upon the serum and liver cholesterol concentrations in rats fed 1% nicotinic, picolinic or pyridine-3-sulfonic acid, 2% cholesterol and 0.5% cholic acid for 3 weeks (table 2) show that all 3 test groups had higher cholesterol levels than did the controls, and the nicotinic acid group also had significantly higher liver cholesterol levels. The serum cholesterol data are in agreement with those of Nath et al. (16) whose experimental design was substantially similar to ours. The control group exhibited a greater weight gain than did any of the test group. Liver weights of the nicotinic and picolinic acid-fed rats were significantly lower than those of the controls.

Duncan and Best (13) observed that the total carcass cholesterol content of rats fed 1% nicotinic acid was the same as that of the controls. The serum-liver cholesterol pools were also similar for the 2 groups. We have calculated serum-liver cholesterol pools (19) for all the groups of rats used in both experiments and these are presented in table 3. The serum-liver cholesterol pools of all the test groups were somewhat lower than that of the control in the series in which cholesterol was present in the diet. With the control diet only the nicotinic acid-fed group had a strikingly smaller serum-liver cholesterol pool. These data show the relatively small contribution of the serum cholesterol to this pool in either diet.

The question of how nicotinic acid affects synthesis of cholesterol by rat liver has been studied by a number of investigators with conflicting results. In table 4 we have attempted to summarize the previous results. Duncan and Best (13) observed a slightly decreased incorporation of acetate-1-C^{14} into the liver cholesterol of rats fed 1% nicotinic acid for 42 days but the difference was not significant. Hardy et al. (22) reported the duration of feeding affected cholesterogenesis. After

TABLE 2

Autopsy data — rats fed cholesterol (2%) cholic acid (0.5%) diet plus 1% of test compound for 21 days

Group	Survival	Wt gain	Liver wt	Liver cholesterol	Serum cholesterol
		g	*g*	*mg/g*	*mg/100 ml*
Nicotinic acid	8/8	39 ± 4.5 [1,a]	9.1 ± 0.4 [1,b]	2.73 ± 0.21 [1,e]	229.6 ± 26.6 [1]
Picolinic acid	3/8	58 ± 7.2	9.6 ± 0.7 [c]	1.89 ± 0.44	222.3 ± 87.0
Pyridine-3-sulfonic acid	7/8	48 ± 7.0	11.8 ± 0.5 [d]	1.64 ± 0.25	227.0 ± 37.4
Control	7/8	69 ± 8.5	12.8 ± 0.5	2.18 ± 0.15	176.3 ± 9.8

[1] SE of mean.
[a] Significance, control vs. nicotinic acid, $0.01 > P > 0.001$.
[b] Significance, control vs. nicotinic acid, $P < 0.001$.
[c] Significance, control vs. picolinic acid, $0.01 > P > 0.001$.
[d] Significance, pyridine-3-sulfonic acid vs. nicotinic acid, $0.01 > P > 0.001$; pyridine-3-sulfonic acid vs. picolinic acid, $P < 0.05$.
[e] Significance, control vs. nicotinic acid, $P = 0.05$.

TABLE 3

Serum-liver cholesterol pools for rats fed 1% nicotinic, picolinic and pyridine-3-sulfonic acid with normal and hypercholesterolemic diets for 21 days

Group	Normal diet			Cholesterol diet		
	Serum	Liver	Total	Serum	Liver	Total
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Nicotinic acid	6.1	152.2	158.3	13.0	248.4	261.4
Picolinic acid	6.5	182.3	188.8	13.6	181.4	195.0
Pyridine-3-sulfonic acid	6.3	207.4	213.7	13.5	192.3	205.8
Control	5.6	190.1	195.7	11.4	279.0	290.4

TABLE 4

Effect of nicotinic acid (NA) on hepatic cholesterol synthesis in rats

Rat strain	Type of experiment	Indicator	Results	Reference
Sprague-Dawley	intact, NA-fed	acetate-C^{14}	increase	(20)
Sprague-Dawley	liver slices, NA added	acetate-C^{14}	increase	(20)
Long Evans	intact, NA-fed	biliary excretion	no change	(14)
Holtzman	intact, NA-fed	acetate-C^{14}	no change	(13)
Hooded	liver slices, NA added	acetate-C^{14}	decrease	(21)
Holtzman	liver slices, NA pre-fed	acetate-C^{14}	increase	(22)
Holtzman	liver slices, NA added	acetate-C^{14}	no change	(22)
Long Evans	liver slices, NA added	acetate-C^{14}	decrease	(7)
Long Evans	liver slices, NA added	mevalonate-C^{14}	decrease	(7)

3 days of nicotinic acid administration cholesterol synthesis was below that of the controls as it was after 7 days but at 14, 21 or 28 days there was an increase in cholesterogenesis.

The experiments of Hardy et al. (22) were carried out using livers from rats fed a diet containing 10% lard which may in itself affect cholesterol biosynthesis (23, 24). Addition of nicotinic acid directly to the incubation mixture generally exerted little effect. Bizzi and Grossi (7) observed that nicotinic acid added at a level of 2 μmole increased cholesterol synthesis from acetate but not from mevalonate; but at 20 μmole nicotinic acid inhibited biosynthesis from either precursor.

Of the experiments in which compounds related to nicotinic acid were used Perry (21) and Hardy et al. (22) observed that nicotinamide had no effect upon cholesterol biosynthesis when added to the incubation mixture. In rats pre-fed the test compound for 3 weeks neither nicotinamide nor isonicotinic acid enhanced hepatic cholesterol synthesis to the extent that 1% nicotinic acid did. The increases observed with these compounds were 127% for nicotinamide, 146% for isonicotinic acid and 231% for nicotinic acid (22). Pyridine-3-acetic acid (7) was active to the same extent as nicotinic acid in inhibiting biosynthesis of cholesterol from acetate or mevalonate.

We administered sodium acetate-1-C^{14} intraperitoneally (1 μc/100 g) to each of the rats fed the cholesterol-free diet after 3 weeks, killed them 6 hours later and assayed the liver cholesterol-C^{14} as the digitonide. Our observations are detailed in table 5. The biosynthesis of cholesterol was significantly reduced in the groups fed nicotinic or pyridine-3-sulfonic acid. The hepatic synthesis of cholesterol is affected by the cholesterol content of the liver (25, 26). In our series of experiments the average liver cholesterol content of the nicotinic acid-fed group was lower than that of the controls. If the percentage of C^{14} incorporation is multiplied by the total liver cholesterol content some idea of how liver cholesterol affects biosynthesis might be obtained.

The results of such a calculation in the groups described in table 5 would be: nicotinic acid, 73.1; picolinic acid, 133.1; pyridine-3-sulfonic acid, 77.8 and control, 195.0.

TABLE 5

Biosynthesis of cholesterol from acetate by rats maintained with 1% nicotinic, picolinic or pyridine-3-sulfonic acid for 21 days

Group	No.	Incorporated C^{14}	P [2]
		%	
Nicotinic acid	4	0.480 ± 0.071 [1]	$0.05 > P > 0.01$
Picolinic acid	4	0.730 ± 0.192	N.S.[3]
Pyridine-3-sulfonic acid	5	0.375 ± 0.019	$0.05 > P > 0.01$
Control	5	1.026 ± 0.204	—

[1] SE of mean.
[2] Significance compared with control.
[3] Not significant.

In the other experiments carried out in which cholesterol biosynthesis was determined in intact rats fed nicotinic acid, Merrill (20) fed 0.8% nicotinic acid and assayed biosynthesis after 8 days and Friedman and Byers (14) fed 1% of this compound and measured biosynthesis at 2 weeks. Duncan and Best (13) noted a slight, but not significant, increase in hepatic cholesterogenesis in rats fed 1% nicotinic acid for 8 days and a non-significant decrease in rats fed 1% nicotinic acid for 42 days. In view of the observations of Hardy et al. (22) on the time effect of nicotinic acid on cholesterol biosynthesis, this factor should be taken into consideration in comparing data. Clinically, occasional liver dysfunction has been noted after prolonged nicotinic acid therapy (27) and this may aid in explaining the variability of results obtained in different laboratories.

LITERATURE CITED

1. Parsons, W. B., Jr. 1961 Treatment of hypercholesteremia by nicotinic acid. Progress report with review of studies regarding mechanism of action. Arch. Int. Med., *107:* 639.
2. Parsons, W. B., Jr., and J. H. Flinn 1959 Reduction of serum cholesterol levels and beta-lipoprotein cholesterol levels by nicotinic acid. Arch. Int. Med., *103:* 783.
3. Miller, O. N., J. G. Hamilton and G. A. Goldsmith 1960 Investigation of the mechanism of action of nicotinic acid on serum lipid levels in man. Am. J. Clin. Nutrition, *8:* 480.
4. Altschul, R., A. Hoffer and J. D. Stephen 1955 Influence of nicotinic acid on serum cholesterol in man. Arch. Biochem. Biophys., *54:* 558.
5. Kritchevsky, D., M. W. Whitehouse and E. Staple 1960 Oxidation of cholesterol-26-C^{14} by rat liver mitochondria: Effect of nicotinic acid. J. Lipid Res., *1:* 154.
6. Ratti, G., and E. DeFina 1959 Effect of 3-pyridineacetic acid on serum lipids. Lancet, *2:* 917.
7. Bizzi, L., and E. Grossi 1961 The evaluation of 3-pyridineacetic acid as hypocholesteremic substance. Arzneimittel-Forsch, *11:* 265.
8. Kritchevsky, D., and S. A. Tepper 1962 Influence of nicotinic acid homologs on oxidation of cholesterol 26-C^{14} by rat liver mitochondria. Arch. Int. Pharmacodyn., *138:* 349.
9. Kritchevsky, D., and S. A. Tepper 1964 Oxidation of cholesterol-26-C^{14} by rat liver mitochondria: effect of compounds related to nicotinic acid and nicotinamide. Arch. Int. Pharmacodyn., in press.
10. Mann, G. V. 1961 A method for cholesterol in serum. Clin. Chem., *7:* 275.
11. Zlatkis, A., B. Zak and A. J. Boyle 1953 A new method for the direct determination of serum cholesterol. J. Lab. Clin. Med., *41:* 486.
12. Shapiro, I. L., and D. Kritchevsky 1963 Radioassay of cholesterol-C^{14} digitonide. Anal. Biochem., *5:* 88.
13. Duncan, C. H., and M. M. Best 1960 Lack of nicotinic acid effect on cholesterol metabolism in the rat. J. Lipid Res., *1:* 159.
14. Friedman, M., and S. O. Byers 1959 Evaluation of nicotinic acid as an hypocholesteremic and anti-atherogenic substance. J. Clin. Invest., *38:* 1328.
15. Gaylor, J. L., R. W. F. Hardy and C. A. Baumann 1960 Effects of nicotinic acid and related compounds on sterol metabolism in the chick and rat. J. Nutrition, *70:* 293.
16. Nath, N., A. E. Harper and C. A. Elvehjem 1959 Diet and cholesteremia. IV. Effects of carbohydrate and nicotinic acid. Proc. Soc. Exp. Biol. Med., *102:* 571.
17. Hartroft, W. S. 1958 Animal fat transport. Diabetes, *7:* 221.
18. Schön, H. 1958 Effect of nicotinic acid on the cholesterol content of rat livers. Nature, *182:* 534.
19. Kritchevsky, D., J. L. Moynihan and M. L. Sachs 1961 Influence of thyroactive compounds on serum and liver cholesterol in rats. Proc. Soc. Exp. Biol. Med., *108:* 254.
20. Merrill, J. M. 1958 Effect of nicotinic acid on the incorporation of radiocarbon into cholesterol. Circulation Res., *6:* 482.
21. Perry, W. F. 1960 Effect of nicotinic acid and nicotinamide on incorporation of acetate into cholesterol, fatty acids and carbon dioxide by rat liver slices. Metabolism, *9:* 686.
22. Hardy, R. W. F., J. L. Gaylor and C. A. Baumann 1960 Biosynthesis of sterols and fatty acids as affected by nicotinic acid and related compounds. J. Nutrition, *71:* 159.
23. Linazosoro, J. M., R. Hill, F. Chevallier and I. L. Chaikoff 1958 Regulation of cholesterol synthesis in the liver: Influence of dietary fat. J. Exp. Med., *107:* 813.
24. Wilson, J. D., and M. D. Siperstein 1959 Effect of saturated and unsaturated fats on hepatic synthesis and biliary excretion of cholesterol by the rat. Am. J. Physiol., 196: 599.
25. Alfin-Slater, R. B., M. C. Schotz, F. Shimoda and H. J. Deuel, Jr. 1952 Effect of low fat and high fat diets on the synthesis of cholesterol in rats. J. Biol. Chem., *195:* 311.
26. Frantz, I. D., Jr., H. S. Schneider and B. T. Hinkelman 1954 Suppression of hepatic synthesis in the rat by cholesterol feeding. J. Biol. Chem., *206:* 465.
27. Parsons, W. B., Jr. 1961 Studies of nicotinic acid use in hypercholesteremia. Arch. Int. Med., *107:* 653.