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SAMUEL ERODY

SAMUEL BRODY
— *A Biographical Sketch*

(February 8, 1890 — August 6, 1956)

Sometimes there looms among scientists a figure who is a philosopher and humanist, as well as a dedicated and undeviating devotee of his own scientific field. Such a man can look with compassion and understanding on the successes and failures of his fellow men, of himself and of his students and colleagues, and humbly count himself blessed in their companionship. Such a figure was that of Samuel Brody, biochemist, physicist, dairy husbandry specialist and master of bio-energetics. He chose his field of research early in his career and, although sometimes tempted by promising leads into other types of problems, he remained true to his original choice.

The problems of bio-energetics, concerned with energy transformations in living things, he conceived to be generalized by the first and second laws of thermodynamics. Relatively few investigators were concerned with this broad field when Dr. Brody entered it and, indeed, relatively few joined him in it during the years of his career.

Samuel Brody was born in Lithuania in 1890. In 1906 he emigrated to Canada where he had been preceded by an older brother. He made his way as a miner, peddler, and briefly a commercial fisherman, moving then to New Hampshire where he had another older brother, I. A. Brody, still living. Several months as a machinist convinced him that he wanted to find work which would bring him into closer contact with living plants, animals and humans. Alone and unaided he made his way to the National Farm School in Pennsylvania. It was here that he heard of the work of T. Brailsford Robertson, then at the University of California in Berkeley, and he set out again to study under this unique scientist. In the meantime he had taught himself to read and write English and was able to enter the University of California by passing the necessary written examinations. He was interested in the physical chemical and thermodynamics researches of Gilbert N. Lewis and in their applications to biological problems, exemplified by the work of Jacques Loeb and T. B. Robertson. The imaginative theories of Robertson as to the dynamics of growth were especially fascinating to Brody and, indeed, it may be fair to say that he never deviated from this early devotion.

He was a member of the first seminar conducted by the writer of this biography in Berkeley in 1916 and here began his active interest in nutrition. The suggestion was made at some meeting of the seminar that the determination of the pH of blood and of the mechanisms for its maintenance was a problem susceptible of solution by physical chemical means. Within a few months Brody had developed apparatus for the determination of the pH of blood and had produced some fruitful speculations as to the role of buffers in this homeostatic mechanism.

In 1917 he received the A.B. degree with a major in biochemistry and, in 1919, the M.A. degree. In the meantime he had joined the U. S. Air Force where he served in both the aviation and chemical warfare service until the end of the war. The following year he served as assistant biochemist in the University of California Medical School. In 1920 he was nominated by W. R. Bloor, who had succeeded Robertson on the Berkeley campus, for a position on the staff of the Department of Dairy Husbandry at the University of Missouri. The appointment was made by Professor A. C. Ragsdale, who remained as administrator and colleague to Brody for the 36 years the latter spent on the Missouri campus. As Professor of Dairy Hus-

bandry, Brody also taught courses in the Department of Agricultural Chemistry and cooperated in the research program of that department.

In 1920 Dr. Brody married Sophie Edith Dubosky of Berkeley, who went with him to Columbia. They had two sons, Dr. Eugene B. Brody and Dr. Arnold J. Brody.

The tradition of scholarship and fundamental research was strong in the Dairy Husbandry department at Missouri for it had been cultivated by Dean H. J. Waters, C. H. Eckles and L. S. Palmer. Brody found it easy and natural to take up the studies of growth and energetics which he had begun at California. His first publications on growth of dairy cows and domestic fowls appeared in the Journal of General Physiology as early as 1921. Practical problems on the effect of age, stage of gestation and weight of dairy cows, upon the composition and volume of milk secreted were included in these early studies.

In 1928, after study at the University of Chicago during sabbatical leave from Missouri, he completed the requirements for the Ph.D. degree under the direction of A. J. Carlson. In 1930 and 1931 he studied in five European universities on a Guggenheim Foundation Fellowship. His leaves of absence were always used for scholarly purposes.

In 1926 the Herman Frasch Foundation became interested in Brody's fundamental studies and sponsored his research in growth and development of domestic and laboratory animals for many years. Altogether 66 Agricultural Experiment Station bulletins were issued under Dr. Brody's co-authorship detailing the results of the growth and development series. In 1946 a new phase of the work was entered, the study of the influence of climatic factors on physiological reactions and productivity in farm animals. In the next 10 years Brody published 39 bulletins in this field of environmental physiology and shelter engineering.

The U. S. Department of Agriculture, the National Research Council, the U. S. Navy and the Atomic Energy Commission were government agencies which contributed to the support of Brody's work. He never lacked for financial support of his researches.

In addition to his tremendous output of Experiment Station bulletins, Brody was author or co-author of 92 journal articles which appeared between 1921 and 1957 dealing chiefly with growth, growth curves, milk secretion, energy metabolism, thyroid function, aging, evaporative cooling and effect of ambient temperature and wind on dairy cows. Some philosophical and wide ranging articles also dealt with population control and the food supply, charts for estimating profit per cow and per unit milk, and the relativity of physiologic time and weight. He contributed widely to encyclopedias and reviews, usually on growth and nutrition. On the latter subject he reviewed extensively, in the 1934 Annual Reviews of Biochemistry, progress on the specific dynamic effect, net energy and feeding standards, the efficiency of animals and humans as transformers of energy, basal metabolic rates and the cost of work. The following year he discussed human adult nutrition and longevity with attention to atherosclerosis and diabetes, the setting of liberal standards for food intakes, problems in energetic efficiency with relation to body size, paired feeding and the relationship of endogenous nitrogen to basal metabolic rate. Many of Brody's speculations and suggestions in these reviews have pungent appropriateness even at the present time.

The most ambitious *tour de force* of Brody's career, however, was the compilation of his book, Bio-Energetics and Growth, with Special Reference to the Efficiency Complex in Domestic Animals, published in 1945 by the Reinhold Publishing Corporation of New York.

This volume consisted of 1023 pages made up of 25 chapters carrying more than 2000 references, more than 500 illustrations and some 113 tables of data. Much of this material was taken from the Experiment Station bulletins dealing with growth and development published during the author's first 25 years in the field. Brody avowedly strove for an integrating principle "which would show by a word or a phrase the interrelatedness of all the phases of growth, development and aging with the energetic efficiencies and

profits in milk, meat, eggs, and muscular work production through generalizing equations," so-called laws for integrating unwieldy bodies of data. Such a principle might be comparable with the theory of evolution. The nearest to such an integrating principle found by Brody was the ancient, perhaps vaguely felt, concept of physiological self regulation propounded by Claude Bernard and more recently designated as homeostasis by W. B. Cannon. This principle, in Brody's opinion, should apply to social as well as physiological self regulation, including that of human society.

Brody defined the "living field" as the totality of the interactions in the living system with the environment, internal and external. The living field pattern thus resembles the electromagnetic field pattern of the physicist, and the self regulation principle of Claude Bernard resembles the theorem of LeChatelier for non-living systems. The universality of homeostasis is illustrated in many forms, homeothermy, the maintenance of a constant level of oxygen and carbon dioxide in the blood during heavy muscular work, homeostasis of metabolism in relation to surface area and change in form. Homeostasis of growth is established through the observations that growth proceeds as if the "normal" condition were that of mature size and that the rate of growth tends to be proportional to the distance from the mature size. Senescence is defined as the gradual failure of homeostasis, and pathology as the attempt to maintain homeostasis under conditions of injury. Reproduction may be a compensatory mechanism against senescence, maintaining constant the internal social environment in spite of the aging of its constituent members. Body weight, body water, carbohydrate level, calcium level, fat level, oxygen and acid level are all cited at length as examples of physiological homeostasis.

Social homeostasis is extended from the mores of social insects to the finer integration of human society, apparently now in a transition period due to the unbalanced development of techniques. During growth and development the living "field" is thought of as a process which organizes the diverse elements into an integrated unit. This field is stable in a dynamic rather than a static sense. The individual units, atoms, molecules and tissues are undergoing continuous change but the pattern, "the field," remains until the homeostatic mechanism breaks down and the organism dies. This is a brief, and perhaps unfairly generalized, statement of Brody's integrating principle as set forth in his book.

If some of the recent discoveries had come sooner Brody undoubtedly would have modified some of his speculations on the future of human society, American agriculture and industry. He decided that we are approaching the end of the "free-energy" oil period and that aside from the renewable vegetation energy, free-energy sources of the future must be sought chiefly in hydro-electric developments and in water power from tides. The utilization of direct solar heat and of interatomic energy he deprecated as having little likelihood of producing practical results at least in the foreseeable future. He was somewhat pessimistic also about the inevitable decline in soil fertility, the unbridled growth of population in underdeveloped countries and the failure of increase in birth rate in this country during and following the depression of the 1930's. He described war as a blind social homeostatic mechanism, agricultural and industrial mechanization as a source of free-energy expenditure and deplored the dim prospect of change or remedy. But these speculations form only a very small fraction of the discussions and convincing expositions which characterize this remarkable book.

Probably the most useful feature of the book is the detailed exposition of methods of measuring heat production and of establishing prediction norms for it. The review of early literature is extensive and the description of metabolism apparatus, underlying assumptions, equations and calculations is complete. The metabolism in relation to body weight and surface area of all species is covered in much detail with recurrent conclusion that the "surface law" first proposed by Rameaux and Sarrus in 1838 is sound and derivable, not from area of cooling surface, but from physiologic bases, such as intensity of

blood flow, oxygen consumption, respiration rate, pulse rate and heat loss. Brody derived the relationship that basal metabolism of all species varies not with surface area directly, that is, $W^{2/3}$, but with $W^{0.73}$, or more simply, $W^{0.7}$, representing metabolically-effective body weight. He cites numerous concordant data from his own and other publications on many species from mice to elephants. He added that this reference base, $W^{0.7}$, may have still broader implications since it can be used also for endogenous urinary nitrogen and urinary neutral sulfur. Urinary creatinine, he thought, reflects the mass of muscle rather than the total of visceral organs and so varies more nearly with $W^{1.0}$ than with $W^{0.7}$. The quantity of milk-energy and egg-energy production likewise tends to vary with $W^{0.7}$. The latter might, therefore, Brody suggests, be adopted as the reference base for the prediction of all these transformations.

Growth rates were discussed at length in his book and the conclusion reached that the Robertson-Ostwald autocatalytic monomolecular equation, $dW/at = KW\ (A - W)$, is not applicable to the whole growth cycle of animals. The actual growth curves invariably show breaks, often of metamorphosis-like character. Brody suggests that the growth cycle be broken up into its constituent parts and each segment defined by separate equations. He separated Robertson's curve into two parts, $dW/dt = K_1W$ and $dW/dt = K_2(A - W)$, and successfully applied these to many growth curves for animals and humans. The wide range of illustrations is a striking feature of this, as of other sections of the book.

The unusual feature of Brody's treatment of bio-energetics is the extension of his efficiency calculations into the realm of practical agricultural economics, especially of milk and egg production. He discussed the varied aspects of milk production and utilization including nutritional, social and physiological phases and followed this by consideration of its monetary economy. The gross energetic efficiency of milk production he believed to be essentially independent of live weight of the cow and to be superior to that of other feed transformations, that is, 33 to 50 per cent, about twice the efficiency of egg production and five times that of meat production. Monetary efficiency, on the other hand, he shows to be governed by something he called the "dairy merit" or gross energetic efficiency of the lactation process of the cow; that is, the percentage of feed energy that is converted into milk energy. The capacity factor, actual weight of milk produced, is apparently proportional to body size, $W^{0.7}$. The formula,

$$\text{dairy merit ratio} = \frac{61\ \text{FCM}}{\text{FCM} + 0.173\ W^{0.73}},$$

is offered as a yardstick for judging the value of dairy animals. This is based on the division of the energy of the total digestible nutrients (TDN) energy between fat-corrected milk, 4% fat, (FCM) production and maintenance as follows:

$$\text{TDN} = 0.305\ \text{FCM} + 0.053\ W^{0.73}.$$

When asked some years ago by a newspaper correspondent to name any results of the growth and development experiments that might be useful to a farmer, Dr. Brody readily dictated the following examples:

"The analyses of our data through the years have brought to light many bits of agriculturally useful information. For example, how the costs of the animal's feed conversion processes (metabolism) and maintenance vary with weight and age; how temperature influences productive processes; how to evaluate the partition of feed consumed between maintenance, growth and milk or egg production; how the plane of nutrition influences the efficiency and profit of milk production; and the relative energetic efficiency of meat, milk and egg production.

"To elaborate on the last point, milk production is the most efficient of the three processes. Up to 50 percent of the total digestible nutrients consumed may be converted into milk; and that is twice as high as the maximum efficiency of egg production and several times as high as that of beef production. The efficiency of meat production is fairly high, about 30 percent, when the animal is very young; but it declines rapidly with increasing age. These are not arguments in favor of milk or against eggs and beef production, because

the consumption of eggs and beef is not on the basis of economy alone.

"Also, we found that the productive level is a very important aspect of the profitableness of any animal enterprise. While the maximum efficiency of milk production is 50 percent, most 'good' cows produce milk at an efficiency of only 25 percent, which just about pays for the dairyman's labor, feed and other expenses. An efficiency of 33 percent indicates a 'superior' dairy cow and a profitable one. But the profit still is complicated by body size, age and related factors."

A typical review of Brody's book is that of F. S. Hammett, editor of Growth, the periodical to which Brody contributed so much from its inception. Hammett commented as follows:

"These thousand pages packed with charts, tables, illustrations, references to some three thousand investigators, and the scholarly analysis and comment of the author, mark the product as a classic. It is a competent, efficient, comprehensive, practical, philosophical job. It is competent because it presents the data fully, concisely, logically. It is efficient because the reader can find what he wants clearly stated. It is comprehensive because it not only covers the central theme, but also the ramifications thereof. It is practical because it integrates maintenance, growth, and the products of growth with the cost thereof. And it is philosophical because it indicates how bio-energetics and growth are related to all our social, economic, political, military and associated problems. Moreover, Dr. Brody does it calmly with no drum- or breast-beating: no belaboring of any pet thesis, but just simply and convincingly. This man is a scientist."

This biographer can only say amen to Hammett's review of the book. Seldom has so comprehensive, scholarly, and original a tome been added to our scientific heritage. No rival in its field has appeared and Brody's book will doubtless long remain the reference book *par excellence* on bio-energetics and growth.

Some attention is given in this book to environmental influences on energetic efficiency but most of Brody's work in this field was done after the book was published. In 1946 Brody took on a new project with strong Federal support, the study of the effect of climatic conditions on the efficiency of animal energy transformations. The Psychroenergetic or Climatic Laboratory, built in 1948 on the University of Missouri campus, provided for control of temperature, humidity, air movement, light and ventilation rate independently in the two chambers, each of which housed six mature cows or nine calves for the experimental studies. The performance of Jersey, Brown Swiss and Holstein was compared with that of Texas-bred Brahman cows under varying conditions of temperature, light and wind exposure. This project was a cooperative one between the U. S. Department of Agriculture and the departments of Dairy Husbandry and Agricultural Engineering and is still in operation.

The efficiency of heat production and heat elimination, of feed and water consumption, and of milk production was examined at temperatures ranging from 8° to 105°F. The European-bred cows exhibited the best efficiencies at about 50°F. The Brahman cows showed decline in milk yield only above 90°F. Water consumption was irregular but feed consumption, thyroid activity, heat production, and body weight decreased in the European-bred cows at the higher temperatures. Rectal temperature rose 5 to 8°F. when the ambient atmosphere was above 70°F. The amount of heat dissipated by vaporization and insensible weight loss was found to rise sharply at temperatures above 92°F. The effect of high temperatures upon reflection of visible radiation from the hair of Brahman and Brown Swiss cows showed much more rapid increases in the hair of the Brahmans at high temperatures. Changes in blood composition were detected only above 65°F. In all the cows creatinine levels were doubled, carbon dioxide combining capacity, ascorbic acid and cholesterol levels were halved at the high temperatures. The causes of these changes were stress and reduced food intake. The distress symptoms caused by the high temperatures were most marked in the Holstein cows, next in the Jerseys, Brown Swiss and were least marked in the Brahmans. Declining temperatures (0°F.) affected most adversely heat production

and feed consumption in the low producing small Brahman cows and least adversely the European-bred cows. The low heat tolerance of the cows was thought to be due to their low moisture vaporization for heat dissipation and high heat production per unit surface area. Although neither the European nor Indian cattle "sweat," the Brahman cows withstood the high temperatures better than the others, probably because of their lower heat production, lower basal metabolism and lower milk production. These low initial levels provide a greater range for increase during stress. Brody recognized that the low productivity of the Indian cattle counterbalanced their heat tolerance. He recommended selective breeding to obtain more sweat-producing, water-drinking, heat-tolerant cows of high productivity. Practical suggestions on engineering of shelters to shield the cattle from direct sunlight, and to provide sprinkling systems and similar evaporative devices were also elaborated.

No review of the scholarly work of this man can convey the quality of his mind and personality. His colleagues at Missouri recognized his unique ability to call forth loyalty from students and also to stimulate them to strive for the best possible performance. He criticized them sternly, encouraged them to try their own ideas, but never failed to show them how to improve their product. Above all he scorned mediocrity.

He had a remarkable sense of humor of the broad homely type characteristic of Abraham Lincoln. He never failed to enliven his lectures, whether to a class of students or to a learned society, with an unexpected and often earthy simile. There were those who misunderstood these sallies and resented them, but Brody never changed his style nor apologized for what he knew to be human and honest.

He was of a philosophical turn of mind, scornful of materialism, and, in his later years, much concerned with religious and ethical issues. He was naively modest and almost childlike in his life long search for truth; that is, for a unifying principle, not only in the energetics of living things, but in the life of man. He sought for, and sometimes thought he found, biological and evolutionary significance in man's religious and ethical beliefs.

He cared little for material things, for adulation or social prestige. He travelled on buses and coaches in order to meet and enjoy "the real people." This genuine humility shone through his everyday actions and illuminated his relations with students and colleagues. "Not Dr. Brody's laboratory, *our* laboratory" was his reminder to any new office assistant. This modest genius so impressed his students that, as one of them wrote, "Dr. Brody will be my unseen co-author for the rest of my life."

The driving force of Brody's life was his scientific ambition. Indeed, he left himself little time for anything else than his work, even during the last 20 years of life which he lived calmly and patiently under the threat of recurrent attacks of angina pectoris. He could be found in his office or laboratory seven days a week, often late at night, standing at his drawing board, writing, calculating or drawing diagrams. The routine of laboratory measurements and techniques he left largely to his assistants and students although he was sharply critical of both methods and results. Much of his genius lay in the ability to coordinate seemingly unrelated data and to see a logical pattern leading to mathematical development of natural relationships. He adopted the use of statistical devices, well ahead of the majority of biologists. His great ambition was to uncover a universal principle in biology, perhaps relating homeostasis to the principles of thermodynamics somewhat like Hamilton's principle of least action or a philosophic insight such as Occam's Razor. He came near to his goal and if he failed in attaining it, the failure was indeed a magnificent one. No lesser man could have envisaged this ambition.

The end came on August 6, 1956, when Dr. Brody, alone in his workrooms in Eckles Hall on the University campus at Columbia, succumbed to coronary occlusion. Only the week before, he had returned from a trip West, full of plans and

ideas for new researches. The manner of his going accorded well with his unpretentious, often lonely life.

ACKNOWLEDGMENTS

I am happy to acknowledge the important help given me by Samuel Brody's colleagues and former students in writing this sketch. In particular, assistance was offered by A. G. Hogan, R. E. Stewart, A. C. Ragsdale, J. H. Longwell, C. F. Winchester, U. S. Ashworth, L. E. Washburn, H. E Dale, and D. E. Worstell. In addition, valuable data were provided by Eugene B. Brody, W. R. Bloor and H. H. Mitchell.

AGNES FAY MORGAN
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Maintained Pregnancy in the Rat as Associated with Progesterone Administration and Multiple-Nutrient Deficiency

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Previous studies have shown the beneficial effect of ovarian hormones upon the maintenance of fetal life in the rat pregnant for 18 to 23 days, when fed diets deficient in pyridoxine (Nelson, Lyons and Evans, '51), protein (Nelson and Evans, '54), thiamine,[1] or potassium.[2] Other investigators have reported a similar effect on the vitamin A-deficient rabbit (Hays and Kendall, '54, '56) by progesterone.

The ability of ovarian hormones to aid in the maintenance of pregnancy, when any one of the foregoing dietary deficiencies occurs, raised a question whether fetal life could be maintained with the aid of ovarian hormones, providing the diet fed was deficient in more than one essential nutrient.

The observations reported here were made to determine whether fetal life, through the aid of ovarian hormones, could be maintained under conditions of (1) a multiple deficiency diet, and (2) a diet containing a single energy source, sucrose.

EXPERIMENTAL

Fifty-six virgin female rats (Holtzman strain) were placed in 6 groups, on the day of mating, which was determined either by vaginal smears or by the presence of vaginal plugs in the litter trays. The females were individually caged and beginning on the day of mating, fed the special diets for 20 days. Group A received a complete diet (Nelson and Evans, '53). Group B was fed a similar diet except that the 4% mineral mixture was replaced with an equal quantity of sucrose, thus reducing the mineral content of the diet to 0.38% ash. The phosphorus content of the diet was 0.21%. The diet for group C was made up of 92% of sucrose (CP) and 8% of hydrogenated vegetable fat,[3] while groups D, E, and F received sucrose only. Each female of group E received daily injections of 5 mg of progesterone in sesame oil and 0.5 μg of estrone, while those of group F received a daily injection of 5 mg of progesterone. Group A had access to tap water and the others to distilled water.

The females were weighed at 48-hour intervals. On the 20th day after mating, the females were autopsied. Uteri were examined for evidence of pregnancy. *Corpora lutea* were counted and checked against the number of implantation sites. Fetuses were weighed and analyzed for dry matter, total nitrogen and ash. The young in each litter were divided randomly into two groups, with essentially one-half of the fetuses of a litter being analyzed individually for dry matter and ash, with the remaining young within a given litter being individually analyzed for total nitrogen. Dry matter was determined by heating each individual fetus in a crucible to 100°C until constant weight was reached. The dried fetuses were then ashed at a temperature of 575 to 600°C for 8 hours. Total nitrogen was determined by the Kjeldahl method (Association of Official Agricultural Chemists, '55).

Received for publication August 5, 1959.

[1] M. M. Nelson, and H. M. Evans 1954 Effect of thiamine deficiency on reproduction in the rat. Federation Proc., *13:* 470 (abstract).

[2] M. M. Nelson 1955 Interruption of pregnancy by potassium deficiency and correction by ovarian hormones. Federation Proc., *14:* 446 (abstract).

[3] Crisco.

RESULTS AND DISCUSSION

The changes in female weights, the evidence of pregnancy, number of *corpora lutea,* average fetus weights, and average per cent of dry matter, total nitrogen and mineral content of individual fetuses, are presented in table 1.

Females fed the low mineral diet had an average litter size per mating of 9.4 living young, weighing 2.79 gm as compared with the average litter size per mating of 9.8 young weighing 3.78 gm from females fed a complete diet. Limiting the females' diet to sucrose and fat, resulted in an average litter size of 5.0 living young per mating, with an average fetus weight of 2.23 gm. When the diet was limited to sucrose, the number of females carrying viable fetuses declined to one out of 8 with an average of 1.1 young per mating, and a fetus weight of 2.31 gm.

The daily administration of 5 mg of progesterone and 0.5 μg of estrone to females fed sucrose, resulted in 3.8 living young per mating weighing 2.71 gm. However, where progesterone was administered at a level of 5 mg to the sucrose-fed females, an average of 7.1 living young per mating weighing 2.12 gm was observed. Thus, it seems that estrone may have contributed to the decrease in litter size. This possible influence might not have manifested itself had the injections of estrone been started several days after mating.

The body composition of fetuses, produced under these varied and limited dietary regimens, showed a highly significant difference in dry-matter content, but little variation between litters on comparison of total nitrogen and mineral values. This was in marked contrast to the differences observed in litter sizes and fetus weights.

Five of the 8 females fed sucrose only, showed evidence of having been pregnant when killed. The other three females of this group may have had implantations and possibly resorbed the fetuses due to the stress of nutrient deficiency manifesting itself, earlier in some of the females than in others.

Changes in body weights of the females reflected demands upon maternal stores for nutrients for fetal development and maintenance. Although the differences in average weight change between the starting and final weights for the sucrose-fed females in both groups D and F were 60 and 49 gm, respectively, the actual average losses in female body weights were 62.5 and 64.0 gm, in the same order, when the weights of fetuses were deducted from the final weights of the females before killing.

TABLE 1

Evidence of pregnancy as associated with diet and hormone treatment

Animal group	A	B	C	D	E	F
Diet:	Complete	Low mineral	Sucrose + fat	Sucrose	Sucrose	Sucrose
Added treatment:	None	None	None	None	P,[1]E[2]	P[1]
Females:						
Mated	16	8	8	8	8	8
Were or had been pregnant	15	7	8	5	7	8
Weight change, gm	+58	−2	−65	−60	−45	−49
Weight change, %	+24	−0.0	−24	−25	−24	−23
Corpora lutea:	14.9	17.0	15.0	11.8	11 1	11.7
Young:						
Total living/mating	9.8	9.4	5.0	1.1	3 8	7.1
% of implants	100	91	48	16	65	95
Fetus weight, gm:	3.78	2.79	2.23	2.31	2.71	2.12
Fetus composition:						
Dry matter, %	12.9	11.4	11.8	12.3	12.3	11.7
Total nitrogen,%[3]	1.40	1.25	1.36	1.36	1.34	1.37
Mineral, %[3]	1.40	1.32	1.29	1.34	1.36	1.34

[1] Progesterone 5 mg daily. [2] Estrone 0.5 μg daily. [3] Fresh basis.

Nelson and Evans demonstrated the ability of the rat to maintain pregnancy when subjected to diets deficient in pantothenic acid (PA) ('46) or pteroylglutamic acid (PGA) ('49) starting on the day of mating. Their later work (Nelson and Evans, '56) demonstrated the failure of 4 mg of progesterone, either with or without estrone, to maintain pregnancy when the rat was placed on the PA deficient diet, on the day of mating, or 25 to 30 days before mating. This failure of ovarian hormones to maintain pregnancy does not necessarily conflict with the results of the present studies, since in the former work the deficiency stress existed for a longer period, with different levels of hormones, and a different strain of rats being used.

The maintenance of pregnancy in the sucrose-fed rats given 5 mg of progesterone daily as shown in these studies, suggests that the progesterone, in some manner, either directly or indirectly played a role in the mobilization of nutrients from maternal stores for fetal growth and development or contributed to the maintenance of placental function.

Whether the stress of nutrient limitation resulted in the failure of the *corpora lutea* to secrete adequate levels of progesterone (possibly due to a lowered functioning of the anterior pituitary) is not evident from the present studies.

SUMMARY

The feeding of a diet containing 0.38% of minerals but otherwise adequate from the day of mating, until autopsy 20 days later, resulted in normal litter size with an average fetus weight 76% of normal.

When a diet, containing 94% of sucrose and 8% of hydrogenated vegetable fat was similarly fed, the number of living young at autopsy was 47% of normal, with fetus weights averaging approximately 60% of normal.

The daily injection of 5 mg of progesterone was accompanied by an increased litter size, from 1.1 to 7.1 young per mating, when females were fed, for 20 days following mating, a diet consisting solely of sucrose and distilled water. Under these conditions the average fetus weight was reduced to 56% of normal.

The results of these studies suggest that progesterone is related either directly or indirectly to the mobilization of nutrients from maternal stores or to the maintenance of placental function, thus permitting the life of the fetus to be maintained for a period of 20 days.

ACKNOWLEDGMENT

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Porcine Neonatal Nutrition: The Effect of Diet on Blood Serum Proteins and Performance of the Baby Pig[1]

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In our experience it has been difficult to raise pigs taken at birth from their dam (Weybrew et al., '49; Barrick, Matrone and Osborne, '54). Most pigs developed diarrhea around the 4th day, and if unmedicated, approximately 60% died. Those surviving the first two weeks were able to make proper weight gains, provided they remained in isolation (Lecce, '59). Bellis ('57) recently summarized this situation with the statement that "mortality increases with progressively earlier weaning, and the survival of piglets that have not received colostrum is very unlikely . . . ."

Many investigators[2] (Brambell, '58) have shown that certain species of animals (pigs included) are born devoid of gamma globulin, and it is the function of colostrum to supply this missing protein to the newborn animal. Antibody-rich-colostral-globulin is believed to cross unaltered from the gut to the serum of the nursing pig within the first 36 hours. Thus, the offspring is given a ready supply of globulin before its own synthetic mechanisms can fulfill this need. Further, colostral globulin, carrying immune bodies or antibodies, endows the piglet with passive immunity to infectious disease—accounting for the vigor in the pigs fed colostrum. No such resistance to disease is expected in the non-immune, colostrum-free pigs; hence, the difficulty in raising them.

Beyond supplying antibodies, very little has been implied concerning other means whereby colostral proteins might function in disease resistance. No doubt the ease of measuring antibodies, coupled with the traditional role of antibodies in disease resistance, contributed to an emphasis of this aspect of colostral proteins. The fact that colostrum-free pigs can not synthesize their own antibodies before they are approximately 8 weeks old (Hoerlein, '57), and that it was not difficult to raise these antibody-free pigs once they were over two weeks old, suggested that more was involved in this early period than can be explained by immunity imparted by antibodies. The experiments reported herein were designed to obtain some insight into this problem by studying the influence of diet on the reaction of piglets, along with the development of their serum protein patterns.

EXPERIMENTAL

Diets. The following 4 diets were used in this study: (1) Sow's milk—administered by allowing the pigs to nurse their sow from the time of birth; (2) Cow's milk—fortified with 5 ppm of copper, 50 ppm of iron, and cod liver oil; (3) Artificial milk—compounded to simulate cow's milk (table 1); and (4) "Amino acid milk"—the same as artificial milk except that instead of whole casein and lactalbumin, the enzymatically hydrolyzed sources, casein hydrolyzate and lactalbumin hydrolyzate, were used. (Because of incomplete hydrolysis, peptides as well as amino acids are found in these preparations).

Individually-caged piglets were fed the diets (other than sow's milk) 5 times a day from shallow pans. The volume per feeding was the approximate amount the pig would consume in two hours—essentially, non-restrictive feeding.

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[2] ——— 1958 Intestinal permeability to proteins. Nutrition Rev., *16:* 342.

TABLE 1

Composition of artificial and amino acid milk diets[1]

Constituents	Artificial milk	Amino acid milk
	%	%
Casein	2.0	—
Lactalbumin	1.5	—
Casein hydrolyzate[2]	—	2.0
Lactalbumin hydrolyzate[2]	—	1.5
Lactose	2.0	2.0
Glucose	1.5	1.5
Lard	3.0	3.0
Vitamins in glucose[3]	0.5	0.5
Minerals in glucose[4]	0.5	0.5
Cod liver oil	0.05	0.05
Water	89.95	89.95

[1] Diets were homogenized at 3000 pounds pressure.

[2] Enzymatic casein and lactalbumin hydrolyzate, Nutritional Biochemicals Corporation, Cleveland.

[3] For 5 pounds of vitamin mixture: thiamine·HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 gm; Ca pantothenate, 1.42 gm; pyridoxine HCl, 570 mg; folic acid, 57 mg; *p*-aminobenzoic acid, 1.13 gm; inositol, 11.35 gm; biotin, 11.4 mg; choline chloride, 113.45 gm; menadione (2-methylnaphthoquinone), 115 mg; 0.1% vitamin B_{12} (with mannitol), 4.66 gm; α-tocopherol acetate, 570 mg; glucose, 2132 gm. Acknowledgment is gratefully made to Merck and Company for contributing the vitamins.

[4] For 5 pounds of mineral mixture: $CaHPO_4$, 818 gm; KCl, 273 gm; NaCl, 239 gm; $MgSO_4$, 204 gm; $CuSO_4 \cdot 5H_2O$, 893 mg; $FeSO_4 \cdot 2H_2O$, 7648 mg; $MnSO_4 \cdot H_2O$, 1399 mg; ZnO, 2263 mg. $CoCO_3$, 9 mg; glucose, 722 gm.

Experimental pigs. Piglets used were of two types: (1) artificially raised and (2) natural, sow raised. Methods employed for securing and maintaining artificially raised piglets under sanitary and isolated conditions are detailed in another report (Lecce, '59). The sow-raised pigs were procured essentially as the artificially raised pigs, except after the desired number had been obtained, they were simultaneously returned to the sow in an isolation unit.

In preliminary experiments, it was extremely difficult to keep all of the pigs alive long enough to measure dietary effects. For example, it was not unusual to lose approximately one-half of the pigs fed cow's milk, and with the amino acid milk, 92%, of the pigs died. Therefore, the results reported were obtained from pigs under intense medication, that is, either systemic chloromycetin,[3] because it was effective against coliforms, or injections of pooled 10% porcine gamma globulin.[4]

Mainly, the results in this study came from experiments using pigs from two litters. In the first experiment, 6 littermates were assigned the amino acid milk diet and 6 to the artificial milk-diet. Within 8 hours of birth, three pigs on each diet were injected intraperitoneally with 15 ml of pooled porcine gamma globulin. Treatments were assigned randomly. In addition, all pigs were injected intramuscularly with 40 mg of chloromycetin at intervals of one-and-a-half days. In the second experiment, at random, approximately 6 hours after birth, 6 pigs were returned to their sow, and three pigs assigned the cow's milk diet. The three artificially raised pigs were injected at birth with 15 ml of pooled porcine gamma globulin and at one-and-a-half days and 4 days with 40 mg of chloromycetin. Results of observations of individual pigs assigned the same treatment were averaged and reported as such.

Blood chemistry. Serum was obtained from 3 ml of clotted and refrigerated blood by bleeding the pigs from either the heart or the anterior vena cava. Generally, pigs were bled at time of birth and at two, 4, 7 and 14 days.

Relative concentrations of serum proteins, as well as sow's colostral-whey proteins, were determined with commercial paper electrophoretic apparatus according to techniques previously described (Lecce and Legates, '59). The mg/ml of the various protein fractions were calculated by multiplying the relative per cent of a particular fraction by the total amount of serum protein. The total amount of serum protein was estimated by refractive index.

Trichloroacetic acid (TCA) precipitable material was determined by placing 0.05 ml of serum in 4 ml of distilled water, adding one ml of 10% TCA, and recording, after 15 minutes, the optical density (OD) of the resulting precipitate at 540 mμ.

[3] Chloromycetin was contributed by Parke-Davis Co.

[4] Porcine gamma globulin was contributed by Armour and Company, Kankakee, Illinois.

Bacteriology. At each bleeding session, fecal samples were obtained from the pigs' rectum with a cotton swab. Immediately after sampling, the cotton swab was transferred to one milliliter of trypticase soy broth. A loopful of this broth (containing the fecal suspension) was then cultured on a blood plate. The plates were examined after 24 to 48 hours of incubation at 37°C for colonies of bacteria. Representative colonies were selected and identified by standard bacteriological techniques. An attempt was made to identify not only kinds of bacteria, but also relative numbers of bacteria so that shifts in microbial populations might be discerned.

RESULTS

Comparison of the neonatal serum protein pattern with the adult serum protein pattern. The serum electrophoretic pattern of the newborn, unfed pig is of interest. Typical observations are shown in figure 1. Neonatal serum was notable in the following respects: (1) albumin was a minor fraction; (2) a protein migrating at the rate of alpha globulin was the major fraction; (3) gamma globulin was lacking completely; (4) total serum proteins were low; and (5) serum proteins were not precipitable by TCA. The extreme character of this neonatal pattern was made obvious in comparison with its counterpart from adult serum. Here, elevated amounts of albumin, beta and gamma globulin, double the total serum proteins, and TCA precipitable material were found. One may then, for the sake of clarity, characterize the neonatal extreme as an "immature serum pattern" and the other extreme as a "mature serum pattern."

Effect of diets on serum protein patterns. Results of the analysis of serum from nursling pigs and from pigs fed the three experimental diets are shown in figure 2. In pigs being nursed by the sow rather intense and immediate changes in serum proteins were noted as well as a continuous and rapid shift toward the mature serum pattern. For example, after sucking for 48 hours: (1) the total serum proteins doubled; (2) beta and gamma globulin albumin increased; and (3) serum proteins became precipitable with TCA. Subsequently, the amount of albumin and beta globulin increased whereas alpha and gamma globulin decreased. Throughout this period, total serum protein and the amount of TCA precipitable material approached or remained essentially at the normal, mature level. In less than two weeks the serum pattern was similar to a mature serum pattern.

Apparently, pigs fed the amino acid milk lacked the capacity to react to treatment to the extent found in pigs nursing the sow. In contrast to the latter pigs, those fed the amino acid diet showed little tendency to increase total serum proteins, albumin and beta globulin fractions and

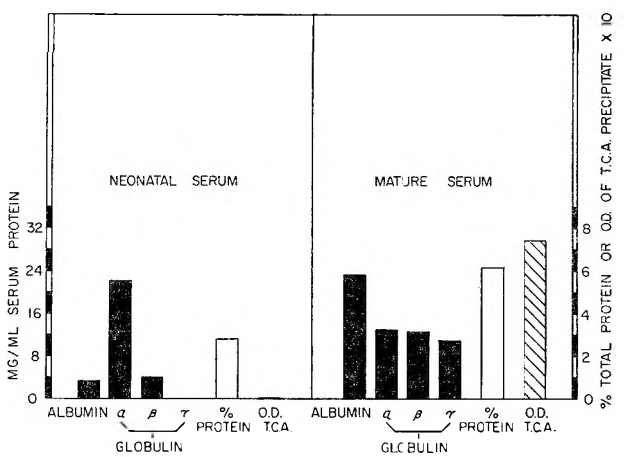

Fig. 1 Serum protein profile, total protein, and optical density of trichloroacetic acid precipitate of neonatal pig serum and mature pig serum.

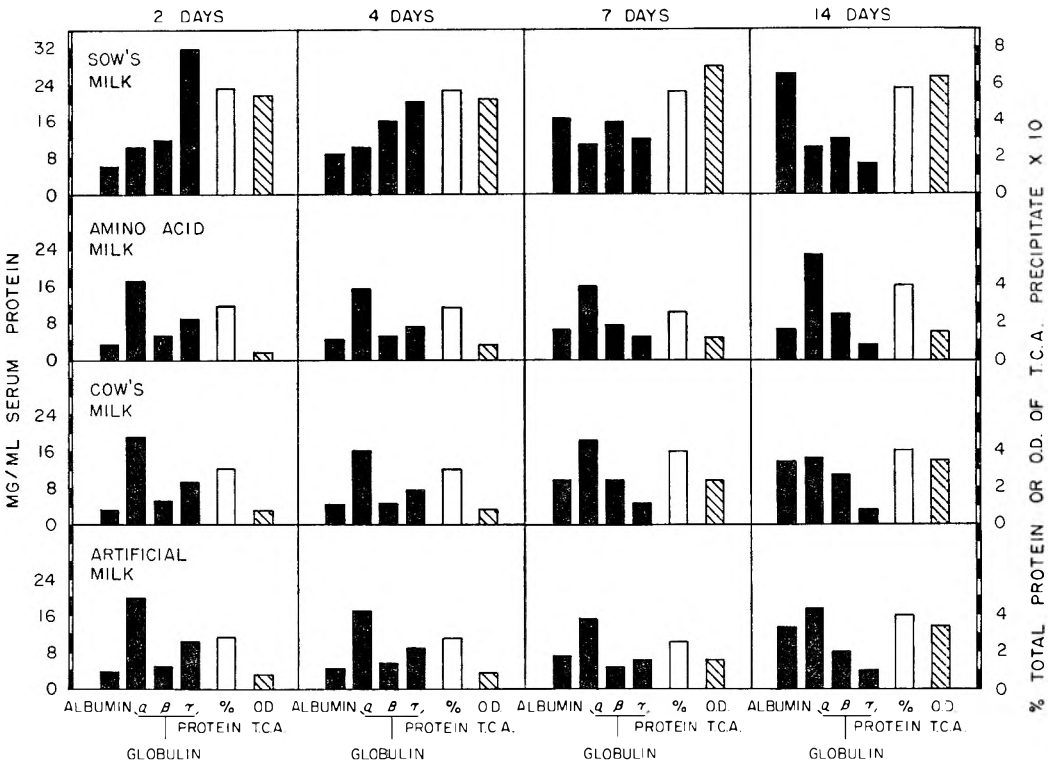

Fig. 2 Effect of diets on serum protein profile, total protein, and optical density of trichloroacetic acid precipitate of serum from pigs from two to 14 days old.

amount of TCA precipitate. In other words, the amino acid diet was remarkable in that it had very little capacity to promote normal changes associated with serum protein development.

Pigs fed cow's milk and artificial milk showed intermediate results between the two extremes of those fed sow's milk and amino acid milk. However, it was of interest to note that in the first 4 days, very little difference was observed between the pigs fed the amino acid milk, artificial milk and cow's milk. Regardless of diet, at the end of 4 days, all pigs showed essentially the same immature pattern present at birth. Thus, for most of this early period, at least, these three diets appeared to be equally inadequate in providing for the protein needs of the piglets. However, from 4 days on, effects of the different diets were detected. Changes observed in pigs fed artificial milk and cow's milk began to simulate those observed with the pigs fed cow's milk (except for a slower rate). In these pigs, albumin and beta globulin, total serum protein, and the amount of TCA precipitable proteins increased, whereas similar changes were not observed in the pigs fed the amino acid milk.

Comparison of sow's colostral-whey proteins with neonatal serum proteins and neonatal serum proteins obtained from pigs that have nursed the sow for 4 days. Figure 3 shows that sow's colostrum contains proteins which migrate at the rate of albumin, beta and gamma globulin. These fractions were low in neonatal serum, but rapidly increased after piglets nursed the sow.

Performance of piglets fed different diets. Only the pigs nursing the sow gained weight rapidly (fig. 4). Pigs fed cow's milk, artificial milk and amino acid milk gained little or no weight the first week, but from then on, weight increases were observed. These changes in weight, as well as vigor, coincided with changes toward

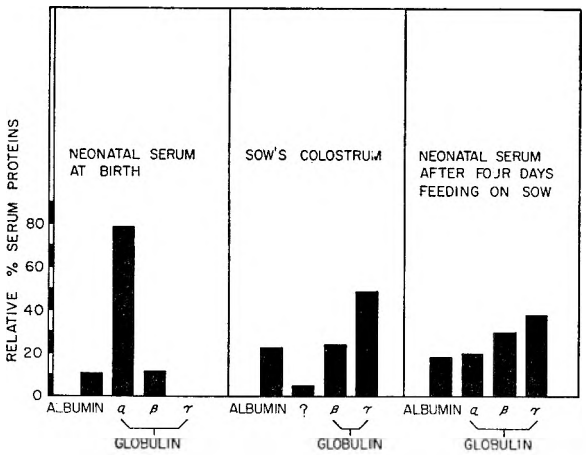

Fig. 3 Serum protein profile of neonatal pigs, sow's colostrum, and pigs nursing the sow for the first 4 days.

the development of a mature serum protein pattern; namely, during the period in which little changes were observed in the neonatal serum pattern, the piglets did not grow, and were most susceptible to diarrhea, bacteremia, and death. Even with the intense treatment (porcine gamma globulin and chloromycetin), three of the 6 pigs fed the amino acid diet died by the 14th day, and the body conformation of those living left much to be desired (fig. 5). Simply, the animals fed amino acid milk continuously for two weeks seemed to be suffering from acute protein malnutrition; blind, approximately 2 cm of subcutaneous edema, cream colored and sparse skeletal muscle, flattened rib cage and seemingly not enough elasticity in the joint cartilage to keep the femoral head in its socket. It is interesting that littermates fed artificial milk (same as the amino acid milk except for whole casein and lactalbumin) did not exhibit these marked abnormalities.

Bacteriology. With our sampling technique, there was no apparent dietary influence on the bacterial flora of the feces. Essentially, with all the diets, coliforms predominated, followed by streptococcae, staphylococcae, proteus and pseudomonas. Sometimes the coliforms were hemolytic, but no pathogenic significance could be attached to this activity.

DISCUSSION

Our results, along with others[2] (Rutqvist, '58; Nordbring and Olsson, '57; Brambell, '58), show that the baby pig is born free of gamma globulin. When viewing the serum protein pattern in its totality, however, other extremes also are impressive: very low albumin, over 75% of the protein an alpha-like protein and the main mass of the protein not precipitated by TCA, (in this respect resembling fetal protein or fetuin (Bettelheim-Jevons, '58.)

As mentioned in the introduction, much of the research with colostral proteins has centered around the effect of colostral-immune globulins on new-born serum antibody titers and resistance to infectious disease. Without minimizing the importance of antibody globulin, the protein-deficient immature pattern described above, plus the parallelism between the development of a mature serum protein pattern and performance, raises a question whether more is required of colostrum, with respect to influences on serum proteins and piglet resistance, than can be accounted for by the antibodies alone. Pigs fed colostrum immediately upon birth effected changes in serum proteins involving albumin, α-, β-, and γ-globulin. Furthermore, the serum protein pattern of pigs fed colostrum changed rapidly from the im-

[2] See footnote 2, page 13.

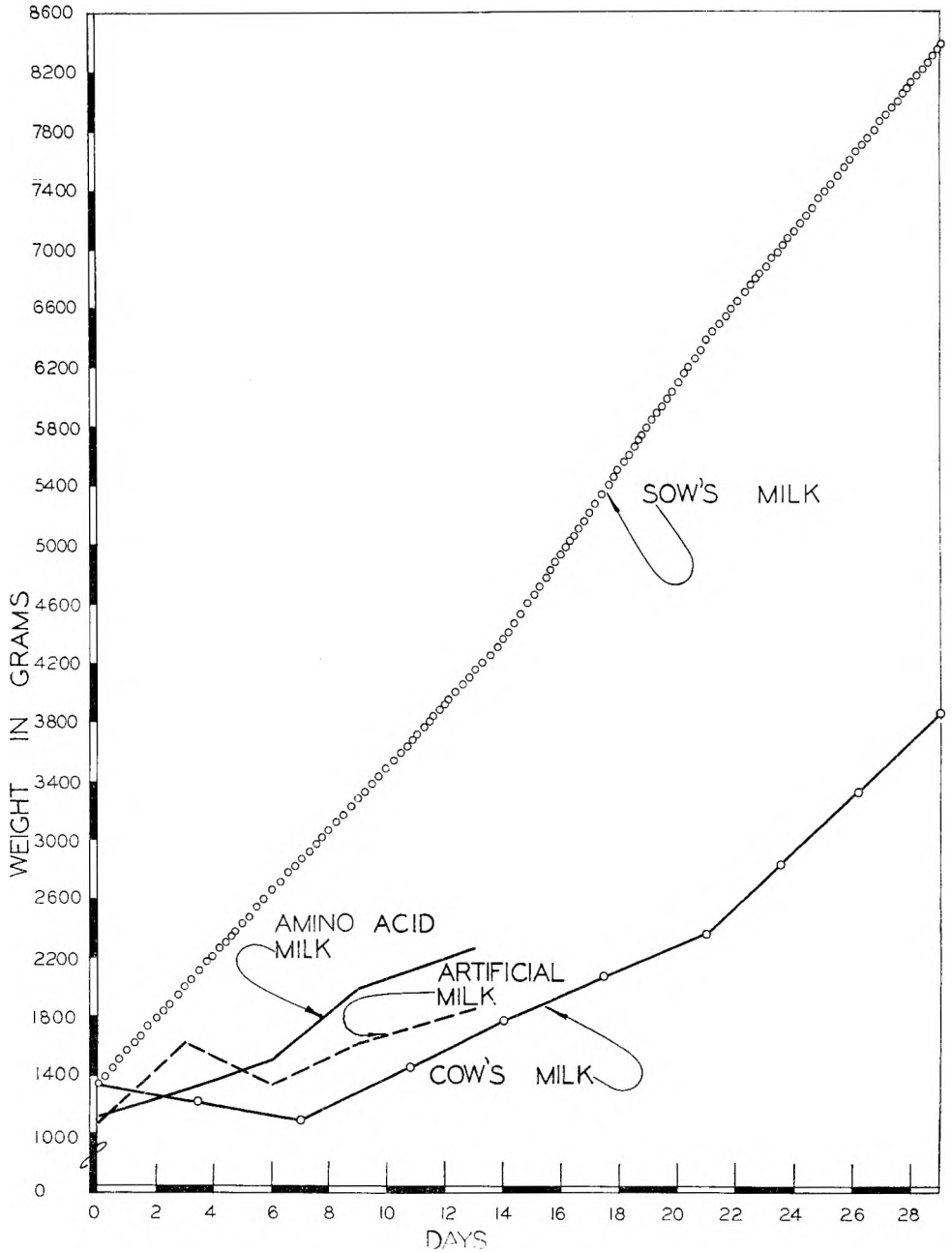

Fig. 4 The effect of diet on weight gains of pigs.

mature pattern to the mature pattern, whereas no immediate change occurred in pigs fed a "balanced diet" such as cow's milk. In other words, those pigs denied colostrum had a latency in the development of a mature serum protein pattern including all the serum proteins. Pigs with the static, immature serum protein pattern were most susceptible to diarrhea, bacteremia and death. Inefficient digestive capacity during the piglets' first few days of life could explain in part this delay in

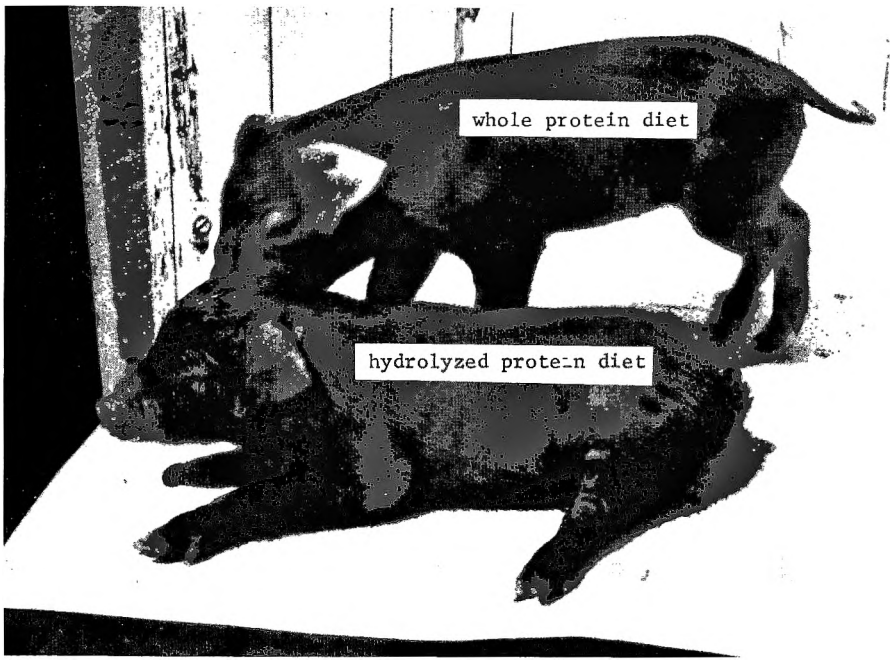

Fig. 5 The effect on body conformation of pigs of feeding the amino acid milk and artificial milk, continuously for two weeks.

the production of serum proteins, and evidence exists that the new-born pig has low proteolytic activity (Catron, Baker, and Hartman, '57; Braude et al., '58). Our results, however, with the amino acid diet suggest that an impairment in synthetic ability might also be operating.

In conclusion, the following hypothesis is offered: the piglet is born with a deficiency in albumin, β and gamma globulin. The sow, with her colostrum and milk, rapidly fills in these deficiencies by supplying proteins which are readily absorbed, unaltered, by the piglet. Also, the sow supplies factor(s) that set in motion the synthetic mechanisms of the piglet. This results in the serum protein pattern of the piglet changing rapidly from an immature pattern to a mature pattern, whereas no such change is found in litter-mates fed a balanced diet such as cow's milk. The piglet making rapid changes in his immature pattern is more vigorous and resistant to disease. This, in part, can be explained by the presence of antibodies in colostral globulin and, in part, by less definitive factors involving "stress" arising in piglets burdened with a latency in the synthesis of serum proteins.

Work underway at present is designed to separate the benefits derived from antibody protein from other benefits in colostral protein; also to study reasons for, and means of overcoming, the seeming latency in the development of protein metabolism in the piglet.

SUMMARY

Neonatal serum protein patterns from unfed pigs were compared with serum from mature pigs. This yielded a profile of an immature serum protein pattern and a mature pattern. The capacity of 4 diets to augment and influence changes from the immature to the mature serum protein pattern was determined. It was found with pigs nursing the sow that there were not only rather intense and immediate changes in serum proteins, but also a continuous and rapid alteration toward the mature serum protein pattern. Pigs fed an amino acid milk had little ability to promote normal changes associated with this serum protein maturation. The observations of

pigs fed cow's milk and artificial milk were intermediate between the two extremes of those fed sow's milk and amino acid milk. That is, in the first 4 to 7 days, little or no change was observed in the serum protein patterns; however, from then on, changes, similar to those seen in the pigs fed by the sow, started to occur, but at a slower rate. The ability of pigs having static, immature patterns was inferior to that of piglets having rapidly maturing patterns, in reacting to treatment.

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Quantitative Studies on the Metabolites of Tryptophan in the Urine of Swine[1,2]

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The urinary excretion of tryptophan metabolites by pyridoxine-deficient swine has been reported (Cartwright et al., '44). The animals were found to excrete kynurenine, xanthurenic acid, and a pigment which resembled urorosein, but very little kynurenic acid. A more recent report indicated that swine excreted some niacin in the form of N^1-methylnicotinamide and N-methyl-2-pyridone-5-carboxamide (Perlzweig, Rosen and Pearson, '50). Draper, Hironaka and Jensen ('58) found xanthurenic acid in the urine of gilts, sows, and growing pigs.

The recent development of relatively specific and convenient methods of determining urinary tryptophan metabolites (Price, '54; Brown and Price, '56; Brown, '57; and Satoh and Price, '58) has made it possible to find the urinary excretion of these metabolites by various species. Brown and Price ('56) reported that man, the dog, and the rat excreted significant quantities of these metabolites in the urine before and after supplementation with L-tryptophan, while the cat failed to excrete significant quantities. After ingestion of tryptophan, the rat and man were found to excrete relatively large amounts of xanthurenic acid, with these species and the dog excreting large amounts of kynurenic acid. Other species differences were observed in the same studies (Brown and Price, '56).

The excretion level of 9 tryptophan metabolites in the urine of swine has now been determined. Growing swine, as compared with the dog, rat, and man were found to excrete large quantities of N^{α}-acetylkynurenine, o-aminohippuric acid and anthranilic acid glucuronide, and relatively small amounts of kynurenine, 3-hydroxykynurenine, kynurenic acid and xanthurenic acid.

EXPERIMENTAL

Animals. Two separate experiments were conducted. Four litter mate cross-bred pigs (two barrows and two gilts), approximately 8 weeks old, were used in experiment 1. These pigs ranged in weight from 9.5 to 15.0 kg at the start and from 15.9 to 22.7 kg at the end of the study, with a range of weight gain of 5.9 to 7.7 kg. In experiment 2, two pairs of litter mates were used. One animal of each sex from a Poland China litter and one of each sex from a Chester White litter was used. These animals weighed from 11.4 to 14.1 kg at the start, and from 15.0 to 25.5 kg at the end of the study with a range of gain in weight of 4.0 to 11.4 kg.

The animals were fed the diets shown in table 1, with water and feed allowed ad libitum.

Urine collections. The animals were housed in individual compartments of a metabolism cage. The bottom of the cages was constructed of 3/8 inch galvanized wire screen, which allowed the urine and part of the feces to drop into a polyethylene-lined trough with sufficient slope to allow the urine to drain readily into collection funnels. Each funnel was covered with a single layer of 36 by 40 mesh cheese cloth to avoid contamination of the urine with feed or feces. Urine was collected in amber-colored bottles under 20 ml of toluene in both experiments. In the second

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[3] American Cancer Society—Charles S. Hayden Foundation Professor of Surgery in Cancer Research.

TABLE 1

Detailed composition of diets[1]

	Experiment 1	Experiment 2
Wheat	300	—
Corn	450	745
Soybean oil meal (44% crude protein)	100	145
Meat and bone scraps (50% crude protein)	30	80
Fish meal	20	—
Dehydrated alfalfa meal (17% crude protein)	90	—
Brewers' yeast	—	10
Steamed bonemeal	5	10
Trace mineral salt[2]	5	8
Antibiotic supplement[3]	1.5	1
Zinc supplements[4]	0.5	0.5
Total	1002.0	999.5

[1] Two and one-quarter million I.U. of vitamin A and 250,000 I.U. of vitamin D were added per half-ton of each ration.

[2] Contained in per cent: sodium chloride, 96.5 to 97.5; iron, 0.3; manganese, 0.15; cobalt, 0.10; copper, 0.06; iodine, 0.007.

[3] Contained 10 gm chlortetracycline per pound.

[4] Supplied elemental zinc in the form of zinc sulfate heptahydrate at the rate of 100 ppm of total ration.

experiment, 60 ml of 33% acetic acid was added as a preservative. The bottles were changed at 24-hour intervals. Urine was refrigerated at 0 to 5°C until analyzed, usually in one to three days. Urine was discarded for the 48-hour period immediately preceding the first collection period. Urine samples were collected and analyzed for each day of each experimental period.

Administration of supplements. The supplements were placed in the upper esophagus of the animals with a small-size balling gun. The L-tryptophan was in the form of 0.5 gm tablets and the nicotinamide in 100 mg tablets. In each experiment, 0.5 gm of tryptophan was administered 24 hours after the urine collections were started, followed 5 days later by 1.0 gm of the amino acid. The nicotinamide supplement (experiment 2) was administered 5 days after the 1.0 gm dose of tryptophan.

Analytical methods. The diazotizable aromatic amines were determined according to the procedure of Brown and Price ('56). N-methyl-2-pyridone-5-carboxamide (pyridone) was determined by the method of Price ('54). Determination of 3-hydroxykynurenine was made by the method of Brown ('57); kynurenic and xanthurenic acids according to Satoh and Price ('58); creatinine by the method of Peters ('42), and 4-pyridoxic acid according to Reddy, Reynolds and Price ('58). Paper chromatography was used to check the quantitative results as previously described (Brown and Price, '56).

Tests for statistical significance were made by analysis of variance, in which the mean for the second day (the initial 24-hour post-treatment period) was compared with the mean for the first day (the 24-hour period immmediately preceding treatment) for each metabolite (Snedecor, '53). First-day means were also compared with third-day means.

RESULTS

Since the results were similar in the two experiments and no evidence was obtained for a sex difference in the response to tryptophan, the data from the two experiments are presented as the average values for the 8 animals (table 2).

Urine was collected and analyzed for each of several days pre- and post-tryptophan. Because of the similarity of the average daily levels of urinary metabolites other than for the first 24 hours post-treatment only the data for one day before and two days after tryptophan were recorded in table 2.

Following the administration of 0.5 gm of tryptophan, a highly significant increase ($P < 0.01$) in the excretion of o-aminohippuric acid and aromatic amine fraction A was observed. Following the administration of 1.0 gm of tryptophan there was a highly significant increase ($P < 0.01$) in the excretion of anthranilic acid glucuronide and a significant increase ($P < 0.05$) in kynurenic acid, xanthurenic acid, aromatic amine fraction A, *o*-aminohippuric acid, acetylkynurenine, and kynurenine. From a quantitative standpoint, o-aminohippuric acid, acetylkynurenine, and the glucuronide of anthranilic acid were the most significant fractions, although considerable increases in the diazotizable amine fraction A were noted.

One animal in each experiment had diarrhea throughout most of the test period and both responded in an anomalous manner. These animals failed almost com-

TABLE 2

Average excretion of tryptophan metabolites in urine of 8 pigs studied. Data (except for creatinine values) are expressed as micromoles excreted per 24 hours. Tryptophan was administered at the end of first day.

Day	Dose of L-tryptophan	Creatinine	Micromoles of metabolite excreted per animal per day[1]							
			MPCA	KA	XA	A	AAG	oAH	AcK	K	HK
	gm	*mg/24 hours*									
1	—	.422	9.0	8.0	14.8	223.5	34.2	47.6	36.2	12.4	12.4
2	0.5	.496	9.4	34.4	27.4[2]	379.0[3]	126.0[2]	294.5[3]	213.5[2]	43.2[2]	16.6
3	—	.520	11.0	12.1	27.0	304.4	44.4	96.8	50.2	18.5	10.6
1	—	.560	13.2	10.2	19.9	291.9	34.9	72.4	45.4	17.5	4.8
2	1.0	.576	17.9	54.4[2]	33.2[2]	592.8[2]	154.0[3]	575.5[2]	344.9[2]	47.8[2]	12.1
3	—	.609	16.2	10.2	18.4	317.9	29.9	56.8	43.5	14.9	10.0

[1] The following abbreviations are used: MPCA, *N*-methyl-2-pyridone-5-carboxamide; KA, kynurenic acid; XA, xanthurenic acid; A, aromatic amine fraction A; AAG, anthranilic acid glucuronide; oAH, *o*-aminohippuric acid; AcK, acetylkynurenine; K, kynurenine; HK, hydroxykynurenine.

[2] Significantly greater than the mean for day 1 at the 0.05 probability level.

[3] Significantly greater than the mean for day 1 at the 0.01 probability level.

pletely to react to the tryptophan supplements. Hence, some question arose whether they actually retained the tryptophan or whether their lack of response to treatment was caused by the diarrhea. However, the low response to the tryptophan supplements for both pigs on both occasions leads one to suspect the diarrhea as the more likely explanation. In any event, summarizing the data for the remaining 6 pigs, the increase in the excretion of xanthurenic acid and aromatic amine fraction A, following the administration of 0.5 gm of tryptophan, was statistically significant ($P < 0.05$); whereas the increase in the excretion of anthranilic acid glucuronide, *o*-aminohippuric acid, acetylkynurenine, and kynurenine was highly significant ($P < 0.01$). Similarly, following the ingestion of 1.0 gm of tryptophan, the increased excretion of xanthurenic acid, o-aminohippuric acid, kynurenine and hydroxykynurenine was statistically significant ($P < 0.05$) whereas the increase in urinary kynurenic acid, aromatic amine fraction A, anthranilic acid glucuronide, and acetylkynurenine was highly significant ($P < 0.01$)

The administration of either 0.5 gm or 1.0 gm of tryptophan to the growing pigs did not result in a significant increase in the urinary excretion of the pyridone metabolite of niacin. However, when 400 mg of nicotinamide was administered to the 4 animals in experiment 2, the urinary excretion of the pyridone increased from a pre-treatment average level of 11 micromoles per 24 hours to 433 micromoles in the first 24 hours after the administration of the supplement and 28 micromoles during the second 24 hours post-treatment.

The urinary excretion of 4-pyridoxic acid was determined on each of two days for each animal. In experiment 1, the average excretion of 4-pyridoxic acid was 6.3 micromoles (range 5.2 to 7.5) per animal per day during the day before and day after the administration of 1.0 gm of tryptophan. In experiment 2, the 4-pyridoxic acid excretion averaged 4.5 micromoles per day (range 2.5 to 7.5) both the day before and day after the administration of the 0.5 gm of amino acid supplement.

DISCUSSION

Detailed studies of urinary excretion of tryptophan metabolites have been conducted on humans, dogs, rats, cats (Brown and Price, '56) and swine, which indicated that the metabolite excretion pattern is different for each species, following the administration of the amino acid. The cat excreted very little tryptophan in the form of the metabolites studied. Kynurenic acid was the chief urinary metabolite in rats and dogs, whereas kynurenine and the pyridone were the most significant metabolites in humans. Swine excreted very small amounts of any of these metabolites, but relatively large amounts of o-aminohippuric acid, acetylkynurenine and anthranilic acid glucuronide. The excretion of the pyridone was not significantly increased in the urine of swine after the ingestion of tryptophan, but the administration of niacin was followed by an increase in the excretion of the pyridone, adequate to account for about 13% of the amount of the vitamin. This result was in agreement with similar data obtained by Perlzweig, Rosen and Pearson ('50). Quantitative studies of Firth and Johnson ('56) have confirmed and extended a number of earlier studies indicating that tryptophan serves as a precursor of niacin in young pigs.

The urinary "kynurenine" determined by Cartwright et al. ('44) was probably actually acetylkynurenine. The investigators emphasized that the method of determination used was probably not very specific; also that relatively little kynurenic acid was present in the urine of pyridoxine-deficient swine, but that significant quantities of xanthurenic acid were noted. In the studies described here, the actual level of kynurenic acid and xanthurenic acid in the urine of normal, growing swine was low, even after the administration of tryptophan. That these metabolites were actually present in pig urine is indicated by the statistically higher values for xanthurenic acid excretion following either a 0.5 gm or 1.0 gm dose, and for kynurenic acid following a 1.0 gm dose of tryptophan. Furthermore, these quinoline compounds have been isolated in crystalline form from the urine of these same pigs (Roy and Price, '59). Draper, Hironaka and Jensen ('58) also detected xanthurenic acid in the urine of growing pigs fed a balanced, purified diet.

In general, the results of the paper chromatographic studies were in agreement with the quantitative analyses.

The nature of the diazotizable components in "aromatic amine fraction A" (Brown and Price, '56) remains unknown. Unlike the species studied previously (Brown and Price, '56), the pig excreted increased quantities of "diazotizable aromatic amine" in this fraction following the ingestion of tryptophan.

SUMMARY

The urinary excretion of tryptophan metabolites by growing swine was determined before and after the administration of 0.5 and 1.0 gm supplements of L-tryptophan.

Kynurenine, 3-hydroxykynurenine, kynurenic acid and xanthurenic acid, which had been found major urinary metabolites of tryptophan in man, the dog and the rat proved to be minor metabolites of the amino acid in swine. The growing pigs excreted large quantities of N^{α}-acetylkynurenine, o-aminohippuric acid, anthranilic acid glucuronide and an unknown diazotizable amine.

When swine were given supplements of tryptophan, no significant change was found in the urinary excretion of N-methyl-2-pyridone-5-carboxamide, although this metabolite appeared in relatively large quantities following the administration of 400 mg of nicotinamide.

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Retention of Fluoride in Soft Tissues of Chickens Receiving Different Fat Diets[1]

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Miller and Phillips ('55) have observed that diets containing 15 or 20% of cottonseed oil increase fluoride toxicity in rats. Similarly, Buttner and Muhler ('57, '58) have reported that semi-purified diets containing 15 or 20% of cottonseed oil will increase skeletal storage of fluoride and, in addition, promote the storage of fluoride in selective soft tissues of the rat. In these latter studies no effect on the serum cholesterol level was noted with the use of either the fat or fluoride supplements. Since more than 30% of the calories of the average American diet are derived from fat sources (U.S. Dept. Agr., '57), a clear understanding of the relationship between dietary fat intake and fluoride retention and metabolism is important. In this study, different fats or fatty acids were studied in relation to their effect on fluoride storage in chickens.

MATERIAL AND METHODS

One-day-old White Leghorn chicks were placed in 5 groups of 30 each. These groups, in turn, were divided into three sub-divisions. The animals of series 1 received a semi-purified sucrose diet which contained no added fat. The group 1 animals in this series were given no added fluoride; each animal in group 2 received by stomach tube 2 mg of fluoride per day as sodium fluoride (NaF); each animal in group 3 was injected intraperitoneally daily with 0.5 mg of fluoride as NaF. Each of the 4 remaining series was divided similarly into three groups, each animal receiving the same amount of NaF as the animals of series 1, either by stomach tube or by injection; however, the dietary fat level was appropriately altered. The composition of the diets is indicated in table 1. All the fluoride solutions were aqueous and unbuffered. The diets and drinking water were available ad libitum.

All animals were housed in raised screen-bottom cages in a temperature- and humidity-controlled room and weighed weekly for the 8-week experimental period. Blood samples were taken by cardiac puncture once each week and serum cholesterol was determined (Buttner and Muhler, '57). At the end of the experimental period the chickens were exsanguinated. The adrenals, thyroid and part of the liver were removed, weighed and fixed for histologic study. Representative soft tissues were also removed for fluoride analysis (heart, kidney, brain, skeletal muscle, liver). The remaining carcasses and femurs were then ashed separately to determine fluoride storage.

RESULTS

Data in table 2 summarize organ and body weights of the chicks in the various groups. The animals receiving the oleic acid and lard supplements gained the most weight, whereas those given supplements of stearic acid gained the least weight. Also, morbidity was the highest in the stearic acid series irrespective of the fluoride supplements. The same effect of stearic acid in diets has already been observed in this laboratory using the rat.

An examination of the adrenal weights of the various groups revealed a significant increase ($P = 0.001$) in adrenal weight in all animals receiving the fat supplements (series 2 to 5). This finding suggested that the level of fat used here is high enough to stimulate gluconeogene-

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TABLE 1

Composition of experimental diets

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	%	%	%	%	%
Sucrose	75	55	55	55	55
Vitamin-test casein (fat free)	18	18	18	18	18
Cottonseed oil	—	20	—	—	—
Oleic acid	—	—	20	—	—
Stearic acid	—	—	—	20	—
Lard	—	—	—	—	20
Cellulose	2	2	2	2	2
Salt mixture[1]	4	4	4	4	4
Vitamin mixture[1]	1	1	1	1	1

[1] Muhler ('54).

TABLE 2

Effect of fat and fluoride upon body weight and organ weights in the chicken

	No. animals	Final body weight	Adrenal weight	Thyroid weight
		gm	*mg/100 gm*	*mg/100 gm*
Series 1 (no fat)				
Control	7	298	8.4 ± 1.3[1]	5.6 ± 1.8
NaF injected, 0.5 mg F/day	8	284	11.1 ± 1.8	6.9 ± 1.9
NaF oral, 2 mg F/day	7	283	11.9 ± 1.3	6.9 ± 1.8
Series 2 (cottonseed)				
Control	6	300	17.1 ± 2.3	4.7 ± 0.6
NaF injected, 0.5 mg F/day	7	301	14.4 ± 2.0	7.6 ± 2.3
NaF oral, 2 mg F/day	7	281	13.4 ± 0.7	5.7 ± 0.8
Series 3 (oleic acid)				
Control	8	333	15.4 ± 0.7	6.2 ± 1.3
NaF injected, 0.5 mg F/day	7	331	12.5 ± 1.3	6.1 ± 1.4
NaF oral, 2 mg F/day	6	361	17.4 ± 3.0	7.2 ± 2.9
Series 4 (stearic acid)				
Control	5	278	14.1 ± 3.0	6.9 ± 2.0
NaF injected, 0.5 mg F/day	5	276	15.4 ± 3.7	5.5 ± 0.6
NaF oral, 2 mg F/day	5	255	18.1 ± 2.4	5.5 ± 0.5
Series 5 (lard)				
Control	7	322	13.2 ± 1.5	9.1 ± 4.5
NaF injected, 0.5 mg F/day	6	357	13.7 ± 2.0	9.5 ± 1.8
NaF oral, 2 mg F/day	8	343	14.7 ± 0.5	14.7 ± 7.7

[1] Standard deviation.

sis. Previous experience in our laboratories had shown that much higher levels of fat or protein were necessary to stimulate gluconeogenesis in the rat. No histologic alterations in the adrenal glands were observed, but the liver sections revealed marked changes. Livers of chickens not given fat supplements (series 1) were depleted of glycogen, and hepatic cells contained largely fat droplets. Analyses of the livers of animals on the high-fat diets (series 2 to 5) varied qualitatively but otherwise were essentially the same; that is, the hepatic cells were filled with what appeared to be glycogen. No glycogen analyses of livers were performed. However, the material was stained a purplish-red by the periodic acid-Schiff method and found to be compatible in histologic appearance with glycogen.

The animals in series 5 had thyroid weights greater than those of controls, but these differences were not statistically significant. Those of the other series fell within the normal range. Histologic examination revealed no differences among

TABLE 3

Fluorine storage in carcass and femurs of chickens receiving different fats and fluoride

	Carcass		Femur	
	Total	Concentration	Total	Concentration
	mg	*μg/gm*	*mg*	*μg/gm*
Series 1 (no fat)				
Control	3.92	378	0.16	404
NaF injected, 0.5 mg F/day	10.87	1225	0.60	1462
NaF oral, 2.0 mg F/day	22.65	3005	1.07	3387
Series 2 (cottonseed)				
Control	2.54	325	0.12	383
NaF injected, 0.5 mg F/day	12.07	1317	0.60	1547
NaF oral, 2.0 mg F/day	23.97	2863	0.89	2963
Series 3 (oleic acid)				
Control	3 84	383	0.11	254
NaF injected, 0.5 mg F/day	12 37	1087	0.56	1323
NaF oral, 2.0 mg F/day	25.60	1900	1.13	2270
Series 4 (stearic acid)				
Control	3.98	408	0.18	459
NaF injected, 0.5 mg F/day	13.70	1470	0.60	1820
NaF oral, 2.0 mg F/day	26.73	4170	1.03	4650
Series 5 (lard)				
Control	4.04	360	0.17	350
NaF injected, 0.5 mg F/day	13.33	1066	0.61	1253
NaF oral, 2.0 mg F/day	23.40	2223	1.20	2690

any of the glands of the chicks in all 5 series.

No significant effect of NaF administration upon serum cholesterol level in any of the 5 series was noted. This observation confirms the data of a previous report using the rat (Buttner and Muhler '57). However, all the animals receiving fat supplements had a higher serum cholesterol level after 6 weeks than the non-fat-supplemented animals (series 1). Interestingly, the animals receiving the lard had the highest serum cholesterol from week to week of all the fat-supplemented groups. The significance of this result is not known. These data are summarized graphically in figure 1.

The data on fluoride storage in the skeleton of chicks in the various series are summarized in table 3. The feeding of fat at a 20% level in the diet did not alter skeletal fluoride storage except in the animals of series 2, receiving cottonseed oil. In this instance, the control chickens which received cottonseed oil and no NaF had less fluoride in the carcass than similar animals receiving either no fat (series 1) or oleic and stearic acids or lard. When the femur was used to evaluate fluoride retention, both the control cottonseed and oleic acid animals had less fluoride in the femur, although the differences were not statistically significant. However, when the NaF input was increased, as in the groups in which fluoride was injected or given by stomach-tube, all the animals in the high-fat series (2 to 5) showed increased retention of fluoride in comparison with animals in the fat-free series (series 1), when total carcass fluoride was used as the criterion. This result confirms the findings of Miller and Phillips ('55) and of Buttner and Muhler ('57, '58).

When the femur was used for comparative purposes, the total fluoride retention was similar in all groups irrespective of the fat used. This difference between fluoride retention in the carcass and in the femur has been observed previously in our laboratories (Buttner and Muhler, '57, '58) and suggests to us that there is more fluoride in the carcass because of the retention of fluoride by soft tissues with the use of certain dietary regimens.

It is interesting to note that the animals receiving 0.5 mg of NaF per day by intra-

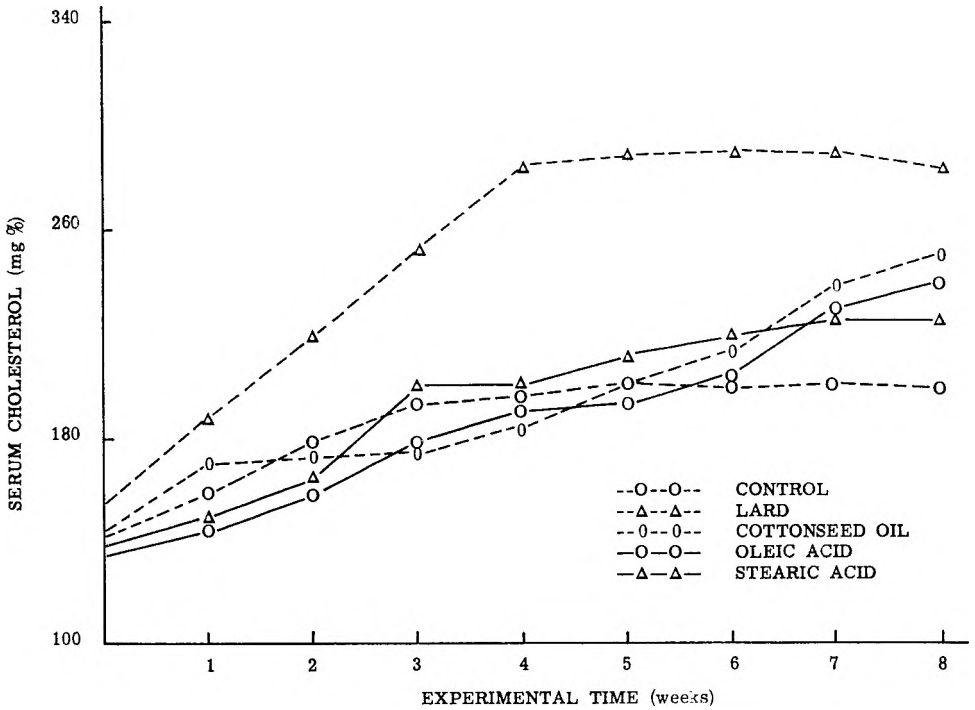

Fig. 1 Effect of fat supplements upon serum cholesterol in the chicken.

peritoneal injection stored one-half as much fluoride as the animals that received 2 mg of NaF daily by stomach tube. This result would seem to indicate that the metabolic availability of fluoride in the chicken is twice as great with parenteral administration as by enteral administration when fed the diet used here. Weddle and Muhler ('54) reported a 50% decrease in fluoride storage in animals receiving calcium salts with orally administered fluorine. Since the experimental diet used in this study contains approximately 0.6% of calcium, a lower metabolic availability of fluoride by the oral route in these studies is not entirely unexpected.

Table 4 shows the fluoride concentration in the various soft tissues. Variations in concentration are high from group to group. Probably this can be explained by the fact that it was necessary to pool individual organs of several animals in order to obtain sufficient material for accurate analysis. Such a procedure precludes a statistical analysis of these data. Nonetheless, some interesting generalities are evident. In every instance, more fluoride is retained in each soft tissue analyzed in the different control groups receiving fat in comparison with the control fat-free group. These findings suggest that increasing the amount of dietary fat results in the retention of a greater proportion of fluoride in soft tissue than occurs in the absence of dietary fat. This observation is in keeping with the hypothesis offered previously for explaining the differences in fluoride retention between femur and carcass.

Similarly, there was generally more fluoride in soft tissues of animals receiving dietary fat plus NaF than in the fat-free groups which received NaF (series 1). The notable exceptions were found when the liver was used for comparative purposes. For example, the cottonseed, oleic acid and lard groups which received NaF had less fluoride in the liver than the fat-free group which received NaF. This was also evident in the stearic acid and lard group when muscle was analyzed and compared with the fat-free group.

It is most interesting to note the high fluoride content of the brain in all the ex-

TABLE 4

Effect of various fats upon soft tissue fluoride storage[1]

	Fluoride concentration (μg/gm)				
	Heart	Liver	Kidney	Brain	Muscle
Series 1 (no fat)					
Control	0.2	0.2	0.2	2.5	0.5
NaF injected, 0.5 mg F/day	0.2	1.3	0.1	4.1	1.3
NaF oral, 2.0 mg F/day	0.8	1.6	3.7	17.5	2.5
Series 2 (cottonseed)					
Control	1.6	0.7	2.9	5.8	3.7
NaF injected, 0.5 mg F/day	2.0	0.5	2.4	7.8	2.6
NaF oral, 2.0 mg F/day	1.2	0.8	1.7	13.0	12.7
Series 3 (oleic acid)					
Control	2.6	0.2	2.0	8.0	1.8
NaF injected, 0.5 mg F/day	1.4	0.4	1.5	5.9	1.4
NaF oral, 2.0 mg F/day	1.4	0.5	1.3	4.6	0.5
Series 4 (stearic acid)					
Control	0.5	2.3	4.4	3.6	0.6
NaF injected, 0.5 mg F/day	2.0	3.3	2.7	4.0	0.2
NaF oral, 2.0 mg F/day	1.8	2.3	7.6	5.2	3.8
Series 5 (lard)					
Control	0.6	0.4	2.9	6.3	0.7
NaF injected, 0.5 mg F/day	0.4	0.6	1.1	8.1	0.6
NaF oral, 2.0 mg F/day	0.7	0.6	2.1	1.7	0.7

[1] Each value represents the mean value obtained by pooling three livers, 6 hearts, 6 brains, 6 kidneys or three pectoral muscles. This was necessary since such small quantities of fluoride are present in soft tissue that accurate individual organ analysis is not possible. Such a procedure prevents statistical evaluation.

perimental animals. Such high concentrations are in marked contrast to those of the other soft tissues analyzed.

SUMMARY

Day-old White Leghorn chickens were divided into 5 experimental series receiving different diets. One series received a fat-free diet and the other 4 were fed different fats at a 20% dietary level as follows: series 2, cottonseed oil; series 3, oleic acid; series 4, stearic acid; series 5, lard. Each series was divided into three groups, one receiving no added fluoride, another, 2 mg of NaF/day by stomach tube and the remaining group of 0.5 mg of NaF/day by injection.

After 8 weeks the animals were killed and the following observations made: (1) no effect of fluoride administration upon serum cholesterol level was noted, although, after 6 weeks, the animals fed the diets containing 20% of fat had consistently high serum cholesterol levels as compared with those fed fat-free diets; (2) increased skeletal retention of fluoride by the fat-supplemented animals when the fluoride was administered either by injection or stomach tube; and (3) increased retention of fluoride in heart and kidney of animals on the fat-supplemented diets.

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Growth Promotion by Invertible D-Amino Acids in Diets Containing Extraneous (Poorly Invertible) D-Amino Acids[1]

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Comparisons with mixtures of the L versus the DL modifications of the 10 amino acids essential for growth in the rat have made it clear that relatively large quantities of the D-amino acids may be included in the diet as components of a DL mixture without retarding the growth rate (Van Pilsum and Berg, '50). On the other hand, the simultaneous replacement by the D counterparts of several of the essential amino acids whose D isomers are invertible (methionine, tryptophan, phenylalanine, histidine and arginine) markedly decreases the rate of growth[2] (Phillips and Berg, '54). This occurs despite the fact that each D isomer serves singly as a fairly effective substitute for its L enantiomorph.

When methionine was fed at approximately the optimal level in a diet containing both the essential and the non-essential amino acids, Wretlind and Rose ('50) obtained as effective growth with the D as with the L isomer. In subsequent tests with mixtures of 9 essential amino acids (arginine excluded) Wretlind ('52) noted that the rate of growth induced by suboptimal levels of D-methionine was inferior to that promoted by the same level of L-methionine. The differences were greater when all of the other 8 amino acids were fed in the racemic form than when only isoleucine, valine, and threonine were so supplied.

The inversion of D-histidine takes place slowly enough to promote appreciably less rapid growth than L-histidine, even at levels optimal for the L enantimomorph (Cox and Berg, '34). It seemed to us, therefore, that interference with its inversion should be readily detectable. The primary purpose of the studies reported here was to compare the growth rate, using a synthetic amino acid mixture composed exclusively of the L-amino acids, with the growth rate upon substituting in this reference mixture the DL forms of the poorly and readily invertible groups of the essential amino acids as well as the non-essential amino acids. An additional purpose was to determine whether the presence of extraneous D-amino acids (the D forms of the poorly invertible group of essential amino acids) might interfere appreciably with the inversion of D-histidine.

EXPERIMENTAL

The amino acids used in these studies were recrystallized products which gave correct amino nitrogen or total nitrogen values. The specific rotations of the L-amino acids and the single D-amino acid used were checked carefully. They are recorded in table 1.

The L-amino acid diet, which served as the basis for reference, contained a mixture of both the essential and the non-essential amino acids at an overall level of 12.1%. Its composition is given in table 2. For convenience, the amino acids are grouped in three categories: group A includes the essential amino acids which are either not invertible or only poorly

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[1] This study was supported in part by Grant-in-aid N-23 from the American Cancer Society. We are indebted also to the Dow Chemical Company, Midland, Michigan for generous supplies of several of the DL-amino acids.

[2] J. F. Van Pilsum 1949 The relative capacities of the DL (racemic) and of the L (natural) modifications of the essential amino acids in promoting growth in the weanling rat. Ph.D. thesis, State University of Iowa.

invertible (lysine, threonine, isoleucine, and valine and leucine); group B consists of the more readily invertible essential amino acids (methionine, tryptophan, phenylalanine, histidine, and arginine); the non-essential amino acids constitute group C.

In addition to the variously modified amino acid mixtures, the diets also contained the following per 100 gm: sucrose, 15; Celluflour,[3] 2; salt mixture (Jones and Foster, '42), 4; corn oil, 2; vitamin A and D concentrate, 0.08;[4] inositol, 0.1; choline chloride, 0.2; liver extract 0.4;[5] and dextrin to make 100 gm. The other vitamin supplements were provided by adding to each kilogram of diet in milligrams: thiamine hydrochloride, 5; riboflavin, 10; pyridoxine hydrochloride, 5.0; nicotinic acid, 5.0; calcium d-pantothenate, 25.0; *p*-aminobenzoic acid, 300.0; α-tocopherol (acetate), 25.0; 2-methyl-1, 4-naphthoquinone, 2.0; and in μg biotin, 100; folic acid, 100; and vitamin B_{12}, 15.

Weanling male rats of the Sprague-Dawley strain, 35 to 50 gm in weight, were used in the tests. They were housed individually in false bottom, wire cages in a room maintained at 80 ± 2°F and allowed to consume food and water ad libitum for 28 days. Food consumption and body weights were recorded every 4 days.

Several series of tests were made at the outset to compare the effects upon growth of varying the composition of the amino acid mixture. In series 1, the reference L-amino acid mixture presented in table 1 was fed. In series 2, the group A amino acids were fed in the DL form at twice the L level indicated in table 1. In series 3, all of the essential amino acids (groups A and B) were fed in the DL form, the amino acids of group A at twice the L level, those of group B at the same level, except for a 25% increase in histidine. In series 4, the group A amino acids were fed in the DL form as in series 2, but the non-essential amino acids were replaced by glycine and ammonium citrate, and in series 5 both the group A and the group B amino acids were fed in the DL form as in series

[3] Obtained from Chicago Dietetic Supply House, Chicago.

[4] Oleum percomorphum, marketed by Mead Johnson and Company, containing per gm, 60,000 U.S.P. units of vitamin A and 8,500 U.S.P. units of vitamin D.

[5] Liver concentrate, N. F., kindly supplied by The Wilson Laboratories through the courtesy of Dr. S. W. Hier.

TABLE 1

Specific rotations of the optically active amino acids used in experimental diets[1]

Amino acids	$[\alpha]_D^{25}$	Solvent used	Sclute/ 100 ml solution
	degrees		*gm*
L-Alanine	+14.3	5 N HCl	4.0
L-Aspartic acid	+23.7	6 N HCl	2.0
L-Arginine monohydrochloride	+20.8	N HCl	1.0
L-Cystine	−213.0	N HCl	2.0
L-Glutamic acid	+30.9	6 N HCl	1.0
L-Histidine monohydrochloride monohydrate	+9.7	N HCl	2.0
L-Isoleucine	+37.6	5 N HCl	1.0
L-Leucine	+15.7	6 N HCl	3.8
L-Lysine monohydrochloride	+20.8	6.08 N HCl	3.0
L-Methionine	+23.2	2 N HCl	2.0
L-Phenylalanine	−34.2	HOH	2.0
L-Hydroxyproline	−75.1	HOH	1.0
L-Proline	−83.0	HOH	1.0
L-Serine	+14.4	5 N HCl	2.0
L-Threonine	−28.0	HOH	2.0
L-Tryptophan	−31.4	HOH	0.5
L-Tyrosine	−7.0	6.08 N HCl	4.0
L-Valine	+26.8	6 N HCl	2.0
D-Histidine monohydrochloride monohydrate	−9.6	N HCl	2.0

[1] In general, the values found under the conditions recorded agree with observations from the literature recorded in Phillips and Berg ('54); Dunn and Rockland ('47), and Greenstein ('54).

TABLE 2

Composition of the L-*amino acid mixture in reference diet*

	gm/100 gm
Group A	
Isoleucine	0.5
Leucine	0.8
Lysine[1]	1.0
Threonine	0.5
Valine	0.7
	3.5
Group B	
Arginine[1]	0.2
Histidine[1]	0.4
Methionine	0.6
Phenylalanine	1.0
Tryptophan	0.2
	2.4
Group C	
Alanine	0.6
Aspartic acid	1.0
Cystine	0.2
Glutamic acid	2.0
Glycine	0.6
Hydroxyproline	0.1
Proline	0.5
Serine	0.2
Tyrosine	1.0
	6.2

[1] Lysine and arginine were provided as the monohydrochlorides, and histidine as the monohydrochloride monohydrate in amounts equivalent to the quantity of free amino acid indicated. The monohydrochlorides contained 0.43 gm of H_2O and HCl. $NaHCO_3$ (0.88 gm) equivalent to the HCl was added.

3, with glycine and ammonium citrate replacing the non-essential amino acids. Series 6 differed from series 3 in that non-essential amino acids were used also in the DL form. The DL-serine and DL-cystine were fed at twice the L level, the DL forms of all of the other non-essential amino acids at the same level as in the reference L-amino acid diet.

Table 3 shows the comparative results obtained in these series of tests. The growth rates averaged 4.3 gm per day for series 1, 3.5 gm for series 2, and 2.1 gm for series 3. Food consumption per day on these three regimens decreased progressively from 11.8 to 10.4 to 7.8 gm. When the non-essential L-amino acids in series 2 and 3 were replaced by glycine and ammonium citrate (series 4 and 5), average growth was slower (3.2 and 1.7 gm per day) and average daily food consumption was smaller (9.6 and 7.0 gm). On the other hand, replacement of the non-essential amino acids by their DL forms, with only serine and cystine doubled, led to a somewhat more rapid average growth in series 6 (2.7 gm per day), with slightly greater food consumption (8.5 gm per day) than observed in series 3. To make certain that the latter observation was not the result of chance, the series 3, 5 and 6 tests were repeated subsequently. The only dietary change in series 3a, 5a and 6a lay in the use of a D-allo, L-isoleucine mixture instead of DL-isoleucine. Similar results to those in the earlier tests were obtained.

To determine the influence of the DL forms of the slowly invertible group of essential amino acids upon the growth promoting capacity of D-histidine, 0.4 and 0.2% of D- and 0.4 and 0.2% of L-histidine were incorporated into diets which contained all of the other amino acids in the L form; and also into diets which contained the poorly invertible amino acids (group A) in the DL form at twice the L level, all others in the L form. In some of the tests, lack of adequate amounts of L-alanine, L-serine or L-isoleucine made it necessary to substitute DL-alanine, DL-serine or L-isoleucine, D-alloisoleucine. The latter two were incorporated at twice the L-level. Since pure DL-isoleucine was no longer available on the market, its replacement by L-isoleucine, D-alloisoleucine also became necessary.

The data obtained are summarized in table 4. The relative growth rates induced by 0.4% of L- and by 0.4% of D-histidine in the L amino acid mixture (series 7 and 8, respectively) were similar to those noted previously with histidine-deficient casein hydrolysates (Cox and Berg, '34; Conrad and Berg, '37). With 0.2% of histidine, the L form (series 11) promoted only slightly less average growth than with 0.4% (4.1 versus 4.5 gm per day), but the D form (series 12) was markedly less effective at the lower than at the higher level (1.0 versus 3.5 gm per day). In the diets in which the poorly invertible group of essential amino acids was incorporated in the DL form at twice the L level, only slightly less growth was obtained with

TABLE 3

The influence of variation in opitcal isomerism and of replacement of non-essential amino acids by glycine and ammonium citrate upon the growth response to amino acid mixtures

Series[1]	Gain in weight	Food eaten	Mixture fed[2]
		gm	
1	120 ± 2.5[3]	327 ± 4.3	A + B + C
2	98 ± 2.7	291 ± 6.9	A_2 + B + C
3	58 ± 3.7	217 ± 9.4	A_2 + B_1 + C
4	90 ± 3.6	269 ± 9.7	A_2 + B + G
5	47 ± 4.3	196 ± 9.1	A_2 + B_1 + G
6	75 ± 2.5	237 ± 7.8	A_2 + B_1 + C_1
3a	60 ± 2.7	244 ± 6.8	A_{2a} + B_1 + C
5a	52 ± 2.6	217 ± 5.7	A_{2a} + B_1 + G
6a	70 ± 1.4	266 ± 3.9	A_{2a} + B_1 + C_1

[1] Six male rats were used in each series.

[2] The letter "A" indicates that the L forms of isoleucine, leucine, lysine, threonine and valine were fed at the levels shown in table 1; A_2 that the DL forms of these amino acids were fed at twice the L level. The A_{2a} mixture differed from the A_2 only in that D-allo-isoleucine, L-isoleucine was substituted for DL-isoleucine.

"B" represents the L forms of arginine, histidine, methionine, phenylalanine and tryptophan, fed as listed in table 1; B_1 the same amino acids fed in the DL form at the same level, except that the DL-histidine was increased 25% to compensate for the slow invertibility of its D component.

"C" indicates the non-essential L-amino acids, plus glycine, in the quantities specified in table 1. C_1 signifies replacement of the L by the DL forms; the DL-serine and DL-cystine were fed at twice the L level, all others at the same level.

"G" represents replacement of the non-essential amino acids by 0.9 gm of glycine and 4.4 gm of ammonium citrate, to provide essentially the same amount of nitrogen.

[3] Standard error of the mean.

TABLE 4

Comparative rates of growth upon D- *and* L-*histidine in diets containing only the* L *isomers, or both the* L *and the* D *forms of the poorly-invertible group of essential amino acids*

Series[1]	Gain in weight	Food eaten	Histidine-deficient mixture fed[2]	Histidine supplement
	gm	*gm*		*gm/100 gm*
7	125 ± 5.8[3]	368 ± 8 8[3]	A + B′ + C	0.4 L
8	97 ± 7.7	302 ± 22.1	A + B′ + C	0.4 D
9	118 ± 6.3	361 ± 15.5	A_{2a} + B′ + C_a	0.4 L
10	28 ± 5.7	161 ± 12 5	A_{2a} + B′ + C_a	0.4 D
11	114 ± 6.7	352 ± 17.5	A_a + B′ + C	0.2 L
12	29 ± 2.8	168 ± 7 1	A_a + B′ + C	0.2 D
13	105 ± 3.9	352 ± 17.0	A_{2a} + B′ + C_a	0.2 L
14	4 ± 0.9	127 ± 1 3	A_{2a} + B′ + C_a	0.2 D

[1] Four male rats were used in each series.

[2] The letter "A" indicates that the amino acids of the poorly-invertible essential amino acid group were fed in the L form in the amounts shown in table 1. A_a differed from A only in that it contained 1 gm of D-alloisoleucine, L-isoleucine instead of 0.5 gm of L-isoleucine. A_{2a} indicates employment of the DL forms of this group of amino acids at twice the L level, with D-allisoleucine, L-isoleucine instead of DL-isoleucine.

"B′" represents the use of the L forms of the "B" group of essential amino acids, minus histidine, at the levels indicated in table 1.

"C" indicates that the L forms of the non-essential amino acids were used at the dietary levels indicated in table 1. The C_a mixture contained 0.6 gm of DL-alanine and 0.4 gm of DL-serine instead of 0.6 gm of L-alanine and 0.2 gm of L-serine.

[3] Standard error of the mean.

0.4 (series 9) and 0.2% (series 13) of L-histidine (4.2 and 3.8 gm per day), but much less growth with 0.4% (series 10) and 0.2% (series 14) of D-histidine (1.0 and 0.14 gm per day). The consumption of the diets which contained the D-histidine and the extraneous D isomers of the poorly invertible group A amino acids was also strikingly less.

DISCUSSION

It seems fair to state that no ideal proportionate pattern of individual amino acids in an amino acid mixture has been established for growth promotion at any age or level of intake, even in the experimental rat. The pattern of the mixture of essential plus non-essential amino acids used in these tests was an arbitrary one, based partly upon the minimal requirements of the essential amino acids for growth in diets providing also the non-essentials (Rose, '37), as well as upon our own experience and that of others.

At the 12.1% level, at which the complete L-amino acid reference mixture was fed (series 1 and 7), the average weight gains of 120 and 125 gm in 28 days were appreciably greater than the average 106 gm- and 100 gm-gains observed previously in 28 days in rats fed 12.5 and 11.9%, respectively, of a fortified casein hydrolysate in otherwise similar diets (Van Pilsum and Berg, '50; Phillips and Berg, '54). Using a diet which contained 12.1% of physiologically active amino acids in a complete amino acid mixture (15.82% of the diet, of which 1.92% represented HCl and $NaHCO_3$, and 1.8% the D forms of 4 essential and two non-essential amino acids), Womack, Snyder and Rose ('57) observed an average gain in weanling rats of 115.4 gm in 28 days. Ramasarma, Henderson and Elvehjem ('49) had obtained an average gain of 114.8 gm in 28 days on a diet containing 16.05% of essential plus non-essential amino acids. This calculation includes the D componnents of DL--serine and DL-alanine, but not those of the 6 essential amino acids fed in the DL-form. The average gain on this mixture was inferior to the 129.1 gm average gain observed in a similar period on a 19% casein diet.

The reason that replacement of the L forms of the poorly invertible group A amino acids, by their DL modifications at twice the L level (series 2 and 9), produced somewhat less growth (98 and 118 gm versus 120 and 125 gm in 28 days) is obscure. Both diets contained the same quantities of the L enantiomorphs. It is possible that small differences in balance were caused by limited inversion of the D components of the DL-valine and the DL-leucine, but it is questionable whether this could have been solely responsible. The use of minimal levels of the essential amino acids in these studies would, however, have made the animals sensitive to any appreciable imbalance.

The still greater growth retardation, observed when the readily invertible amino acids of group B were also replaced by their DL-modifications, as in series 3, strongly suggests inadequate inversion of one or more of the D forms of the invertible group B acids, possibly through competition of the poorly invertible D-amino acids for the enzyme (or enzymes) involved. Of incidental interest in this connection is the observation that the additional provision of the non-essential amino acids in the DL form, with serine and cystine at twice the L level, and all others at the L level, led to an appreciably increased rate of growth (compare series 6 with series 3 and series 6a with series 3a). The reason for the improvement is not obvious. Possibly a more efficient proportion of the non-essential amino acids was effected. In any event, the data seem to indicate that the D forms of the non-essential amino acids do not interfere markedly with the inversion of the D forms of the B group of readily invertible essential amino acids, as do the D-amino acids of the essential group A.

The fairly rapid growth, observed on the diets in which the non-essential amino acids were replaced by glycine and ammonium citrate (series 4 and 5), indicates that the organism's capacity to synthesize the non-essential amino acids was certainly very marked, though not completely efficient.

In the tests of the influence upon the utilization of histidine of the invertible D enantiomorphs, provided when the essen-

tial amino acids of group A were fed in the DL form, the growth upon L-histidine was only slightly less than on the complete L-amino acid mixture, both at the 0.4% and at the 0.2% L-histidine levels. By contrast, the differences in the response to D-histidine were very marked, both at the 0.4 and 0.2% levels. Little doubt exists that one or more of the D modifications of the group A amino acids markedly impairs the utilization of D-histidine.

In the growing rat, D-histidine has been proved to undergo conversion to L-histidine (Conrad and Berg, '37). The slower rate of growth with this isomer has been assumed to be caused by its slow rate of inversion. If this is true, the still further retardation in the presence of other D-amino acids, as in the series 10 and 14 diets, may be tentatively attributed to their interference with the inversion process. The possibility that interference of this type might occur was first suggested by Wretlind ('52). Evidence which we have obtained, both in the growing animal[6] (Phillips and Berg, '54) and at the crude enzyme level[7] seems to lend plausibility to this assumption. Such other possibilities, as the more ready excretion of the D forms and limited food consumption, cannot be ignored as correlative factors, however.

SUMMARY

Excellent growth has been obtained on an L-amino acid diet containing both the essential and the non-essential amino acids. Replacement in this diet of the poorly invertible group of essential amino acids (isoleucine, leucine, lysine, threonine and valine) with their DL forms at twice the L level produced less rapid growth. The additional replacement of the readily invertible essential amino acids (arginine, methionine, histidine, tryptophan and phenylalanine) by their DL forms at the L level led to a much more marked decrease in rate of growth. In either of the two latter diets, replacement of the non-essential L-amino acids by glycine and ammonium citrate led to a further decrease in rate of gain, but their replacement by their DL isomers improved the growth response. It is therefore tentatively concluded that the D forms of the poorly invertible group of essential amino acids interfere with the inversion of the D forms of the readily invertible group. The D forms of the non-essential amino acids apparently do not augment this interference. The excellent, though not quite equal, growth obtained upon replacement of the non-essential amino acids by glycine and ammonium citrate indicates that the synthesis of the non-essential amino acids occurs surprisingly readily. The presence of the poorly invertible group of essential amino acids in the DL form at twice the L level interferes markedly with the utilization of D-histidine. This occurs not only when the latter is fed at a suboptimal level, but also when fed at a level optimal for L-histidine. The probability of interference with inversion is suggested.

[6] See footnote 2, page 31.

[7] Gerulat, B. F., and C. P. Berg 1958 Competitive inhibition of D-amino acid oxidase by D-amino acids. Federation Proc., *17*: 227 (abstract).

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The Utilization of Some B Vitamins Administered to the Rat Apart from the Diet

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Although considerable evidence exists that proteins and amino acids given apart from the other components of the diet may not be efficiently utilized for growth (Henry and Kon, '46; Cannon et al., '47; Geiger, '47, '48a, '48b, '50), little is known of the utilization of vitamins administered separately from the diet. Macko et al. ('56) observed greater weight gains in guinea pigs given ascorbic acid daily than in those receiving it at semi-weekly or weekly intervals. In other studies, Macko ('56) found that rats given vitamin A once a week grew as rapidly as those given the same amount of the vitamin in 7 daily doses. However, he observed that thiamine and vitamin D were better utilized if given daily rather than at weekly intervals.

The present studies were conducted to determine whether limiting amounts of 4 B vitamins, administered apart from the diet each day, are as well utilized for the growth of rats as the same amount of vitamins included in the diet.

EXPERIMENTAL

The basal 18% casein diet (diet 1) used in the two experiments reported herein was similar to that used by Morrison and Sarett ('59), except that 0.063 mg of thiamine, 0.125 mg of riboflavin, 0.063 mg of pyridoxine hydrochloride and 0.5 mg of calcium pantothenate were used per 100 gm to limit growth. These values represent approximately one-half of the levels required by the growing rat (Brown and Sturtevant, '49). The same 4 vitamins were omitted from diet 2. A low level of ascorbic acid, 10 mg per 100 gm, was included in the diets in experiment 1 but omitted in experiment 2. In order to minimize vitamin loss, dietary supplies were kept refrigerated. Previous analyses of this type of diet showed no vitamin loss under these storage conditions. Male weanling rats of the McCollum-Wisconsin strain were used in both experiments. The animals were individually housed in screen-bottom cages kept in an air conditioned room maintained at 74 to 76°F.

Experiment 1. Thirty rats received diet 1 ad libitum during a two-week pretest period. At the end of this time, 10 pairs of animals were selected on the basis of body weight and rate of weight gain. One member of each pair continued to receive diet 1 ad libitum. The second member of each pair received diet 2 ad libitum (diet 1 minus thiamine, riboflavin, pyridoxine and pantothenic acid), and, in addition, the same amounts of the 4 vitamins which had been consumed by its pair-mate the previous day. Vitamins were administered in aqueous solution by stomach tube. To facilitate administration of the vitamins, a solution was prepared containing (per ml) the amount of thiamine, riboflavin, pyridoxine and pantothenic acid present in 10 gm of diet 1. The animals receiving diet 1 (with the B vitamins in the diet) were given an amount of water by stomach tube equal to that received by their pair-mates, to equalize the effect of handling and treatment. Rats were individually weighed, weekly, for three weeks. During this time records were kept of food and water intake. Because of the death of one animal in each group, only 8 pairs of animals remained at the end of the experiment. These were fasted for 24 hours, killed by intraperitoneal injection of pen-

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tobarbital sodium solution,[3] and the livers, kidneys, and adrenal glands were removed and weighed.

Experiment 2. Weanling rats were trained, during a two-week pretest period, to consume their daily diets within a one-hour period. This was done in order to permit study of separate vitamin administration immediately, and several hours after the diet was consumed. Pairs of rats were then selected on the basis of body weight and rate of weight gain. Eight of these pairs were used in part A of the experiment, and the other 8 used for part B. One animal in each of these parts was lost during the experiment.

In part A, animal 1A of each pair was given diet 2 (diet 1, minus thiamine, riboflavin, pyridoxine and pantothenic acid) ad libitum from 8:00 to 9:00 A.M., and at 4:30 P.M. the amounts of the missing vitamins present in an equivalent amount of diet 1 were given by stomach tube. Animal 2A received this amount of diet 1 from 9:00 to 10:00 A.M., followed by 1 ml of water by stomach tube.

In part B, animal 1B of each pair received diet 2 from 8:00 to 9:00 A.M. and immediately thereafter was given by stomach tube the amounts of the 4 B vitamins contained in an equivalent amount of diet 1. Animal 2B was allowed this amount of diet 1 from 9:00 to 10:00 A.M. and then given 1 ml of water by stomach tube.

Records were kept of food and water intake, daily, and of weight, weekly, for three weeks. At the end of this period, the animals were fasted for 24 hours and killed by intraperitoneal injection of pentobarbital sodium solution; livers, kidneys and adrenal glands were removed and weighed. The liver of each animal was analyzed for solids and total lipid content by the method of Sarett and Jandorf ('47).

RESULTS AND DISCUSSION

In experiment 1, the animals receiving the vitamins separately from the diet once

[3] Nembutal, Abbott.

TABLE 1

Data on weight gain, food and water intake, food efficiency values and organ weights of male rats receiving 4 E vitamins in the diet or once daily by stomach tube

	Diet 1 vitamins included in diet	Diet 2 vitamins given by stomach tube
No. of rats	8	8
Initial weight, gm	69	71
Weight gain, gm		
1 week	13	9
2 weeks	17	21
3 weeks	20.0 ± 9.0[1]	28.8 ± 8.8[1]
Food intake, gm	123	135
Food efficiency[2]	16.3 ± 4.9	20.7 ± 4.0
Water intake, ml	143	141
Liver weight		
gm	3.1	3.5
% body weight	3.7 ± 0.3	3.9 ± 0.3
Kidney weight		
gm	0.85	0.91
% body weight	1.05 ± 0.08	1.00 ± 0.06
Adrenal weight		
mg	24.6	25.5
mg/100 gm body weight	30.1 ± 2.6	28.1 ± 5.4

[1] Standard deviation.
[2] Grams gained/100 gm of food consumed.

each day gained somewhat more weight than those receiving the vitamins in the diet (table 1). However, this difference, which was not significant, may have been caused, in part, by the large variation in weight gain found with suboptimal intake of vitamins. A slight loss of B vitamins in the diet may have occurred (Lyman and Elvehjem, '51; Rombouts, '53), although this is not likely under the experimental conditions. The animals receiving the vitamins in the diet ate less than their pair-mates, and also showed lower food efficiency values. The organ weights of the animals receiving the vitamins in the diet were similar to those of the animals receiving the vitamins by stomach tube.

Data on weight gain, food and water intake and efficiency of food utilization in experiment 2 are shown in table 2. In part A of the experiment, group 1A (the B-vitamin-free diet in the morning with the vitamins given separately late in the afternoon) gained 35 gm, with a food efficiency of 28.6 gm gain per 100 gm of food intake. The animals in group 2A (receiving the same amount of diet containing the vitamins) showed a similar weight gain (34 gm) and comparable food efficiency of 28.0 gm gain per 100 gm of food intake. In part B, group 1B which received the B-vitamin-free diet followed immediately by the vitamins, gained 40 gm with a food efficiency of 30.2 gm gain per 100 gm of food intake. The paired animals in group 2B, which received the vitamins with the diet, showed a similar weight gain (41 gm) and food efficiency (33.2).

In both parts of this experiment, the water intake of the animals receiving the vitamins with the diet (groups 2A and 2B) was significantly greater than that of those given the vitamins by stomach tube. This cannot be readily explained. Data on liver, kidney and adrenal weight and liver composition (table 3) showed no significant differences among the groups, other than the relatively smaller kidney weight of the animals in groups 2A and 2B. These differences in kidney weight presumably result from the variation in water intake noted above.

Therefore, B vitamins administered separately from the diet once a day appear to be utilized as well as those incorporated in the diet.

SUMMARY

Two experiments were conducted to determine whether limiting amounts of thi-

TABLE 2

Weight gain, food and water intake and food efficiency values of male rats given 4 B vitamins, in the diet or separate from the diet

	Part A[1]		Part B[2]	
	Group 1A	Group 1B	Group 2A	Group 2B
No. of rats	7	7	7	7
Initial weight, gm	74	74	75	72
Weight gain				
1 week	12	14	15	15
2 weeks	27	27	31	32
3 weeks	34.7 ± 7.5[3]	33.7 ± 10.0	39.9 ± 5.7	41.3 ± 5.3
Food intake, gm	121	120	132	124
Food efficiency[4]	28.6 ± 4.9	28.0 ± 6.6	30.2 ± 2.1	33.2 ± 2.4
Water intake, ml	165	240	189	304

[1] Group 1A—"Vitamin-free" diet ad libitum, 8 to 9 A.M., vitamins by tube at 4:30 P.M.
Group 1B—Basal diet, equivalent to 1A, 9 to 10 A.M., water by tube at 10 A.M.
[2] Group 2A—"Vitamin-free" diet ad libitum, 8 to 9 A.M., vitamins by tube at 9 A.M.
Group 2B—Basal diet, equivalent to 2A, 9 to 10 A.M., water by tube at 10 A.M.
[3] Standard deviation.
[4] Grams gained per 100 gm of food consumed.

TABLE 3

Data on final body weight and organ weight of male rats given 4 B vitamins in the diet, or separate from the diet

	Part A		Part B	
	Group 1A	Group 1B	Group 2A	Group 2B
No. of rats	7	7	7	7
Final body weight, gm	107	106	115	113
Liver weight				
gm	3.6	3.6	4.1	3.7
% body weight	3.3 ± 0.1[1]	3.4 ± 0.2[1]	3.6 ± 0.5[1]	3.3 ± 0.2[1]
Kidney weight				
gm	0.91	0.79	1.04	0.79
% body weight	0.85 ± 0.08	0.75 ± 0.07	0.90 ± 0.10	0.70 ± 0.02
Adrenal weight				
mg	28.5	26.1	26.4	29.5
mg/100 gm body weight	26.9 ± 4.6	26.8 ± 4.0	23.0 ± 1.7	26.4 ± 3.5
Liver composition				
solids, %	29.4 ± 1.2	27.9 ± 1.1	28.1 ± 1.0	29.0 ± 0.2
total lipid, %	4.2 ± 0.2	3.8 ± 0.3	4.0 ± 0.2	4.0 ± 0.2

[1] Standard deviation.

amine, riboflavin, pyridoxine and pantothenic acid are used as well by growing rats when given apart from the diet as when given with the diet. In the first experiment, one group of animals received these vitamins in the diet throughout the day, while a second group received an equivalent amount of the vitamins once a day by stomach tube. The results showed no significant difference in average weight gain or efficiency of food utilization.

In the second experiment, the animals were first trained to consume their daily food within one hour to permit administration of B vitamins apart from the diet (either immediately after eating or several hours later). The weight gain of these animals was similar to that of the pair-mates which received the vitamins in the diet. Significantly more water was consumed by rats receiving the vitamins in the diet, and these animals had relatively smaller kidneys than those receiving the vitamins separately. B vitamins administered separately from the diet once each day appear to be as well utilized as those incorporated in the diet.

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The Metabolism of Methionine by Single Comb White Leghorn and Black Australorp Chicks

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In 1941, Klose and Almquist reported that methionine was an essential dietary ingredient for growth promotion in the chick. Since then, many studies have been conducted to determine the sulfur amino acid requirements of the chick. The methionine requirement has been shown to be affected by the cystine, choline (Grau and Almquist, '43, Almquist and Grau, '45), protein (Rosenberg and Baldini, '57) and energy content of the chick's diet (Baldini and Rosenberg, '55). With substantially no cystine in the diet the results of feeding studies (Grau and Almquist, '43) have demonstrated that the chick utilizes methionine for cystine synthesis at a rate rapid enough to meet physiological needs for growth. Since the amount of cystine in the diet affects the chick's need for methionine, this requirement cannot be considered without consideration of cystine content.

Recently, McDonald ('57, '58) reported a breed difference in the metabolism of methionine. He found that Single Comb White Leghorn chicks, but not Australorp, gave a growth response to methionine supplementation of the basal diet. However, the Australorp chicks responded to as little as 0.078% of supplemental dietary cystine. Biochemical studies of the liver cysteine content of Single Comb White Leghorn and Australorp chicks, indicated that the Australorp chicks could not synthesize cystine at a rate that would meet physiological need for growth.

The current studies were initiated to determine the effect of cystine supplementation on the growth of Black Australorp and Single Comb White Leghorn chicks fed diets having a low cystine content, and also to study the utilization of methionine S^{35} by the Black Australorp and Single Comb White Leghorn chick.

EXPERIMENTAL

All chicks used in these experiments were obtained from outbred, closed flocks of the Breeding Section of the Poultry Research Branch at the Agricultural Research Center. Chicks were housed in electrically heated chick batteries having raised, wire floors. Feed and water were supplied ad libitum. Basal diets used are presented in table 1. Supplementation of the basal diet with methionine and cystine was made at the expense of cornstarch. The cystine and methionine content of the diets was determined microbiologically.

The method of Horn and Blum ('56) was used for the cystine microbiological assays except for the following two modifications: feed samples were hydrolyzed in an autoclave at 15 pounds pressure for two hours, and the sterilization time for the media was reduced to 2.5 minutes. Methionine was assayed by the method of Williams ('55) using a 24-hour reflux hydrolysis. *L. mesenteroides* P-60 (ATCC no. 8042) was used as the assay organism for both methionine and cystine.

The effect of cystine supplementation of basal diets on growth rate

Experiments 1 and 2. Day-old straight-run chicks were placed randomly on the various dietary treatments outlined in table 2 for a 4-week experimental period.

Experiment 3. During the first week, the chicks were fed basal diet 3, supplemented with 0.3% of DL-methionine and 0.2% of L-cystine. At one week of age the chicks were placed randomly on the various treatments given in table 2, using duplicate groups of both breeds per treatment. The experiment was terminated when the chicks were 4 weeks old. Statistical analy-

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TABLE 1

Composition of basal diets

Ingredient	Diet		
	1	2	3
	%	%	%
Alfalfa meal, 17% protein	—	5	5
Isolated soybean protein[1]	10	12	17
Casein, 88% protein	10	10	5
Gelatin	5	5	5
Fish solubles	3	3	3
Ground cellulose	3	3	1
Corn oil[2]	6	10	10
Mineral mix[3]	4.76	4.76	4.76
B-vitamin mix[4] (triturated in sucrose)	0.5	0.5	0.5
Vitamin A concentrate (10,000 I.U./gm)	0.05	0.07	0.07
Vitamin D_3 concentrate (15,000 I.C.U./gm)	0.003	0.003	0.003
Vitamin E concentrate (44 I.U./gm)	0.01	0.01	0.02
Choline chloride supplement (25% choline chloride)	0.5	0.6	0.8
Chlortetracycline	—	0.002	0.002
Antibiotic supplements[5]	—	—	0.02
DL-methionine	0.3	—	—
Cornstarch	56.87	46.59	47.84
Total	100	100	100
Protein % calculated	22.1	25.3	25.3

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

[2] Stabilized by adding Tenox R, 500 mg/kg of corn oil, Eastman Chemical Products, Inc., Kingsport, Tennessee.

[3] Mineral mix supplied the following per cent of minerals in the diet: tri-calcium phosphate, 3.0; potassium phosphate dibasic, 0.9; sodium chloride, 0.5; magnesium carbonate, 0.262; ferric citrate, 0.05; zinc carbonate, 0.01; potassium iodide, 0.004; cupric acetate, 0.005; manganese chloride, 0.037; sodium selenate, 0.0004.

[4] Vitamin mix supplied the following vitamins in mg/kg of diet: thiamine·HCl, 30; riboflavin, 15; calcium pantothenate, 100; vitamin B_{12}, 0.008; pyridoxine·HCl, 10; folic acid, 4; inositol, 50; menadione, 2.5; niacin, 100; biotin, 0.1.

[5] Contained zinc bacitracin, 25 gm of antibiotic/pound. Commercial Solvents Corp., New York.

TABLE 2

Fourth-week body weights of Single Comb White Leghorn and Black Australorp chicks as affected by methionine and cystine supplementation of low-cystine basal diet

Basal diet no.	Supplements added		Experiment 1[1]		Experiment 2[2]		Experiment 3[3]			
	DL-methionine	L-cystine	SC White Leghorn	Black Australorp	SC White Leghorn	Black Australorp	SC White Leghorn		Black Australorp	
							1	2	1	2
	%	%	gm	gm	gm	gm	gm	gm	gm	gm
1	—	—	281	292						
	—	0.3	275	272						
	—	—			249	340				
	0.30	—			300	360				
2	0.30	0.20			321	338				
	0.40	—			306	357				
	0.40	0.20			304	324				
	—	—					226	177	258	279
	0.15	0.15					290	257	336	336
3	0.30	—					295	274	344	339
	0.30	0.20					296	258	346	348
	0.50	—					303	253	326	349

[1] Experiment 1, day-old chicks, 15 per treatment; basal diet 0.54% of L-methionine 0.10% of L-cystine by microbiological assay.

[2] Experiment 2, day-old chicks, 12 per treatment; basal diet 0.45% of L-methionine 0.11% of L-cystine by microbiological assay.

[3] Experiment 3, week-old chicks, 10 per treatment (each treatment replicated); basal diet, 0.40% of L-methionine, 0.11% of L-cystine by microbiological assay.

ses (analysis of variance and Duncan's new multiple range test) were made on growth data according to the methods outlined by Li ('57).

Utilization of L-*methionine* S^{35}

Using sulfur^{35}-labeled L-methionine, tracer studies were conducted of the amount of methionine utilized for cystine synthesis by each of the two breeds. Commercially obtained L-methionine S^{35} was freed of radioactive impurities by paper chromatography before use.

Experiment 4. Six Single Comb White Leghorn and 6 Black Australorp chicks were fed basal diet 1 from one day of age. Starting the second day, they were injected intramuscularly with carrier-free L-methionine S^{35} (20 microcuries per chick per day) for 4 consecutive days and killed on the 5th day. The droppings were collected daily and the total S^{35} content determined[1] (table 3). The carcasses were pooled by breed and homogenized in a Waring blender. To provide an optimum hydrolysis period for methionine liberation with least destruction of cystine, the homogenized carcasses were hydrolyzed with 6 N HCl for three hours at 110°C, filtered, residue rehydrolyzed for 7 hours and filtered; three-hour and 7-hour filtrates for each breed were combined and reduced in volume *in vacuo*. Aliquots of the hydrolysates were used for isolation of methionine S^{35} and cystine S^{35} by means of ion exchange resins. One-milliliter aliquots of the hydrolysates were placed on 0.9 by 100-cm columns of Dowex 50 (hydrogen form) and eluted according to the procedure of Stein and Moore ('50). One-milliliter aliquots of the fractions collected from the ion exchange columns were evaporated to dryness and counted in a windowless gas-flow counter. Corrections were made for self-absorption and decay. Identity of the methionine and cystine peaks was confirmed by paper chromatography.[2]

Table 4 (experiment 4) shows the per cent of total carcass sulfur S^{35} found in the cystine fraction. In order to determine the specific activity of the cystine S^{35} found in the carcass, larger columns (5 by 50 cm) of Dowex 50 resin (hydrogen form) were used to isolate larger quantities of cystine S^{35}. The cystine S^{35} thus obtained was recrystallized three times and checked for purity by microbiological assays and paper chromatography. The specific activity of the cystine S^{35} isolated from the carcass hydrolysate of the two breeds of chicks is given in table 4 (experiment 4).

[1] Droppings homogenized in Waring blender were made up to volume and aliquots wet-ashed with concentrated nitric acid, residue taken up in 10 ml of H_2O. One-milliliter aliquots of the ashed solution were dried in stainless steel planchets and counted.

[2] Solvent systems for paper chromatography; methyl ethyl ketone, propionic acid, water (75, 25, 30) and *tert.* butanol, formic acid and water (75, 15, 15).

TABLE 3

Retention and excretion of L-*methionine* S^{35} *administered intramuscularly to Single Comb White Leghorn and Black Australorp chicks*

Experiment 4

Breed	Days after initial dose				Total[1]
	1	2	3	4	
	%	%	%	%	%
	S^{35} content of droppings				
SCW Leghorn	15.19	12.76	9.34	7.26	44.55
Black Australorp	12.34	11.43	9.64	8.68	42.09
	S^{35} content of carcass[1]				
SCW Leghorn		56.73			
Black Australorp		56.90			
	Recovery of S^{35} administered[1]				
SCW Leghorn		101.28			
Black Australorp		98.99			

[1] Per cent of total dose administered.

TABLE 4

Conversion of carrier-free L-methionine S^{35} to cystine S^{35} by Single Comb White Leghorn and Black Australorp chicks

Experiment no.	Hydrolysate	Resin column no.	%[1] recovered as cystine S^{35}	Average %	Specific activity cpm/mg
	Carcass hydrolysate				
4	SCW Leghorn	L 1B	33.90		
4	SCW Leghorn	L 1E	37.52	35.71	
4	SCW Leghorn	L 1D			93,811
4	Black Australorp	1A	31.72		
4	Black Australorp	1B	29.75	30.75	
4	Black Australorp	1C			81,683
5	SCW Leghorn	SB	32.39		
5	SCW Leghorn	SD	35.24		
5	SCW Leghorn	SF	30.57	32.73	
5	Black Australorp	SA	27.28		
5	Black Australorp	SC	26.63		
5	Black Australorp	SE	24.67	26.19	
	Liver hydrolysate				
6	SCW Leghorn	LIA	27.30		
6	SCW Leghorn	LIC	30.86	29.08	
6	Black Australorp	AIA	21.53		
6	Black Australorp	AIB	22.18	21.85	

[1] Per cent of total radioactivity recovered as cystine S^{35} from carcass- or liver-tissue hydrolysate.

Experiment 5. Three Single Comb White Leghorn and three Black Australorp two-day-old chicks were injected intramuscularly with carrier-free L-methionine S^{35} (22.64 microcuries per chick per day) for 4 consecutive days and killed on the 5th day. These chicks had been fed basal diet 3 from one day of age. The entire chick carcass was homogenized separately in a Waring blender, hydrolyzed and fractionated on ion exchange resins by the same procedure described in experiment 4. The per cent of total radioactivity found in the cystine fraction for each chick is given in table 4 (experiment 5).

Experiment 6. Two Single Comb White Leghorn and two Black Australorp three-week-old chicks were injected intramuscularly with carrier-free L-methionine S^{35} (28.28 microcuries per day per chick) for 4 consecutive days and killed on the 5th day, having been fed basal diet 2 from one day of age. Livers were removed, hydrolyzed and fractionated on ion exchange resins as described in experiment 4. The per cent of the total radioactivity found in the cystine fraction for each liver hydrolysate is shown in table 4 (experiment 6).

RESULTS AND DISCUSSION

Growth studies outlined in the first three experiments (table 2) show no growth response with either breed of chick when cystine was added to diets containing adequate methionine. The data were analyzed statistically, with no significant difference found in the growth of the two breeds. In experiment 2, no growth response was obtained from methionine supplementation of the basal diet fed to Black Australorp chicks. It appears, therefore, that basal diet 2 contained an adequate amount of sulfur amino acids to meet the requirement of the Black Australorp chick but not of the Single Comb White Leghorn chick.

In experiment 3, the diet was modified to reduce the sulfur amino acid content in the basal diet. Here, a significant difference (5% level of significance), was

obtained when the growth of both breeds fed the basal diet was compared with the growth of chicks receiving supplementary methionine or methionine plus cystine. These results are contrary to those obtained by McDonald ('57), who found that the addition of methionine to his basal diet used for Australorp chicks resulted in a growth depression rather than a growth response. However, the results of the tracer studies with L-methionine S^{35} show that less methionine is converted to cystine by the Australorp chicks than by Single Comb White Leghorn chicks. The cystine S^{35} isolated from the Black Australorp carcass hydrolysate had a lower specific activity than the cystine S^{35} isolated from the Single Comb White Leghorn carcass hydrolysate. This indicates that a definite breed difference exists in utilization of methionine for cystine synthesis. Even though the rate of conversion of methionine to cystine was not as great for the Black Australorp chicks as for the Single Comb White Leghorn chicks, the growth studies indicate that the rate was adequate to meet physiological needs.

SUMMARY

The utilization of methionine for synthesis of cystine by Single Comb White Leghorn and Black Australorp chicks was studied, using a low-cystine content, semipurified diet. A growth response was obtained from methionine supplementation of the basal diet. With either breed of chick no additional growth response was obtained from cystine supplementation of a diet containing adequate methionine.

Experiments using L-methionine S^{35} to study the utilization of methionine for cystine synthesis showed a breed difference in the amount of methionine converted to cystine. More of the radioactive methionine was converted to cystine S^{35} by Single Comb White Leghorn chicks than by the Black Australorp chicks. The cystine S^{35} isolated from the carcass hydrolysate of Single Comb White Leghorn chicks had a higher specific activity than that isolated from the carcass hydrolysate of the Black Australorp chicks. Because of the good growth rate obtained with methionine supplementation of a low-cystine content basal diet, the conversion of methionine to cystine appears to be adequate to meet the physiological needs for growth in both breeds studied.

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Normal Growth and Development of Female Chickens without Dietary Vitamin E or other Antioxidants[1]

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Many studies have shown that under practical feeding conditions an adequate source of vitamin E or other antioxidant is essential in the diet of chicks. However, several reports have indicated that under some conditions, when purified diets are fed, no vitamin E is required by growing chicks (Dam, '44; Zacharias et al., '50; Miller et al., '55). If chicks can be raised successfully without vitamin E, such an observation suggests that α-tocopherol may have no biochemical function other than that of an antioxidant.

With the knowledge that selenium can replace α-tocopherol in the prevention of exudative diathesis (Patterson et al., '57; Schwarz et al., '57), and that sulfur amino acids, independent of any possible selenium content, can partially replace α-tocopherol in the prevention of muscular dystrophy in the chick (Dam et al., '52), the study of the biochemical function of vitamin E should take on clearer perspectives. In the present report it is shown that chicks can indeed be raised to maturity without dietary vitamin E and that such chicks are devoid of the vitamin as far as sensitive analytical methods can detect. The implications of these observations on the metabolism of tocopherol and its biochemical role other than that of an antioxidant are discussed.

EXPERIMENTAL

Purified diets were used in all studies. New Hampshire, female, day-old chicks from a commercial hatchery were kept in electrically heated brooders with raised, wire floors until 7 weeks of age. They were then transferred to a larger, unheated battery. Because of space limitations, the number of birds was reduced to 4 to 6 per group. The animal room was maintained at 76°F ± 2° and at 45 to 55% relative humidity.

The composition of the diets is given in table 1. It should be emphasized that no antioxidants were added to the diets. The only dietary variation worthy of special note was the vitamin A supplement. In the first study, crystalline vitamin A acetate dissolved in ethanol was added to the diets (30 mg/kg). However, a rapid destruction of the vitamin in the diets without tocopherol was found and consequently in the second experiment the vitamin A was added weekly to the drinking water (750 μg/l; Bieri, '57). When the birds were 18 weeks old, analyses of the livers from two hens that died revealed a vitamin A content bordering on a deficiency. Vitamin A was then given per os in aqueous dispersions at a dosage of 750 μg weekly.

Vitamin E in plasma or serum was determined by the micromethod of Quaife et al. ('49). In the early stages of these studies, tocopherol in tissues was determined by the procedure of Swick and Baumann ('52). When it became apparent that this method was inadequate for tissues having low concentrations of tocopherol, a more sensitive paper chromatographic method was adopted (Pollard and Bieri, '59). Briefly, the tissues were saponified for 15 to 20 minutes in the presence of pyrogallol and the unsaponifiable matter extracted in-

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[1] Preliminary reports of portions of this study have been presented before the Poultry Science Association, 1956 annual meeting (Briggs et al., '56) and before the American Institute of Nutrition, 23rd annual meeting: Bieri, J. G., and G. M. Briggs 1959 Non-essentiality of vitamin E for normal growth and development of the chick. Federation Proc., *18*: 517 (abstract).

to hexane. After washing and drying, the unsaponifiable material was concentrated to dryness under nitrogen and redissolved in methanol. Sterols were removed by two or three freezings in dry ice-ethanol. The desterolized, concentrated material was then analyzed by chromatography on zinc carbonate impregnated paper as described by Green et al. ('55).

RESULTS AND DISCUSSION

First experiment. Preliminary studies over a 6-month period with chicks fed fat-free diets without vitamin E (Briggs et al., '56; Bieri et al., '57) showed that they developed normally whether or not vitamin E was in the diet. Although it is well known that the vitamin E requirement is related to the unsaturated fat content of a diet, these long-term results were of considerable interest.

In the next study, the results of feeding vitamin E-free diets with and without fat were compared. The basal diet (C24A, table 1) contained purified casein as the protein source. The results (table 2) showed that not only chicks on a fat-free diet would survive for as long as 24 weeks without vitamin E, but also chicks fed 10% of stripped lard in the diet would develop normally without the vitamin. The appearance of these hens was normal and the weights were superior to those of hens ingesting 4% of corn oil. Deaths occurred in three of the groups, including those receiving tocopherol, between the 18th and 24th weeks. The causes of death could not be determined with certainty. Analyses of the serums for total tocopherols were performed on three of the groups after 4 weeks on the diets. As seen in table 2, no tocopherol was detectable in chicks receiving the vitamin E-free diets, whereas high tocopherol levels were observed in the supplemented group.

TABLE 1

Composition of basal vitamin E-free diets

Ingredient	Diet C24A	Diet C47E[1]
	%	%
Purified casein[2]	20.0	—
Gelatin	8.0	—
Soybean protein[3]	—	30.0
Vitamin E-free lard[4]	—	4.0
Salt mix A[5]	6.0	6.0
Glucose[6]	65.5	59.6
Vitamins[7]	0.2	0.1
L-Cystine	—	0.3
DL-Methionine	0.3	—

[1] Thirty per cent of feed-grade Torula yeast, in place of glucose, was added during the first 6 weeks. Aureomycin, 0.2 gm/kg, was added from the 18th to the 23rd week. Ten per cent of cellulose was included from the 23rd week. Selenium, 0.5 mg/kg as sodium selenite, was added from the start of the experiment.

[2] General Biochemicals, Inc.

[3] Drackett C-1, later renamed Archer-Daniels Midland Company Assay Protein.

[4] Distillation Products Industries.

[5] Briggs et al. ('52).

[6] Cerelose.

[7] Fox et al. ('55). Diet C24A contained 0.2% of choline whereas C47E contained 0.1%. The amounts of vitamins K, D and B complex were the same in the two diets. Vitamins E and A were omitted. Vitamin A acetate was given in the diet or in the drinking water as described in the text.

TABLE 2

Growth of chicks fed a vitamin E-free purified casein diet (C24A)

Additions to basal diet[1]	Deficiencies in diet	Average weights			Serum vitamin E at 4 weeks
		4 weeks	8 weeks	24 weeks	
		gm	*gm*	*gm*	*mg %*
None	Vit. E and fat	302(6)[2]	810(5)	2081(2)	0.0
10% Vit. E-free lard	Vit. E	343(6)	1019(5)	2552(3)	0.0
100 mg α-Tocopheryl acetate/kg	Fat	275(6)	846(5)	1744(2)	1.8 ± 0.37
4% Corn oil, 100 mg α-tocopheryl acetate/kg	None	318(6)	808(6)	2030(4)	—

[1] All diets supplemented with 200 mg aureomycin/kg after the 9th week.

[2] Number of chicks started was 6/group. These were reduced to 4 after the 8th week. Figures in parentheses represent the number remaining.

At no time did symptoms of exudative diathesis or encephalomalacia appear in the vitamin E-free chicks. Subsequent studies of these two syndromes revealed the reasons for this. The purified casein contained sufficient factor 3 (biologically active selenium) to prevent exudative diathesis. The level of lard employed, 10%, did not cause sufficient stress to provoke encephalomalacia; we have found that at least 15% of lard is required under the conditions of our experiment. Furthermore, the assurance of an adequate vitamin A intake in such diets has been found to reduce the incidence of apparent encephalomalacia (unpublished observation).

Second experiment. In this longer term study, diet C47E (table 1) was used which contained 30% of purified soybean protein and, for the first 6 weeks, 30% of Torula yeast. Sodium selenite at a concentration of 0.5 mg selenium/kg (0.5 ppm) of diet was incorporated to prevent exudative diathesis. The diet contained 4% of stripped lard which, as noted above, was not a sufficiently high level to cause encephalomalacia. In the absence of the selenium, however, the diet regularly produced an 80% incidence of exudative diathesis by 4 weeks, with high mortality (Bieri et al., '58). Because of the excessively watery condition of the excreta from chicks ingesting this diet, the Torula yeast was omitted after the 6th week. Since in a previous study air sac infection had been the cause of death in chicks maintained on purified diets for prolonged periods, 0.2 gm of aureomycin/kg of diet was added at the 18th week. After several weeks the excreta became very watery, consequently the aureomycin was eliminated from the diet and 10% of cellulose was added. This change was beneficial and the cellulose was continued in the diet for the remainder of the experiment.

Two groups of 12 chicks each were studied; one was given no vitamin E and the diet of the other was supplemented with the vitamin. At intervals after the 4th week, chicks were killed occasionally in order to analyze the tissues for tocopherol and also to assay various enzyme systems in which vitamin E has been implicated. The results of these latter studies have appeared elsewhere (Pollard and Bieri, '59).

Growth and egg laying. The growth rates of the two groups were similar as indicated in table 3. As in previous experiments, the appearance of the birds in the vitamin E-free group was indistinguishable from that of the supplemented birds. Egg laying began simultaneously in the two groups during the 19th week. Two artificial inseminations produced fertile eggs, but poor incubation facilities probably prevented hatching. Several embryos from both the depleted and control groups survived for 17 to 19 days. At about the 32nd week, coincident with moving and construction in the animal room, all hens abruptly ceased laying. Egg production resumed slowly about the 36th week but at the time of writing (50th week) has been poor in both depleted and control hens.

Hematology. Limited blood analyses were performed several times during the study. Table 4 indicates that at 19 weeks of age the hematocrit and hemoglobin values were similar for the two groups. When the chicks were 32 weeks old, their serums were analyzed by paper electrophoresis. Although considerable variation was found in the electrophoretic patterns, no marked differences were apparent be-

TABLE 3

Growth of chicks fed a vitamin E-free purified soybean protein diet (C47E)

Group	Average weights (weeks)				
	6	12	18	26	45
	gm	*gm*	*gm*	*gm*	*gm*
No vitamin E	634(7)[1]	1580(6)	2415(5)	2671(4)	2882(4)
50 mg α-tocopheryl acetate/kg	619(10)	1490(4)	2001(4)	2223(3)	2528(1)

[1] Figures in parentheses indicate number of chickens remaining at each period.

tween the E-free and control serums. The total serum proteins were also similar (table 4).

TABLE 4

Hematology of vitamin E-depleted and control chicks

Chick no.	Hematocrit[1]	Hemoglobin[1]	Total serum protein[2]
	%	%	%
	Vitamin E-free		
22	25.0	7.9	5.0
27	28.0	8.3	4.8
28	24.5	7.7	3.8
31	31.5	9.7	—
	Plus vitamin E		
38	25.0	7.7	4.5
41	27.0	7.9	4.3
42	28.0	8.7	—

[1] At 19 weeks of age.
[2] At 32 weeks of age.

Analyses of tissues for tocopherol. It was reported previously that when day-old chicks are fed a variety of vitamin E-free diets the rate of depletion of tocopherol from the tissues is very rapid and is not influenced by the composition of the diet (Bieri et al., '58). Within 3 to 4 weeks the apparent total tocopherols in the liver fall to a low level of 2 to 5 μg/gm. A similar observation was made by Bunnell et al. ('56) who pointed out that the same low concentration of tocopherol in the liver was attained at the same time regardless of the initial liver storage. The rate of decrease in chick liver of apparent tocopherol and also of reducible material which reacts like tocopherol has also been reported (Pollard and Bieri, '59). From our studies it became apparent that regardless of the duration of vitamin E depletion, tissues always contained a "background" level of 2 to 5 μg of "tocopherol"/gm when analyzed by the method of Swick and Baumann ('52). This low concentration of apparent tocopherol was also evident in the data of Bunnell et al. ('56) who used a molecular distillation prior to the Emmerie-Engel reaction. Since it appeared incongruous that this constant amount of apparent tocopherol should remain indefinitely, extracts of tissues from vitamin E-depleted chicks were subjected to the paper chromatographic procedure of Green et al. ('55). Although the Emmerie-Engel reacting material which migrated with the same R_f as α-tocopherol at first appeared to indicate that vitamin E was actually present, further study showed that when the sterols in the extracts were removed, the R_f was reduced almost to zero. This slowly moving material did not correspond to β-, γ-, or δ-tocopherols. Part of it was found to be reduced ubiquinone or coenzyme Q (Pollard and Bieri, '59). By the use of this modified paper chromatographic procedure it was possible to detect as little as 0.25 μg of α-tocopherol/gm of tissue.

Analyses of liver, heart and breast muscle from the vitamin E-depleted chicks, killed at varying intervals during this experiment, showed that after 4 or 5 weeks on the vitamin E-free diet the α-tocopherol content was essentially zero and never more than 0.5 μg/gm. After 7 weeks of depletion α-tocopherol was never observed in the extracts, when the total unsaponifiable material from 2 to 4 gm of tissue was analyzed by chromatography. Tissues from the control chicks receiving vitamin E always contained α-tocopherol which was estimated usually to be in excess of 10 μg/gm. Similarly, the eggs from E-free hens contained no detectable α-tocopherol when analyzed as described, whereas eggs from the control hens had considerable amounts of this tocopherol.

Bouman and Slater ('57) have reported that essentially all of the α-tocopherol in tissues is in the mitochondria, and we have found this to be true in both rat and chick liver. In order to determine the possible presence of traces of tocopherol in the tissues from E-depleted chicks with greater sensitivity, the mitochondria from 4.3 gm of heart muscle from chicks receiving the deficient diet for 5 weeks were analyzed as described above. The paper chromatogram of the unsaponifiable material had no Emmerie-Engel reacting material. There were in such chromatograms, however, often as many as three colorless zones which quenched and one which fluoresced in ultraviolet light. These materials, present in varying quantities, had different R_f's from α-tocopherol and were seen routinely in chromatograms from both normal and vitamin E-depleted tissues.

Possible tocopherol content of the deficient diet. Although we were unable to

detect any traces of vitamin E in the tissues of the depleted chicks, it appeared desirable to determine whether they were obtaining small amounts of the vitamin in the diet. The only possible sources of tocopherol in diets C24A and C47E were the purified casein or soybean protein and the stripped lard. Since the longest study was conducted with the C47E diet, attempts were made to find α-tocopherol in the soybean protein. Analyses in which the unsaponifiable matter obtained from 4 gm of protein, after acid hydrolysis and extraction, was analyzed by paper chromatography in one direction as described above did not reveal any α-tocopherol. A very small amount of material was noted near the origin, however, which could possibly be β- + δ-tocopherol, (soybean oil contains about 80% of its total tocopherols as β- + δ- (Green et al., '55)). The contribution of α-tocopherol by this component of the diet appears to be essentially zero.

According to the manufacturer's label on the stripped lard, this material contained not more than 5 μg tocopherols/gm. We were unable to find even one-tenth this amount (0.5 μg/gm). If, as appears reasonable, it is assumed that all of the α-tocopherol present in this diet was from the lard, and the amount was the high figure of 5 μg/gm of lard, then for a hen consuming 70 gm of this diet daily the maximal theoretical α-tocopherol intake would be about 14 μg. It is well-known that a diet such as C47E containing stripped lard readily becomes rancid. Furthermore, the autoxidation of unsaturated fatty acids in the digestive tract and probably also in the tissues is accelerated by such a diet. This combination of autoxidation in the diet and in the body effectively destroys tocopherol. The question, then, of whether chickens depleted of vitamin E for almost a year on such a diet may still have functional trace amounts of α-tocopherol in their tissues becomes more or less academic. Since the most sensitive analytical method available to us did not reveal any α-tocopherol, it appears highly unlikely that functional traces were still present.

Implications of these studies on the nutritional and biochemical roles of α-tocopherol. These experiments could be interpreted as evidence that chickens do not need vitamin E in their diet. It should be pointed out, however, that the baby chicks used in these studies came from hens fed a complete, practical diet and consequently the chicks had body reserves of tocopherol when hatched. These reserves were rapidly exhausted, however, until no α-tocopherol could be found in the tissues after 4 to 7 weeks. Significantly, this absence of tocopherol from the body in no way impaired normal growth and development.

Two problems are involved when both the nutritional and biochemical implications of these studies are considered. From the nutritional standpoint, with diets such as those used here, evidently no antioxidants are required, provided special precautions are taken to insure an adequate intake of vitamin A. Several times in these studies during the early growth period, the E-depleted chicks appeared to respond to higher supplements of vitamin A. This observation, together with the finding of very low liver reserves in chickens which died, led to the subsequent use of relatively massive oral doses of vitamin A. Apparently, then, the primary function of vitamin E in these diets is that of an antioxidant to protect vitamin A. The unsaturated fatty acid content was low enough so that the accumulation of autoxidation products did not produce encephalomalacia. Even though the diet did not contain antioxidants, some compounds in the diet or, more probably, compounds produced in the body apparently have a significant antioxidant function. We have observed (Bieri, '59) that tissue homogenates from chicks receiving adequate dietary vitamin E do not give a positive peroxide test with thiobarbituric acid, even after incubation in air. An occasional homogenate of tissue from tocopherol-deficient chicks has given a positive test without incubation; however, *all* deficient tissues give positive reactions after a short incubation. These observations indicate that the polyunsaturated fatty acids in vitamin E-deficient tissues have some degree of protection in the animal by nontocopherol antioxidants, but either the protection is insufficient, or the antioxidants are lost when the tissues are exposed to air.

From the biochemical viewpoint, the fact that chicks function normally when no α-

tocopherol is detectable in their tissues would seem to rule out any vital role for tocopherol in intermediary metabolism. When the possible concentration of tocopherol in depleted tissues is calculated from the limits of sensitivity of the analytical method, the molar concentration is considerably below that of other essential metabolites with which tocopherol has been associated. For example, with respect to the suggested role for tocopherol in electron transport (Nason et al., '57), calculations show that α-tocopherol would be present in vitamin E-depleted chick heart muscle at no more than 0.002 μmole/gm. Although no data are available on the cytochrome C content of chick heart, the value for normal rat heart is more than 10 times this value (Kampschmidt et al., '59). These considerations, together with the finding that reduced diphosphopyridine nucleotide-cytochrome C reductase in heart muscle preparations from vitamin E-depleted chicks was similar to that of preparations from E-supplemented chicks (Pollard and Bieri, '59), appear to eliminate any role for tocopherol in such enzyme systems.

SUMMARY

Two studies are reported in which chicks were fed from hatching, purified, vitamin E-free diets containing no added antioxidants for 6 to 12 months. Chemical analyses indicated that α-tocopherol disappeared from the tissues of the chicks after 5 weeks. When compared with control chicks receiving vitamin E, the depleted chicks appeared normal, grew at the same rate and layed eggs at the same time. The possible tocopherol content of the diet, and the implications of these results on the nutritional and biochemical functions of vitamin E, are discussed.

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Zinc Requirement and Balance Studies with the Rat

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The recent upsurge of interest in zinc metabolism of swine[1] (Smith et al., '58; Bellis and Philp, '57; Tucker and Salmon, '55; Newland et al., '58; Luecke et al., '58; Lewis et al., '57) and of poultry (Young et al., '58; Morrison and Sarett, '58; Pensack et al., '58; Moeller and Scott, '58; Kratzer et al., '59; O'Dell and Savage, '57; O'Dell et al., '58) has shown that the metabolism may be modified by such factors as dietary calcium level (Bellis and Philp, '57; Newland et al., '58; Lewis et al., '57) and presence of soybean protein (O'Dell and Savage, '57; Moeller and Scott, '58; Morrison and Sarett, '58; and Kratzer et al., '59). The present series of investigations was initiated to elucidate the zinc requirement of the rat and to study the excretory pattern of dietary zinc under varied conditions of calcium and protein supply.

METHODS

In all experiments reported in this paper the diets fed contained the basic ingredients shown in table 1. The protein sources were fed at a level to provide about 15% of dietary protein, with calcium and zinc, as the carbonates, supplemented as indicated. The animals were given distilled water ad libitum and were kept in individual cages of Pyrex glass, aluminum and stainless steel. All animals were males of the Sprague-Dawley strain and weighed about 50 gm at the start, except those in experiment 4. Collections of excreta were made with appropriate attention to avoid, as far as possible, contamination with zinc. All analyses for zinc were conducted by the Vallee-Gibson procedure (Vallee and Gibson, '48). It was found necessary to remove iron from excreta samples prior to completion of zinc analysis. This was done by extracting the mixed dithizonate of zinc and iron with two portions of 0.01 N HCl, thus breaking up the zinc-dithizonate complex and transferring the zinc to the aqueous layer, leaving the interfering iron dithizonate in the CCl_4 layer.

TABLE 1
Basal diet composition

	%
Protein sources[1]	15
Corn oil	10
Cellulose[2]	3
Vitamin-glucose mix[3]	5
Mineral mix[4]	3.7
Vitamin A and D concentrate[5]	0.5
Glucose	62.8

[1] Protein sources were one of the following: Labco casein containing 30 ppm zinc; C-1 Assay Protein containing 30 ppm zinc (Archer-Daniels Midland Co., Cincinnati); laboratory-prepared dried egg white containing 2.4 ppm zinc.

[2] Solka Floc. Brown Company.

[3] Vitamin-glucose mix, mg/kg of mix: thiamine·HCl 200; riboflavin, 120; pyridoxine·HCl, 80; Ca pantothenate, 320; biotin, 4; nicotinic acid, 500; folic acid, 10; vitamin B_{12}, 0.4 (in mannitol); choline chloride, 30,000; menadione, 6.6; chlortetracycline, 1,100; Cerelose added to make 1000 gm.

[4] Mineral mix composition by parts: $CaHPO_4$, 708; NaCl, 101; K_2CO_3, 136 3; $MgCO_3$, 37.4; $FeSO_4 \cdot 7H_2O$, 10.8; $MnSO_4 \cdot H_2O$, 3.266; $CoCl_2 \cdot 6H_2O$, 1.080; $CuSO_4 \cdot 5H_2O$, 1.633; NaF, 0.216; KI, 0.108. All analytical reagent grade.

[5] Vitamin A and D concentrate: 2000 I.U. vitamin A and 250 I.U. vitamin D per gm.

Experiment 1. Twenty-four rats were fed for 6 weeks a 15% casein basal diet supplemented with 0.3% of methionine and containing 0.8% of calcium and 7 ppm of zinc. This diet was modified by additions of zinc and calcium as the carbonate to provide both 0.8 and 1.6% of calcium at zinc levels of 7, 18, 31 and 52 ppm. Three animals were placed on each treatment. Feed intake was equalized for all animals during the first two weeks, and thereafter for all animals receiving more

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[1] D. W. Beardsley 1958 Growth and chemical studies of zinc deficiency in the baby pig. Doctoral thesis, University of Illinois.

than 7 ppm of zinc. During the second and 6th weeks, 7-day collections of urine and feces were made. At the end of the experiment the rats were killed and carcasses saved for zinc analysis. At the start of the experiment 6 rats were killed to provide data from which to judge accretion of zinc.

Experiment 2. A total of 45 rats was used in this experiment in which the protein sources were casein plus methionine, egg white and isolated soybean protein plus methionine. Zinc carbonate was added to these diets to provide zero, 5, 10, 15 and 20 ppm of added zinc. Details of feeding and caging the animals were the same as in experiment 1, although collections were not made. Three animals were used for each treatment.

Experiment 3. This was designed to test further the zinc requirement in the presence of casein and to study the effect of calcium on zinc utilization in isolated soybean protein diets. Twenty-four rats were fed the basal diets described previously. Casein diets were supplemented with zero, 2, 4, 8 ppm of zinc as the carbonate; the soy protein diets were supplemented to provide 10 or 20 ppm of added zinc at levels of 0.8 or 1.6% of calcium. A 7-day collection of feces was made from the animals receiving the soy protein diets.

Experiment 4. This was a balance study designed to investigate further the calcium-zinc interrelationship of rats on soy protein diets, and differed from the preceding test in that zero and 10 ppm of supplemental zinc were tested at 0.8 and 1.6% of calcium, and in that 175-gm rats were used rather than weanling rats.

Experiment 5. This was conducted to provide additional data on weight gain of rats receiving varied levels of zinc added to the egg white protein diet. Seven rats were used, 3 on the basal and 2 each on 15 and 25 ppm of zinc.

RESULTS AND DISCUSSION

A summary of the 42-day weight gains in experiment 1 is shown in table 2. Analysis of variance applied to these data shows highly significant effects of zinc level and of calcium level but no significant interaction between calcium and zinc. Deficiency symptoms, coarseness and loss of hair, mild dermatitis, scaling and cracking of paws, erratic appetite, were not influenced by calcium level and were present only in the animals on the basal diet containing 7 ppm of zinc.

TABLE 2

Effect of varied levels of dietary zinc and calcium on 42-day weight gains (gm) of rats fed casein diets in experiment 1

Zn in diet	Dietary calcium	
	0.8% level	1.6% level
ppm	*weight gain (gm)*	
7 (Basal)	102	78
18 (Basal + 11 ppm Zn as $ZnCO_3$)	183	170
31 (Basal + 24 ppm zn as $ZnCO_3$)	192	174
52 (Basal + 45 ppm Zn as $ZnCO_3$)	185	168

TABLE 3

Influence of dietary zinc and calcium levels on percentage of zinc intake excreted by rats fed casein diets in experiment 1

Treatment	% Zn intake excreted			
	Collection 1		Collection 2	
	Urine	Feces	Urine	Feces
0.8% Ca[1]	7.8	39	5.0	43
1.6% Ca[1]	7.8	40	4.6	48
7 ppm Zn[2]	17	15	12	18
18 ppm Zn[2]	7.2	31	3.2	37
31 ppm Zn[2]	4.3	48	2.4	55
52 ppm Zn[2]	2.7	65	1.3	74

[1] Including all levels of dietary zinc.
[2] Including both levels of dietary calcium.

The data from the zinc balance studies of experiment 1 are shown in table 3. The conclusions drawn from these data were based on results of statistical analysis. Zinc excretion, expressed as a per cent of the intake, was less in period 2 urine than in period 1. The reverse was true of fecal zinc excretion. The total excretion of zinc did not differ between the two periods.

The presence of additional calcium had no effect on zinc excretion by way of urine and feces. The absolute amount of zinc excreted in urine was not influenced by zinc intake. This is reflected in the decreasing percentage of zinc intake excreted by this pathway as zinc intake in-

TABLE 4

Influence of dietary zinc and calcium levels on liver and carcass zinc (ppm, fresh basis) of rats fed casein diets in experiment 1

Treatment	Zn in liver	Zn in carcass less liver
	ppm	*ppm*
0.8% Ca[1]	17	22
1.6% Ca[1]	17	21
7 ppm Zn[2]	16	18
18 ppm Zn[2]	16	22
31 ppm Zn[2]	16	23
52 ppm Zn[2]	19	24
Initial	43	26

[1] Including all levels of dietary zinc.
[2] Including both levels of dietary calcium.

creased. However, zinc excretion by way of the feces increased both in absolute and in percentage terms, as dietary zinc concentration increased.

Table 4 shows liver and carcass zinc concentrations. Analysis of livers of newly weaned rats showed 43 ppm of zinc on the fresh basis, whereas about 17 ppm were found in livers of animals fed for 6 weeks, with no effect of calcium or of zinc except at the highest zinc level. Carcass analyses showed 26 ppm of zinc in newly weaned rats, 18 to 24 ppm in those fed for 6 weeks, the variation noted being related to zinc intake but not to that of calcium. The total retention of zinc in the carcasses increased rapidly as dietary zinc increased from 7 to 18 ppm, but only slowly from 18 to 52 ppm. This is primarily a reflection of the larger feed intake and weight increase in rats fed the second and higher levels in comparison with the basal level of zinc.

In that portion of experiment 2 dealing with requirement studies of rats consuming casein diets, only the basal group appeared to be receiving less than an optimum supply of zinc; thus an additional experiment was required to establish the slope of the response to suboptimum zinc levels. This was conducted as a portion of experiment 3, and the data were combined. Since the food intake varied widely among the adequately-fed rats between the two experiments, analysis of covariance was used to minimize the effect of this variable and the adjusted weight gains of the rats are shown in figure 1. The lines shown in the figure intersect

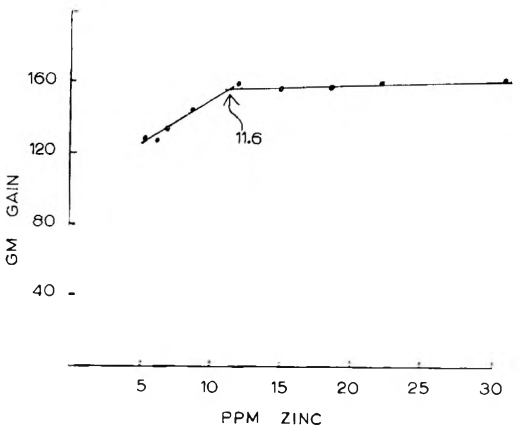

Fig. 1 Adjusted 42-day weight gains of rats fed casein diets and varying levels of zinc. Each dot represents the average gain of a group of three rats.

at 11.6 ppm of zinc (5 ppm added), thus establishing this level as that required for optimum weight gain of the young male albino rat whose basal dietary zinc is present in casein. The data obtained from feeding varied levels of zinc to rats consuming the isolated soy protein diets show (fig. 2) that the requirement for optimum weight gain is about 18 ppm (11 ppm added) when the basal dietary zinc is derived from isolated soybean protein. In the experiment employing egg white as the protein source, since insufficient evidence for higher levels of zinc was obtained, a supplementary experiment (no. 5) was conducted under similar condi-

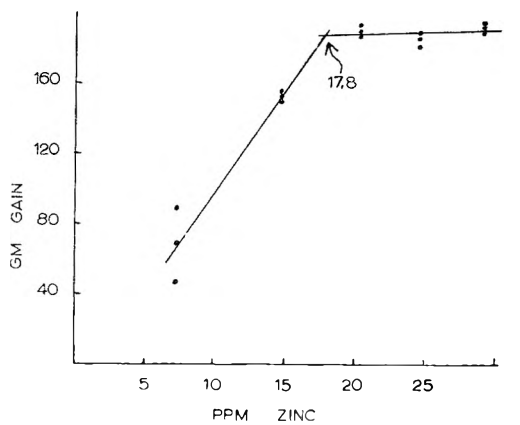

Fig 2 Individual 42-day weight gains of rats fed isolated soybean protein diets and varied levels of zinc.

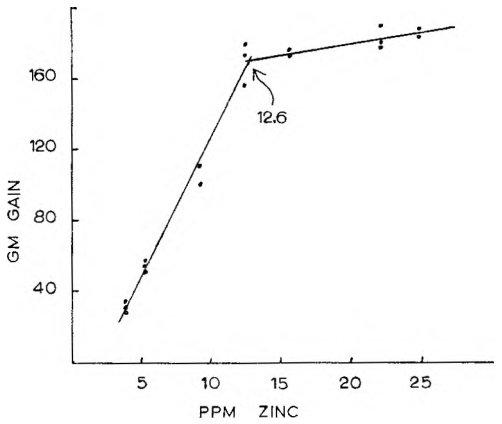

Fig. 3 Individual 35-day weight gains of rats fed egg-white protein diets and varied levels of zinc.

TABLE 5

Effect of dietary zinc and calcium on weight gain (gm) and percentage of zinc intake excreted in feces of rats fed isolated soy protein diets

Treatment	Weight gain	Zn intake excreted in feces
	gm	*%*
Experiment 3 (40 days)		
Basal + 12 ppm Zn	187	48
Basal +12 ppm Zn + 0.8% Ca	154	48
Basal + 22 ppm Zn	186	65
Basal + 22 ppm Zn + 0.8% Ca	159	49
Experiment 4 (32 days)		
Basal (7.7 ppm Zn)	73	56
Basal + 0.8% Ca	65	54
Basal + 12 ppm Zn	108	51
Basal + 12 ppm Zn + 0.8% Ca	97	59

tions, and these data were combined with the results shown in figure 3, in which the intersection of the lines is at 12.6 ppm of zinc.

The data obtained in the requirement studies clearly demonstrate that the minimum concentration of zinc required by the young rat approximates 12 ppm of the diet. This figure is considerably higher than that inferred from the earlier investigation of Hove, Elvehjem and Hart ('37). The recent report by Moeller and Scott ('58) employing chicks, indicates a similar figure.

Our data with isolated soy protein are qualitatively similar to the several reports of investigation with poultry (O'Dell and Savage, '57; Morrison and Sarett, '58; Moeller and Scott, '58) although the ultimate requirement figure is not as high, presumably because we have used lower protein levels.

Investigators in poultry science have inferred that the zinc of soy protein is less readily absorbed than that in casein. Our zinc balance data from experiments 1 (table 3), and 3 and 4 (table 5) show this to be the case.

These data show that the apparent absorption of dietary zinc is much inferior from the basal diets containing isolated soy protein (table 5) compared with those containing casein (table 3), and that the presence of calcium has no definite effect on absorption of dietary zinc in the presence of either protein. Thus, the investigations we have conducted with the rat have failed to show a specific effect of calcium on zinc absorption or on the zinc requirement. This is not in agreement with observations with practical swine rations in which marked improvement in condition has been observed as a result of feeding 0.5 or 0.8 rather than 1.6% of calcium in diets containing 30 to 40 ppm of zinc (Luecke et al., '58; Lewis et al., '57). Lewis et al. ('57) also noted a marked effect of calcium level on zinc concentration of several tissues, including liver. Our data showed no such trend. However, the data from experiments 3 and 4 are in agreement with those of experiment 1 in so far as the effect of calcium on weight gain is concerned. In all of this work there is a highly significant depression of weight gain of animals fed 1.6 versus 0.8% of calcium, and no interaction with zinc level occurs. Thus, the depression in gain appears to be related to the unfavorable Ca/P ratio of 2.3/1 in the diets. That such a ratio is wider than the optimum for weight gain of rats may be seen in data from Bethke ('32) and from Toepfer and Sherman ('36).

Further, it may be calculated by the usual difference procedure that the zinc carbonate added to supply a total of 20 ppm of zinc in casein and in soy protein diets was absorbed to the extent of 52 and 50%, respectively. If one employs

the above absorption data together with the requirement data given, the requirement in terms of absorbable zinc is 8.2 ppm from the casein data and 8.6 ppm from the soy data. The good agreement between these figures lends support to the validity of the balance trials and the estimations of requirement from the growth studies.

SUMMARY

In a series of growth and balance studies concerned with investigating factors affecting the zinc requirement of the young albino rat, increasing calcium level from 0.8 to 1.6% of the diet was observed to depress weight gain at all levels of zinc fed both in diets containing casein and those containing isolated soybean protein. Apparent absorption of dietary zinc and its urinary excretion were not affected by increased calcium. Zinc requirement was 18 ppm with 7 ppm derived from soy protein and the remainder from $ZnCO_3$; 12 ppm with 7 ppm supplied from casein and 5 from $ZnCO_3$; and 12 ppm with 2 to 4 ppm derived from egg white and the remainder from $ZnCO_3$. Apparent absorption of zinc from casein, $ZnCO_3$ and isolated soybean protein was 84, 51 and 44%, respectively.

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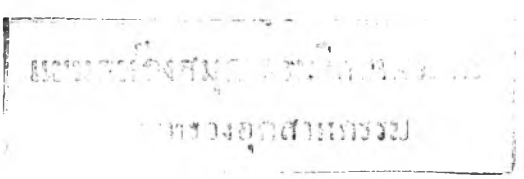

The Availability of the Phosphorus in Dicalcium Phosphate, Bonemeal, Soft Phosphate and Calcium Phytate for Mature Wethers[1]

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Despite the current interest in availability of phosphorus from various supplements and feeding trials which have been conducted upon them, few measurements have been made of actual absorption of phosphorus from the intestinal tract. The relative availability of phosphorus from various substances has been determined for poultry (Gillis et al., '54; Wilcox et al., '54) using growth and bone ash as criteria of availability. Ammerman et al. ('57) studied the utilization of inorganic phosphates by the ruminant. Dicalcium phosphate, bonemeal and soft phosphate were studied resulting in poor utilization of the soft phosphate. No direct measures of absorption were made, however. Kleiber et al. ('51) adapted an isotope dilution method for use with dairy cows measuring the absorption of phosphorus from a mixed hay and concentrate ration. The development of isotope dilution techniques has made it possible to measure availability in terms of absorption, or true digestibility as it is classically referred to in contrast to apparent digestibility—not a valid measure of absorption because of re-excretion of phosphorus into the intestinal tract.

Dicalcium phosphate, bonemeal and soft phosphate with collodial clay have all been used as phosphorus supplements for ruminants. Since little is known of the actual amount of phosphorus which will be absorbed from these supplements, it seemed important to study the availability of their phosphorus content. Much of the phosphorus in ruminant feeds is known to be in the form of calcium phytate and apparently a high proportion is hydrolized in the rumen, making the phosphorus available for absorption. No direct measures of absorption have been made, however, on isolated calcium phytate. The studies reported herein were conducted to determine the availability of the phosphorus from dicalcium phosphate, bonemeal, soft phosphate with collodial clay and isolated calcium phytate. Availability is used herein to mean absorption or true digestibility.

EXPERIMENTAL

The 4 phosphorus supplements were added to a basal ration in amounts estimated to approximately double the phosphorus intake. The basal ration alone and together with each of the 4 supplements were fed to 5 mature wethers according to a 5 by 5 Latin square design with each period consisting of a minimum of a 7-day preliminary period and an 8-day collection period, with a constant feed intake being maintained for the entire period. The basal ration consisted of the following percentage composition: alfalfa hay, 14.9; barley straw, 8.9; cottonseed hulls, 4.8; ground barley, 27.8; dried molasses beet pulp, 14.3; cottonseed meal, 14.3; cane molasses, 14.3 and salt, 0.7.

The determined phosphorus contents of the 4 supplements were 19.70, 13.40, 9.77 and 14.75 per cent for dicalcium phosphate, bonemeal, soft phosphate and calcium phytate, respectively.

All wethers were fed twice daily at a maintenance level of energy. The phosphorus supplements were mixed with molasses prior to adding them to the re-

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mainder of the basal ration. This prevented selection of the ingredients of the rations. The supplements were added to the basal ration in the following percentages: dicalcium phosphate, 1.65; bonemeal, 2.45; soft phosphate, 3.39 and calcium phytate, 2.22.

The 5 wethers were assigned at random to ration sequences also chosen at random according to the method described by Fisher and Yates ('49). The isotope dilution method of determining true digestibility was used for phosphorus availability studies with sheep (Lofgreen and Kleiber, '54). The method involves the labeling of body phosphorus by one subcutaneous injection of neutral, isotonic phosphate solution containing P^{32}. After a period of 7 days, a steady state is reached so that the decline in activity of blood and fecal phosphorus is linear through the collection period, during which blood and fecal samples are taken. The proportion of fecal phosphorus of metabolic origin (as distinguished from that passing directly through the tract unabsorbed) is represented by the ratio of specific activity of feces to that of blood plasma. That portion of the fecal phosphorus having passed through the tract, never having been absorbed, is represented by

$$1 - \frac{\text{specific activity of fecal phosphorus}}{\text{specific activity of plasma phosphorus}}.$$

In order to determine the time lag required for a given level of activity in the plasma to be reflected in the feces, fecal samples were collected daily for 4 days following injection. Data from these samples dictated the number of days lag between the taking of the plasma and fecal samples. For example, the data shown in figure 1 show that the peak in fecal specific activity occurred during the second day following injection.

Since peak plasma activity is known to occur within the first half-hour after a subcutaneous injection, a given level of activity in the plasma can be assumed to be reflected in the feces approximately two days later. For the animal for which data are shown in figure 1, the blood specific activity, therefore, from days 6 through 12 would be related to the fecal samples taken on days 8 through 14, with that collected on day 7 being discarded. The lag period was thus determined on all animals during periods one and 5.

Phosphorus was determined on a trichloracetic acid filtrate (TCF) of blood plasma by the method of Sumner ('44). Feed and fecal samples were prepared for phosphorus determination by wet ashing with concentrated sulfuric acid with the last traces of carbon oxidized by use of hydrogen peroxide. Samples were neutralized with ammonium hydroxide and

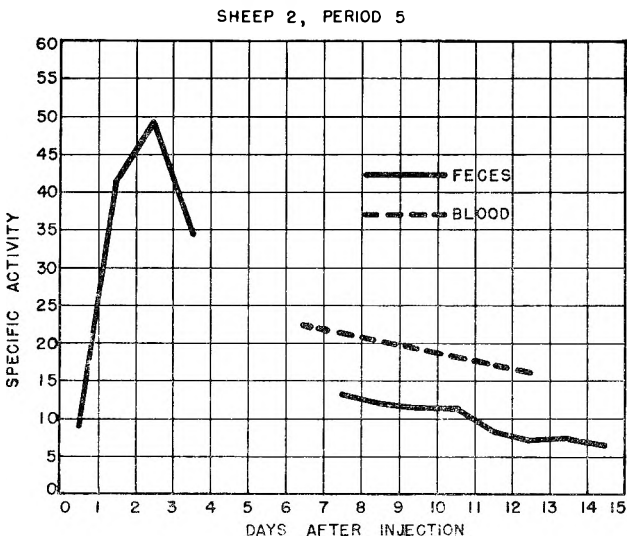

Fig. 1 Fecal and blood specific activities for an individual sheep.

phosphorus determined by the same method used for plasma TCF. Determinations of P^{32} were made after pipetting the plasma TCF or the fecal ash solution onto copper planchets according to the method described by Kleiber ('41).

The levels of P^{32} injected were 3.2, 4.6, 4.0, 5.1, and 3.8 millicuries per animal for periods one through 5, respectively.

RESULTS AND DISCUSSION

The relative specific activities of blood and feces, with the activity of the blood given the value of 100 for each of the 7 days of the collection period are shown in figure 2. The activities have been corrected for the lag in fecal specific activity for each sheep. The more closely the fecal activity approaches the activity of the blood, the greater is the proportion of the dietary phosphorus being absorbed. The reason for this is that if the nonactive feed phosphorus is completely absorbed from the intestinal tract, the only phosphorus found in the tract will be that of metabolic origin (having come from the labeled body phosphorus) and will be labeled with P^{32}. At complete absorption the fecal and blood specific activities would be equal. Conversely, the farther apart the blood and fecal activities, the smaller the proportion becomes of dietary phosphorus being absorbed, since the unabsorbed nonlabeled phosphorus dilutes the activity of that being excreted into the feces from the body phosphorus. Thus, figure 2 indicates that a larger proportion of the phosphorus in the basal ration is being absorbed than from the basal ration plus any of the supplements. The basal ration is followed by this ration plus dicalcium phosphate, bonemeal, calcium phytate and soft phosphate, in that order.

The data in figure 2 include the phosphorus of both basal and supplements. Since the availability of the phosphorus in the basal alone and in the basal plus each supplement is known, it is possible to determine the availability of the phosphorus in each supplement. These data are presented in table 1. When the values are corrected for the phosphorus absorbed from the basal, striking differences are observed in the percentage of phosphorus from the supplement which was absorbed. The phosphorus in the soft phosphate was absorbed at a lower rate (highly significant) than any of the other supplements. That from calcium phytate was significantly lower than dicalcium phosphate or bonemeal and no statistically significant difference was observed in the availability of the phosphorus in dicalcium phosphate and bonemeal.

Although the phosphorus intakes in these studies are considerably above the requirements for mature wethers, all supplements were fed at a level to furnish approximately the same amount of phosphorus. The results, therefore, are a re-

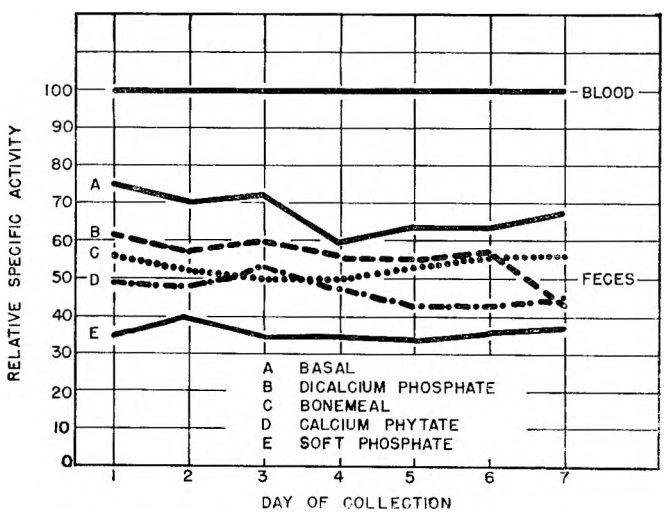

Fig. 2 Mean relative specific activity of blood and feces of all wethers on each treatment.

TABLE 1

Phosphorus intake, excretion and absorption[1]

Category of interest	Basal ration	Phosphorus supplement			
		Dicalcium phosphate	Bone-meal	Soft phosphate	Calcium phytate
Phosphorus intake, gm/day					
From basal	4.69	4.16	4.56	4.31	4.53
From supplement	—	3.43	3.86	3.76	3.91
Total	4.69	7.59	8.42	8.07	8.44
Fecal phosphorus, gm/day	4.25	6.84	7.47	7.16	7.58
Phosphorus apparently absorbed, gm/day	0.44	0.75	0.95	0.91	0.86
Apparent digestibility of P, %	10.4	9.9	11.3	11.3	10.2
Metabolic fecal P					
Per cent of fecal P	66	56	53	36	47
Gm/day	2.81	3.83	3.96	2.58	3.56
Unabsorbed P, gm/day					
From basal	1.44	1.29	1.41	1.34	1.4
From supplement	—	1.72	2.10	3.24	2.62
Total	1.44	3.01	3.51	4.58	4.02
Dietary phosphorus absorbed, gm/day					
From supplement	—	1.71	1.76	0.52	1.29
True digestibility of P of supplement, %	—	50	46	14[2]	33[3]

[1] Each value is a mean of 5 observations.
[2] Statistically significant at the 1% level.
[3] Statistically significant at the 5% level.

flection of the availability of the supplements for absorption by wethers under these conditions. If the absorption of phosphorus is related to the needs of the animal, perhaps all supplements show a lower availability than would be the case with younger animals or when fed as a supplement to a deficient ration. These points need further study. The significantly lower availability of the phosphorus in calcium phytate is noteworthy since large amounts of phosphorus in natural feeds are present in this form and this phosphorus has been commonly assumed to be available to the ruminant animal.

Since unpublished work from this laboratory shows that approximately 99 per cent of the total phosphorus excreted by sheep is excreted in the feces, the phosphorus apparently absorbed is a good indication of the net retention of phosphorus. It is interesting to note that these wethers apparently absorbed approximately twice as much phosphorus on all the supplements as on the basal ration. Since this closely approximates phosphorus retained, this could mean that the basal ration was not furnishing sufficient available phosphorus to fill the needs of the animals. During a short balance study, however, animals are able to retain larger amounts of a given nutrient than would be the case over longer periods. It is also interesting to note that even though only 14 per cent of the phosphorus of the soft phosphate was absorbed, this amount was apparently sufficient to meet the needs of the animals, since the larger amounts absorbed from the other supplements were merely excreted back into the gut as part of the metabolic fecal phosphorus.

The data presented emphasize the importance of continued research on the availability of phosphorus from various feeds and supplements and the conditions which might affect this availability. This is a field which is now possible to explore directly by use of recently-developed isotope methods which are adaptable to routine use.

SUMMARY

Studies are described in which the availability (true digestibility) of the phosphorus in dicalcium phosphate, bonemeal, soft phosphate and calcium phytate was

determined by the isotope dilution method with mature wethers.

The true digestibility of the phosphorus in the supplements was 50, 46, 14 and 33 per cent for dicalcium phosphate, bonemeal, soft phosphate and calcium phytate, respectively.

Although significantly less phosphorus was absorbed from the soft phosphate than from the other supplements, the phosphorus retained from the basal ration plus soft phosphate was equal to the others. The surplus phosphorus absorbed from the rations containing the other supplements was excreted by way of the metabolic fecal phosphorus.

Significantly less phosphorus was absorbed from calcium phytate than from dicalcium phosphate and bonemeal.

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Influence of Excess Dietary Molybdenum on Rat and Calf Liver and Heart Enzymes[1,2]

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The mechanism of the toxicity syndrome accompanying molybdenosis in animals has not as yet been elucidated. Recent reviews dealing with this syndrome (Marston, '52; Dick, '56) have presented theories involving the relationship between molybdenum and copper, sulfur and manganese. One approach to the problem has been to determine the effect of excess dietary molybdenum on enzymes. Van Reen ('54) and Van Reen and Pearson[5] have shown that high levels of dietary molybdenum did not decrease the level of catalase or cytochrome oxidase in rat liver nor the amount of acetylated *p*-aminobenzoic acid excreted in the urine. Williams and Van Reen ('56) found that rats on high dietary levels of molybdenum did not lose their ability to acetylate *p*-aminobenzoic acid or to conjugate benzoic acid with glycine. Liver alkaline phosphatase did increase, whereas the activities of the kidney and intestinal alkaline phosphatase were reduced. Recent studies by Mills and co-workers ('58) have shown that the activity of liver sulfide oxidase was markedly depressed in molybdenum toxicity of the rat. High molybdenum intakes apparently had no effect on the activity of liver cysteine desulfhydrase, kidney aryl sulfatase or the oxidation of L-cysteine sulfinate by liver homogenates.

The experiments to be described were designed to study the effect of excess dietary molybdenum on various enzymes of the liver and cytochrome oxidase of the heart of both a monogastric and a ruminant animal.

EXPERIMENTAL

In the first experiment weanling piebald rats of the Long-Evans strain, 21 to 25 days old, were divided randomly by sex and weight into three treatments and housed in cylindrical cages constructed of sheet aluminum and set upon aluminum wire. They were maintained on a mineralized whole milk diet (Arrington and Davis, '55) to which had been added zero, 500 and 800 ppm of molybdenum (as sodium molybdate) for 5 weeks (males) and 8 weeks (females). The copper content of the diets was 7.5 ppm.

In experiment 2, 24 rats were maintained on a synthetic diet having the following composition in per cent: casein, 25; sucrose, 57; cottonseed oil,[6] 10; nonnutritive cellulose,[7] 3; mineral mixture,[8] 5; and vitamins, [9]; with zero, 250 and 800

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[4] Present Address: U. S. Dept. of Agriculture, Foreign Agricultural Service, American Embassy, Lima, Peru.

[5] R. Van Reen, and P. B. Pearson 1954 Biochemical abnormalities during molybdenum toxicity in rats. Federation Proc., *13:* 314 (abstract).

[6] Wesson oil.

[7] Alphacel, obtained from Nutritional Biochemicals Corp., Cleveland.

[8] Percentage composition of mineral mixture: $CaCO_3$, 21.36; $CaHPO_4 \cdot 2H_2O$, 33.28; $MgCO_3$, 2.00; $MgSO_4 \cdot 7H_2O$, 2.40; NaCl, 10.16; KCl, 1.68; KH_2PO_4 25.20; $FePO_4 \cdot 4H_2O$, 1.60; $MnSO_4 \cdot 4H_2O$, 2.00; KI, 0.048; NaF, 0.083; $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$, 0.017; $CoCl_2 \cdot 6H_2O$, 0.C04; $CuSO_4 \cdot 5H_2O$, 0.128; $ZnSO_4 \cdot 7H_2O$, 0.04.

[9] The vitamins were added in the following mg per cent: thiamine, 1.5; pantothenic acid, 8; *p*-aminobenzoic acid, 1; B_{12}, 0.005; pyridoxine, 0.8; menadione, 2.5; riboflavin, 2; niacin, 5; choline, 100; inositol, 50; folic acid, 0.06; E, 7.5; and A and D, 900 and 90 I. U./100 gm of diet, respectively.

ppm of molybdenum (as sodium molybdate) added for three lots of rats. Males were maintained on the diets for 7 to 8 weeks, females, 9 weeks. The average copper content of the diets was 5.7 ppm. The rats were housed in glass cages, with single-strength window glass as sides and glass tubing for top and bottom.

The diets and de-ionized, distilled water were provided ad libitum. In both experiments the rats were killed at the end of the designated periods by lightly anesthetizing with ether, then by decapitation and exsanguination.

The entire heart and liver were excised and placed immediately into cold, 0.39 molar phosphate buffer (pH 7.4) until thoroughly chilled, then removed from the buffer, blotted free of moisture, weighed and homogenized.

Enzyme determinations made on the tissues of the rats for these two experiments included xanthine oxidase, respiration and copper and molybdenum levels in the liver. In addition, in the first experiment tyrosine oxidase was determined on livers, and in the second experiment, heart cytochrome oxidase and blood uric acid levels were measured.

In the third experiment 9 Jersey bull calves, two to three months old, were divided randomly into three dietary treatments. The composition of the basal ration fed the three control calves follows (in pounds): cracked corn, 1.5; crimped oats, 1.5; dry skim milk, 0.25 and timothy hay, 4.0. The other two lots of three calves each, received, in addition, molybdenum in the respective amounts of 200 and 400 ppm of the ration, added as sodium molybdate to the dry skim milk. The timothy hay was fed in the morning and 3 pounds of the concentrate were fed in the evening. The daily intake of hay and concentrate supplied 9.9 ppm of copper. The animals were individually housed in wooden pens and given water and salt ad libitum. After intervals of three to 6 months on the regimen, the calves were slaughtered by severing the jugular vein and exsanguinating. Liver samples were taken from 4 lobes of the liver, right, middle, left and caudate; heart samples were taken from the ventricles and auricles. The samples were placed immediately into cold, 0.039 molar phosphate buffer (pH 7.4) and when thoroughly chilled, removed from the buffer, blotted free of moisture, weighed and homogenized.

Determinations made on calf livers included xanthine oxidase, respiration, copper and molybdenum. Cytochrome oxidase activity in tissues from each of the 4 sections of the calf hearts was determined.

Enzyme determinations on the rat and calf tissues were made on the day the animals were killed. Liver xanthine oxidase was determined by the method of Axelrod and Elvehjem ('41); liver tyrosine oxidase by the procedure of Harper, et al. ('53); liver respiration by measuring the 30-minute endogenous oxygen uptake during the xanthine oxidase determination; and heart cytochrome oxidase by the technique of Stotz ('39). Blood uric acid was determined by the method of Brown ('45). Liver copper (Rush and Yoe, '54) and molybdenum (Sandell, '50) were determined on the liver homogenates. Weights of the homogenates were determined after drying at 100°C to a constant weight.

RESULTS AND DISCUSSION

In the first experiment the rats fed diets containing high levels of molybdenum showed a decrease in growth rate, marked diarrhea and emaciation. The effects of these diets on concentrations of molybdenum, copper and uric acid and activities of enzymes in the rats are shown in table 1. The level of molybdenum in the liver increased in the high-molybdenum rats, approximately the same levels of molybdenum being found in the livers of rats on both the 500 and the 800 ppm diets. Liver tyrosine oxidase activity was not influenced by either of the high-molybdenum diets. Alterations in the copper content of the liver were found, but these were inconsistent, an increase being observed in the rats fed 500 ppm molybdenum and a decrease in those fed 800 ppm. Reduced activity of xanthine oxidase ($P < 0.05$ and < 0.01 for the 500 and 800 ppm diets, respectively) and respiration ($P < 0.01$ for both diets) was observed.

TABLE 1

Dietary molybdenum level and enzymatic activities, uric acid, copper and molybdenum in rats

Mo level	Liver xanthine oxidase[1]	Liver respiration[2]	Liver tyrosine oxidase[3]	Heart cytochrome oxidase[4]	Blood uric acid[5]	Liver copper[6]	Liver molybdenum[7]
ppm			*Experiment 1*				
0	227.5 ± 38.6	1467.6 ± 148.5	679.2 ± 138.8			77.8 ± 36.5	14.7 ± 5.1
500	151.0 ± 76.4[7]	1155.1 ± 123.2[8]	605.3 ± 89.1			58.9 ± 40.8	24.1 ± 7.3[8]
800	119.4 ± 94.9[8]	1068.5 ± 193.1[8]	671.9 ± 147.3			87.0 ± 62.4	25.9 ± 5.5[8]
			Experiment 2				
0	190.1 ± 25.0	1282.5 ± 83.5		24.2 ± 4.7	2.34 ± 0.36	36.7 ± 9.0	11.6 ± 5.7
250	181.8 ± 35.2	1352.4 ± 135.9		20.0 ± 5.8	2.12 ± 0.41	41.2 ± 12.8	18.3 ± 3.2[8]
800	157.6 ± 27.0	1182.4 ± 146.4		20.1 ± 5.1	1.93 ± 0.32	44.9 ± 8.5	25.8 ± 2.8[8]

[1] μl O_2/gm of dry homogenate/20 min.
[2] μl O_2/gm of dry homogenate/30 min.
[3] μl O_2/gm of dry homogenate/10 min.
[4] μl O_2/mg of dry homogenate/60 min.
[5] Mg/100 ml of whole blood.
[6] μg/gm of dry homogenates.
[7] Difference from basal group significant at $P < 0.05$.
[8] Difference from basal group significant at $P < 0.01$.

It is felt that the diarrhea accompanying the molybdenosis was severe enough to reduce protein availability and utilization. The decrease in liver respiration is believed to be the result of an overall depression of liver enzyme activities caused by this decrease in available protein. Miller ('48) and Wainio et al. ('53) have shown that starvation will cause a decrease in liver xanthine oxidase. Other investigators (Westerfeld and co-workers, '50; Meiklaham et al., '51) noted the same effect from the ad libitum feeding of a low (8%) protein diet. The question arises whether the decrease in xanthine oxidase activity observed in these rats was caused entirely by the reduced protein availability resulting from diarrhea, or whether the level of molybdenum in the liver was also a factor. To investigate this question, rats were fed a high-molybdenum diet (experiment 2) which reduced the growth rate and caused a build-up of molybdenum in the liver but did not cause diarrhea. Since apparently an antagonism exists between molybdenum and copper in molybdenum toxicity, in this experiment we studied the enzyme cytochrome oxidase, which is adversely affected by a copper deficiency (Schultze, '39).

The data obtained in the second experiment with respect to liver xanthine oxidase and respiration, heart cytochrome oxidase, blood uric acid and liver copper and molybdenum are presented in table 1. A slight depression of xanthine oxidase and respiration activities in the liver of rats on 800 ppm of dietary molybdenum was evident, as well as a lowered blood uric acid value. A statistical analysis showed no correlation between body size (weight) and xanthine oxidase activity. No difference in heart cytochrome oxidase was found between the rats on the control diet and those on the high-molybdenum diets. Liver copper storage in rats on high dietary molybdenum remained normal. As in the first experiment, increased ($P < 0.01$) storage of molybdenum was observed in the liver. Further, the concentration of molybdenum in the livers of rats fed 250 ppm was lower than that found for the rats on 800 ppm in both experiments and on 500 ppm in the first

TABLE 2

Dietary molybdenum level and liver respiration, xanthine oxidase, copper and molybdenum in calves

Experiment 3

Mo level	Calf no.	Respiration[1]	Xanthine oxidase[2]	Copper[3]	Molybdenum[3]
ppm					
	943	1112.7 ± 16.4[4]	147.9 ± 23.1	283.9 ± 7.7	12.9 ± 3.9
	949	700.5 ± 51.5	218.9 ± 30.7	230.1 ± 10.8	6.0 ± 0.9
0	950	852.9 ± 111.9	150.5 ± 31.8	266.8 ± 20.6	13.5 ± 3.2
	Av.	888.7	172.4	260.3	10.8
	931	700.6 ± 124.9	234.3 ± 9.3	85.1 ± 9.9	50.0 ± 6.2
	947	896.6 ± 114.1	213.8 ± 34.5	170.2 ± 18.3	41.1 ± 7.8
200	967	1035.5 ± 54.1	69.0 ± 4.6	247.1 ± 6.7	36.2 ± 4.1
	Av.	877.6	172.3	167.3	42.4
	988	932.2 ± 77.0	150.5 ± 28.9	187.2 ± 16.0	41.4 ± 4.4
	960	753.4 ± 31.3	106.2 ± 8.6	228.2 ± 28.3	33.9 ± 1.7
400	975	952.9 ± 99.7	242.1 ± 55.7	303.7 ± 25.4	49.9 ± 3.7
	Av.	879.5	166.3	239.7	41.7

[1] μl O_2/gm of dry homogenate/30 min.; av. of 4 lobes.
[2] μl O_2/gm of dry homogenate/20 min.; av. of 4 lobes.
[3] μg/gm of dry homogenate. Av. of 4 lobes.
[4] Standard deviation.

experiment. It appears that the level of dietary molybdenum required to produce a "saturation" level of molybdenum in the rat liver lies between 250 and 500 ppm.

Since the high-molybdenum rats in experiment 2 had a slightly lowered liver xanthine oxidase activity without diarrhea, and therefore presumably with normal protein utilization, molybdenum accumulation in the liver may apparently, in itself, cause some decrease in xanthine oxidase in rat livers.

Calf experiment. The results showing the effect of 200 and 400 ppm of dietary molybdenum on its concentration in the liver and on calf liver xanthine oxidase, respiration and copper levels are presented in table 2. Xanthine oxidase, respiration and cytochrome oxidase were not adversely affected by the ingestion of high levels and increased liver storage of molybdenum. Although differences in xanthine oxidase and respiration values were found among the lobes of the liver, as indicated by the somewhat large standard deviations, an analysis of variance showed that no one lobe consistently had higher activity than another.

Average concentrations of copper in the livers of the calves fed both high-molybdenum diets were lower than concentrations found in control animals. Individual variations were so large however, that the finding is not considered significant. The concentration of molybdenum in the livers of the calves fed the 400 ppm molybdenum diet was no higher than in the livers of those fed 200 ppm, indicating, as in the case with the rats, "saturation" at the lower level of intake.

Findings on heart cytochrome oxidase activity in the tissues from the 4 sections of the calf heart, presented in table 3, show that cytochrome oxidase activity in both ventricles was slightly more than twice as high as that in the two auricles.

Although molybdenum accumulated in the liver, no definite effect on copper storage or on the enzymes was observed. Dick ('56) states that for sheep the magnitude of molybdenum suppression of copper storage depends upon the inorganic sulfate content of the diet. Further, neither molybdenum nor sulfate alone interferes with copper retention, and the effectiveness of either increases to a maximum as the intake of the other is increased. He observed that the toxicity of molybdenum to rats on a low-copper diet was greatly enhanced by an increased intake of inorganic sulfate. It was found that 1000 μg of molybdenum daily could be given with a low-

TABLE 3

Dietary molybdenum level and heart cytochrome oxidase of calves

(Experiment 3)

Mo level	Right ventricle[1]	Right auricle[1]	Left ventricle[1]	Left auricle[1]
ppm				
0	17.1 ± 1.0[2]	7.5 ± 2.8	19.5 ± 5.0	9.4 ± 0.9
200	10.8 ± 1.8	4.9 ± 0.2	18.1 ± 5.7	8.2 ± 0.8
400	21.0 ± 2.8	6.3 ± 1.4	19.8 ± 2.2	8.1 ± 1.3
Av.	16.3	6.2	19.1	8.6

[1] μl O_2/mg of dry homogenate/30 min.; av. of three animals.
[2] Standard deviation.

sulfate diet, whereas 100 µg of molybdenum proved toxic when sulfate was added. On the other hand, Miller, Price and Engel, ('56) have reported that severe growth depression occurring in rats on dietary levels of 75 to 100 ppm of molybdenum on a low-sulfate diet could be overcome by the inclusion of sulfate at a level of 2200 ppm. Van Reen and Williams ('56) also noted a protective effect from feeding sulfur compounds. They report that when either methionine, cystine, sodium thiosulfate or sodium sulfate was fed in a diet containing a toxic level of molybdenum, a definite improvement of the enzymatic anomaly was observed in molybdenum toxicity. Other investigations[10] have not borne out this finding.

It is evident that additional information is required for a logical explanation of the mechanism of molybdenum toxicity. A definite tie-up among molybdenum, copper, sulfate and manganese and possibly other minerals or biochemical compounds appears to exist. Furthermore the effect of sulfate on molybdenum toxicity in monogastric animals needs clarifying before the results can be applied to another species.

SUMMARY

Experiments were designed to study the effect of excess dietary molybdenum on various enzymes of the liver and cytochrome oxidase of the heart of a monogastric and a ruminant animal in an attempt to gain information as to the mechanism of molybdenosis in animals.

1. Rats fed diets containing added molybdenum at 500 and 800 ppm showed molybdenum toxicity symptoms, including diarrhea and decreased rate of growth. These diets did not affect tyrosine oxidase activity or concentration of copper in the liver, but caused an increase in the concentration of molybdenum in this organ and a reduction in liver respiration and liver xanthine oxidase.

2. A second group of rats was fed high-molybdenum diets which raised the concentration of molybdenum in the liver but did not cause diarrhea. These rats also showed a reduction in liver xanthine oxidase activity. Thus, it appears that possible decreased utilization of protein, resulting from diarrhea as exhibited by the first group of rats, was not solely responsible for the reduced xanthine oxidase activity noted. Also, increased liver molybdenum concentration *per se* can influence the level of the enzyme. A slight lowering in blood uric acid concentration was observed in these rats. The cytochrome oxidase activity in the heart was not affected.

3. The concentration of molybdenum found in the liver upon feeding 500 ppm molybdenum in the diet was not exceeded when 800 ppm was fed. It appears that there may be a liver "threshold" for molybdenum of 500 ppm or less; above this dietary level, liver storage is not increased.

4. Calves maintained on diets containing 200 and 400 ppm molybdenum were observed to have increased concentrations of molybdenum in the livers, but no decrease in liver respiration or xanthine oxidase or in heart cytochrome oxidase.

[10] J. T. McCall 1958 Personal communication.

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Dental Caries in the Albino Rat Fed High Sucrose Diets of Relatively High and Low Pyridoxine Content[1]

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Pyridoxine as a dietary supplement is believed by Strean and his co-workers to have a suppressive effect on the initiation and spread of dental caries. This deduction has been drawn from experiments using hamsters, as well as children. Hamsters fed a cariogenic diet containing pyridoxine in a concentration of 10 ppm had fewer dental caries than a similar group fed the same diet with a concentration of 0.5 ppm (Strean, Gilfillan and Emerson, '56; Strean, Bell, Gilfillan, Emerson and Howe, '58). The later report included an experiment using 28 children divided into two groups of 14 each. One group received a lozenge containing 3 mg of pyridoxine three times a day after meals. At the end of a year this group, on the basis of their DMF (decayed, missing, filled) rating, had a lower caries score than the control group. In a preliminary report of a study scheduled to be conducted over a period of several years, Cohen and Rubin ('58) observed a slightly favorable trend in caries experience of a group of children receiving pyridoxine-containing lozenges as compared with another group receiving a placebo.

Strean ('57) has postulated that pyridoxine may effect a change in the oral flora whereby the lactobacilli requiring this vitamin as an essential nutrient, and not associated with dental caries, would multiply at the expense of *L. acidophilus, S. salivarius, S. mitis* and S. *mitior*, which some believe to be associated with tooth decay.

The interesting suggestion has been offered (Strean, Bell, Gilfillan, Emerson and Howe, '58) that the difference in cariogenicity of two high-sucrose diets containing the same amount of sugar which has been reported from our laboratories (Wynn, Haldi, Shaw and Sognnaes, '53) may have been due to a difference in the concentration of pyridoxine in the two diets. The experiments reported in this paper were undertaken to test the validity of this supposition.

PROCEDURE AND RESULTS

The Harvard high-sucrose diet, the composition of which is given elsewhere (Sognnaes, '48; Wynn, Haldi, Shaw and Sognnaes, '53) was used in all experiments. This diet contains 3.5 ppm of pyridoxine. Two sets of experiments were conducted: one on sialoadenectomized albino rats of our Emory-Wistar strain which has proved to be fairly resistant to dental caries, and another on intact animals of a more susceptible strain. In the first experiment 8 groups of triplicate litter mates were selected at weaning and sialoadenectomized by our usual procedure (Haldi, Wynn, Shaw and Sognnaes, '53). Whereas intact animals of this strain are fairly resistant to dental caries, when sialoadenectomized they develop caries on a cariogenic diet within a relatively short time. Beginning immediately after weaning, the three groups of litter mates were fed throughout the experiment the Harvard diet to which was added for each group, respectively, 1, 10 and 100 ppm of pyridoxine. At the end of a 70-day feeding period, the animals were killed and the teeth scored for dental caries by the method customarily followed in our laboratories (Haldi and Wynn, '52).

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TABLE 1

Carious lesions and caries scores of sialoadenectomized caries-resistant albino rats fed a cariogenic diet with different concentrations of pyridoxine

No. animals	Days on diet	Additional pyridoxine	Carious lesions[1]	Caries score[1]
		ppm		
8	70	1	24	59
8	70	10	24	61
8	70	100	22	55

[1] Average per animal.

The results of this experiment are presented in table 1.

From these data it is obvious that the addition of pyridoxine in a relatively large amount to a cariogenic diet did not affect its cariogenicity when fed to sialoadenectomized albino rats of the Wistar strain.

Strean, as stated previously, believes that pyridoxine may bring about a change in the oral flora. In this connection, it may be noted that the addition of pyridoxine to a synthetic medium increases the population of *L. casei* proportional to the quantity of this vitamin added to the medium (Bohonas, Hutchings and Peterson, '42) and that *L. casei* is the test organism for the biological assay of pyridoxine (Rabinowitz, Mondy and Snell, '48).

In view of the possibility that pyridoxine, once absorbed, may be returned to the mouth by way of the salivary glands, thereby having a more prolonged effect on the oral bacteria, we decided to conduct another experiment on intact rats of a strain more susceptible to dental caries. For this purpose we used albino rats from a colony developed from animals that had been supplied to us several years ago by the laboratories of the Naval Medical Research Institute. We had found that these animals, when fed a cariogenic diet with their salivary glands intact, develop caries in a relatively short time.

The animals were selected at weaning, using triplicate litter mates, with 20 animals placed in each group, and fed immediately the prescribed diets. The control group was fed the Harvard diet without supplementation and the other two groups, the same diet to which was added, respectively, 10 and 100 ppm of pyridoxine. When the animals had been fed the diets 120 days, they were killed and the teeth were given a score for dental caries in the same manner as in the preceding experiments. The results are given in table 2. In these experiments, as in those on the sialoadenectomized rats of the Emory-Wistar strain, the addition of pyridoxine to the diet did not reduce its cariogenicity.

TABLE 2

Carious lesions and caries scores of intact caries-susceptible albino rats fed a cariogenic diet with different concentrations of pyridoxine

No. animals	Days on diet	Additional pyridoxine	Carious lesions[1]	Caries score[1]
		ppm		
20	120	0	17	40
20	120	10	18	35
20	120	100	18	38

[1] Average per animal.

DISCUSSION

In our previous studies, we found that the high-sucrose diet referred to as the Harvard diet is much more cariogenic than the Emory diet which contains the same amount of sucrose. These two diets differ in their pyridoxine content with a concentration of 3.5 ppm in the Harvard and 10 ppm in the Emory diet. If Strean's supposition were correct that the difference in pyridoxine concentration may account for the difference in cariogenicity of the two diets, one would reasonably expect that the addition of pyridoxine to the Harvard diet would make it less cariogenic. This proved not to be the case. The cariogenicity of the Harvard diet was not diminished even though the pyridoxine content was brought well above the level of that which obtained in the Emory diet.

The results of our experiments are not in agreement with those of Strean and his co-workers ('56; '58). These investigators found that increasing the pyridoxine concentration in the diet from 0.5 to 10 ppm resulted in a decrease in the cariogenicity of the diet. It is possible that the response of the hamster to the addition of pyridoxine to the diet may differ from that of the albino rat.

Another possibility should also be taken into account, namely, that the diet fed the hamsters, containing 0.5 ppm of pyridox-

ine, may have been deficient in this factor. If this were the case, the higher incidence and greater extent of caries observed when using this diet, as compared with the one containing 10 ppm, may have been related to a pyridoxine deficiency. It seems unlikely, however, that the hamsters suffered from a pyridoxine deficiency inasmuch as they survived over an experimental period of 7 to 10 months. Furthermore, the authors did not report acrodynia or other signs of pyridoxine deficiency. In our experiments, the basic Harvard diet provided 35 to 50 μg of pyridoxine in the normal daily food intake of 10 to 15 gm which doubtless satisfied the rat's pyridoxine requirements. Lepkvosky ('38) obtained optimal growth in rats with a daily dose of 10 μg. Sure ('40) reported later that 10 to 25 μg/day are adequate for continuous growth.

CONCLUSION

The addition of pyridoxine in relatively large amounts to a synthetic high-sucrose diet, originally adequate in its pyridoxine content, did not reduce the cariogenic properties of the diet when fed to sialoadenectomized albino rats of a fairly resistant strain nor to intact rats of a much more susceptible strain.

The difference in the cariogenicity of two high-sucrose diets containing the same amount of sugar, previously reported from our laboratories, cannot be attributed to the difference in pyridoxine concentration of the two diets.

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Further Studies of the Lipotropism of Diethylstilbestrol in Choline-Deficient Weanling Albino Rats[1]

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The addition of diethylstilbestrol (DES) to a choline-deficient and low-methionine diet has been previously reported to produce a lipotropic action in weanling albino rats as well as to protect such rats against death (Miller et al., '58). The following studies were conducted to determine the influence of various concentrations of DES upon the lipides of the livers and adrenals of weanling albino rats fed the choline-deficient and low-methionine diet; also to determine the minimal oral dose of DES that would prevent death.

EXPERIMENTAL

Weanling albino rats (about three weeks old) from a stock colony were placed in individual cages, with tap water and experimental diets provided ad libitum. Five animals of each sex were given each experimental treatment. The diet contained the following in per cent composition: soy protein,[2] 20; sucrose, 68; salt mixture—U.S.P. XIV, 4; cellulose, 3; and refined cottonseed oil,[3] 5. Vitamins and DES were administered as previously reported (Miller et al., '58). The growth rate and food consumption of each animal were measured throughout the experimental period. Illness of the rats was determined by observation and growth-rate depression. The latter occurred between 8 to 12 days after diet initiation.

Livers and adrenals were removed from the animals after 42 days and stored at −25°C for analyses. Livers were extracted, and the percentage of liver lipides determined as reported by Miller et al. ('58). Adrenals of animals in specific dietary groups were extracted by the procedure of Duncan ('57).

Lipide iodine values were determined by the procedure of Byrne and Johnson ('56), and polyenoic fatty acids were determined by the micro-procedure of Herb and Reimenschneider ('53). Determination of lipide phosphorus was made by the procedure of Chen et al. ('56). The liver lipides were pooled separately by sex and by dietary group before the above determinations were made. Total cholesterol was determined by the procedure of Pearson and co-workers ('54). Statistical values were determined according to Li ('57).

RESULTS AND DISCUSSION

The liver lipide percentages shown in table 1 indicate that liver lipide accumulation was not altered in choline-deficient males or females when DES was fed at a concentration of 0.1 ppm. However, this accumulation was significantly decreased when DES was fed at a level of 0.5 ppm ($F = 10.29$ with 1 and 8 degrees of freedom for males; and $F = 30.58$ with 1 and 8 degrees of freedom for females). Increasing the concentration of dietary DES to 1.0 and to 10.0 ppm caused a greater reduction in the liver lipide accumulation. The feeding of 100, 500, or 1000 ppm of DES did not significantly reduce the accumulation below that effected by 10.0 ppm. In addition, 10.0 ppm of DES produced a significantly greater decrease in liver lipide accumulation than 1.0 ppm ($F = 8.10$ with 1 and 8 degrees of freedom for males; and $F = 55.12$ with 1 and 8 degrees of freedom for females). Therefore, it could be assumed that the concentration of DES which reduced liver lipide accumulation to the lowest level was between 1.0 and 10.0 ppm for both males and females.

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[2] Sodium Soybean Proteinate, Archer-Daniel Midland, Minneapolis.

[3] Wesson Oil.

Also, even high concentrations of DES (100 to 1000 ppm) did not reduce the lipide content of the livers of choline-deficient rats to the level observed in the livers of the choline-supplemented rats (table 1).

The concentration of dietary DES that most effectively reduced liver lipide accumulation (1.0 to 10.0 ppm) was much higher than the minimal concentration needed to protect males (0.01 to 0.1 ppm) and females (0.001 to 0.01 ppm) against illness and death (table 2). This would suggest that lipotropism is not the sole action of DES in choline deficiency.

The percentage of liver lipide in choline-supplemented male rats receiving no DES was significantly lower ($F = 36.38$ with 1 and 8 d.f.) than that of the corresponding female rats (table 1). Deuel ('57) has pointed out that male rats have a lower content of liver fat than female rats. The presence of 1.0 or 500 ppm of DES in the diet of choline-supplemented male rats significantly increased the percentage of liver lipide compared with that of choline-supplemented male rats receiving no DES ($F = 28.14$ with 1 and 8 d.f.; $F = 23.26$ with 1 and 8 d.f., respectively). The presence of DES in the diet of choline-supplemented female rats had no significant effect upon the percentages of liver lipide when these values were compared with those of choline-supplemented female rats receiving no DES.

Plagge et al. ('58) suggested that the lipotropic action of estradiol on nutritional fatty livers may be due to a sparing action of choline by growth inhibition. It is apparent from table 1 that a concentration of 0.1 ppm of DES produced a marked growth inhibition in choline-deficient male and female rats but not a decrease in liver lipide accumulation. On the other hand, a concentration of 10 ppm of DES caused a considerable decrease in liver lipide accumulation in choline-deficient male and female rats, yet it produced a growth inhibition approximately equivalent to that obtained with 0.1 ppm of DES. There-

TABLE 1

Weight gain and percentages of liver lipide

Choline	DES	Male		Female	
		Liver lipide[1]	Weight gain	Liver lipide[1]	Weight gain
	ppm	%	*gm/day*	%	*gm/day*
0	0.0	70 ± 2[2]	2.3 ± 0.3	71 ± 2	2.0 ± 0.3
0	0.1	73 ± 6	1.6 ± 0.2	75 ± 3	1.6 ± 0.2
0	0.5	54 ± 4	1.8 ± 0.1	56 ± 2	1.4 ± 0.1
0	1.0	41 ± 2	1.9 ± 0.2	45 ± 5	1.4 ± 0.2
0	10.0	30 ± 6	1.5 ± 0.1	25 ± 3	1.3 ± 0.1
0	100.0	28 ± 5	1.6 ± 0.0	30 ± 3	1.4 ± 0.0
0	500.0	21 ± 2	1.7 ± 0.1	29 ± 3	1.4 ± 0.1
0	1000.0	32 ± 6	1.4 ± 0.1	33 ± 7	1.5 ± 0.1
+	0.0	16 ± 0.2	3.3 ± 0.4	18 ± 0.4	2.9 ± 0.4
+	1.0	18 ± 0.1	1.9 ± 0.2	19 ± 1.0	1.6 ± 0.2
+	500.0	18 ± 0.3	1.9 ± 0.1	19 ± 0.0	1.4 ± 0.1

[1] Expressed on a dry-weight basis.
[2] Standard deviation.

TABLE 2

Survival rates of rats as influenced by concentration of DES

Choline	DES	Male		Female	
		Ill and recovered	Mortality	Ill and recovered	Mortality
	ppm	%	%	%	%
0	0.00001	0	100	100	0
0	0.001	0	100	40	20
0	0.01	60	30	0	0
0	0.1	0	0	0	0

fore, it follows that the lipotropism of DES in choline deficiency is not due solely to a depression of growth.

From table 3 it is evident that the percentages of liver lipide phosphorus in choline-deficient male and female rats increased as the concentration of dietary DES increased until a maximal level of liver lipide phosphorus was reached. Table 1 shows that the percentage of liver lipide of choline-deficient rats decreased in a corresponding manner. Therefore, DES appears to have caused a decrease in the non-phosphorus-containing lipide fraction of the liver; and this agrees with the results reported by Miller et al. ('58).

TABLE 3

Percentages of liver lipide phosphorus[1]

Choline	DES	Lipide phosphorus	
		Male	Female
	ppm	%	%
0	0.0	0.2	0.2
0	0.1	0.2	0.2
0	0.5	0.4	0.4
0	1.0	0.6	0.8
0	10.0	1.5	1.6
0	100.0	1.3	1.1
0	500.0	2.0	1.3
0	1000.0	1.1	1.5
+	0.0	2.4	2.1
+	1.0	2.4	2.2
+	500.0	2.2	2.2

[1] Liver lipides extracted and pooled by sex and by dietary group for these determinations.

The iodine value of the liver lipides of rats receiving some of the experimental diets, as well as the percentage of the polyenoic fatty acids in the lipide (table 4), indicate, in general, that (1) increasing concentrations of DES caused a decrease in the iodine value of the liver lipides of choline-deficient male and female rats; and (2) the liver lipides of choline-deficient male and female rats receiving no added DES contained considerably more dienoic fatty acids than those of the corresponding choline-supplemented rats. These results show that the accumulated liver lipide was more unsaturated than normal liver lipide, this increase in unsaturation being caused by a higher content of dienoic fatty acids. Table 4 also gives the iodine value and polyenoic fatty acid composition of the dietary lipide, which contained almost 60% of dienoic

TABLE 4

Liver lipide iodine values and percentages of polyenoic fatty acids[1,2]

Choline	DES	Male				Female			
		Iodine values	Dienoic	Trienoic	Tetraenoic	Iodine values	Dienoic	Trienoic	Tetraenoic
	ppm		%	%	%		%	%	%
0	0.0	96	25.0	2.3	9.7	98	22.8	2.4	8.7
0	0.5	87	—	—	—	91	—	—	—
0	1.0	79	20.3	2.6	8.2	83	19.6	2.5	6.9
0	10.0	78	—	—	—	75	—	—	—
0	500.0	65	10.4	2.8	8.0	81	17.6	2.1	6.1
+	0.0	68	9.2	1.9	9.9	74	9.5	2.1	10.8
+	1.0	66	8.8	2.5	10.5	68	10.5	2.6	9.8
+	500.0	63	10.0	1.7	9.5	75	9.1	2.5	9.4
Dietary cottonseed oil		104	56.0	3.0	0.1				

[1] Liver lipides extracted and pooled by sex and by dietary group for these determinations.
[2] The percentages of polyenoic fatty acids are expressed as per cent of lipide.

fatty acids. Therefore, under the conditions of these experiments, the accumulated liver lipide in choline deficiency was at least partly derived from dietary lipide.

Estrogens are known to cause enlargement of the adrenal cortex, and are believed to cause a release of ACTH[4] by the hypophysis which stimulates the adrenal cortex to secrete its hormones in amounts larger than normal (Turner, '55). Recently, Barnett and Teague ('58) confirmed that DES treatment of rats produced liver and adrenal enlargement, moderate depletion of adrenal ascorbic acid, maximal depletion of adrenal cholesterol and elevated liver glycogen levels. The authors stated that this observed stimulation of activity in the rat adrenal cortex was probably caused by the release of ACTH. Schroder and Hansard ('58) also reported that stimulation of adrenal secretion was produced by DES treatment of wether lambs. However, these workers found that the DES treatment produced an increase in adrenal cholesterol but had no effect on adrenal weight. Therefore, DES apparently produces opposing effects on the adrenal cholesterol levels of rats and of wether lambs. This is interesting because DES reduces true growth in rats but increases the rate of gain in fattening lambs, heifers, and beef steers (Andrews, '58).

Table 5 gives the percentage of cholesterol in the adrenals of choline-deficient and choline-supplemented rats receiving zero, 1.0 and 500 ppm of DES. The adrenals of choline-deficient male rats receiving 1.0 and 500 ppm of DES contained significantly less cholesterol than those receiving no DES ($F = 496.00$ with 1 and 2 d.f.; $F = 300.00$ with 1 and 2 d.f., respectively). The same was true of the adrenals of choline-deficient female rats ($F = 69.23$ with 1 and 2 d.f.; $F = 78.77$ with 1 and 2 d.f., respectively). Likewise, the adrenals of choline-supplemented male rats receiving 1.0 and 500 ppm of DES contained less cholesterol than those of their corresponding controls ($F = 33.33$ with 1 and 2 d.f.; $F = 33.32$ with 1 and 2 d.f., respectively). This was also true of the adrenals of choline-supplemented females ($F = 73.14$ with 1 and 2 d.f.; $F = 92.57$ with 1 and 2 d.f., respectively). It is evident that treatment with DES caused a striking decrease in the percentages of adrenal cholesterol in both choline-supplemented and choline-deficient rats. Therefore, in agreement with Barnett and Teague ('58), these observations suggest that treating rats with DES caused an increase in adrenal-cortex activity.

TABLE 5

Percentages of adrenal cholesterol[1]

Choline	DES	Male	Female
	ppm	%	%
0	0.0	4.6 ± 0.2[2]	4.9 ± 0.3[2]
0	1.0	1.4 ± 0.0	1.8 ± 0.0
0	500.0	2.1 ± 0.1	1.6 ± 0.6
+	0.0	5.0 ± 0.3	5.2 ± 0.6
+	1.0	2.0 ± 0.6	2.0 ± 0.3
+	500.0	2.1 ± 0.6	1.6 ± 0.1

[1] Expressed as per cent of fresh-tissue weight.

[2] Standard deviation.

Stimulation of the adrenal cortex by DES cannot explain completely the lipotropism of DES in choline deficiency. Deuel ('55) has reported that (1) adrenalectomized animals stored less lipide in the liver after fat ingestion than controls; (2) lipide absorption in the intestines was inhibited by adrenalectomy; (3) injection of adrenocortical extracts into rats caused increased content of liver lipide; (4) injection of ACTH into male rats, force fed a fluid containing carbohydrate, caused an increase in liver lipide; and (5) fatty livers cannot be produced in adrenalectomized animals. In fact, Le Breton ('56), suggested that nutritional fatty livers may be caused by a prolonged hyperfunction of the adrenal glands and that any factor inhibiting the functioning of the adrenals would counteract the development of fatty livers. Therefore, stimulation of the adrenal cortex by DES does not appear to be involved in the lipotropic action of DES. It may well be that DES is acting through enzyme systems since Villee and Hagerman ('58) have shown that there appears to be an estrogen-sensitive transhydrogenase in human placenta, while Mason and Gullekson ('58) have reported that the disulfate of DES inhibits pyridoxal phosphate-dependent enzymes.

[4] Adrenocorticotropic hormone.

The concentration of DES required to prevent death, as pointed out previously, was much lower than that required to decrease liver lipide accumulation in choline-deficient rats. Therefore, it is still possible that stimulation of the adrenal cortex by dietary DES may be responsible for the prevention of death due to choline deficiency.

SUMMARY

The concentration of dietary diethylstilbestrol (DES) that most effectively reduced liver lipide accumulation in choline-deficient rats was much higher than the concentration required to prevent illness and death.

The growth depression caused by 0.1 and 10 ppm of DES was essentially the same, but the liver lipide accumulation in choline-deficient rats receiving 0.1 ppm of DES was considerably larger than that of rats receiving 10 ppm.

The increased unsaturation in the liver lipide of choline-deficient rats appeared to be due to a higher content of dienoic fatty acids, which made up approximately 60% of the dietary lipide.

The presence of dietary DES significantly reduced the adrenal cholesterol content in rats when this value was compared to that of rats receiving no DES. This was true for both choline-deficient and choline-supplemented rats.

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The Effect of Cold on the Weight, Food Intake and Acetylating Activity of Pantothenic Acid-Deficient Rats[1]

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It is fairly well established that pantothenic acid improves the ability of animals to withstand cold stress. Ershoff ('53) has reported an average survival time of only 11.8 hours in pantothenic acid-deficient rats exposed to 2°C. Arnrich et al. ('56) have described a derangement of carbohydrate metabolism in pantothenic acid-deficient dogs subjected to swimming stress in moderately cold water. Large doses of pantothenic acid were found by Ralli ('54) to improve the swimming time of rats in cold water and to modify some of the responses of men placed under stress by immersion in cold water. Prolonged survival was observed by de Bias et al. ('57) in cold-stressed, adrenalectomized rats given calcium pantothenate and a small amount of hydrocortisone. Weiss ('57) concluded that a deficiency of pantothenic acid increases the sensitivity of undernourished rats to cold.

Imposing a stress (such as cold) on a deficient animal, then, may lead apparently to a variety of suboptimal responses. Reports from this laboratory (Vaughan and Vaughan, '57, '59), however, indicate that thiamine- and riboflavin-deficient animals are able to grow and survive almost as well at 5°C as at 25°C if they become adjusted to cold *before the onset of the deficiency*. The present report contains data which indicate that the severity of a pantothenic acid deficiency, when induced in cold-adjusted rats, is not greatly increased during cold exposure.

EXPERIMENTAL

Forty-two male Sprague-Dawley rats, ranging in weight from 150 to 180 gm were divided into two groups of 21 each. One group was placed in individual cages in a cold room held at 5 ± 2°C; the other group remained in an animal room at 25°C. Wire-bottom cages were used, rats being placed randomly as to position in the cage rack. Both groups were given a pantothenic acid-deficient basal diet[2] of the following per cent composition: vitamin test casein, 18; sucrose, 68; vegetable oil, 10; U.S.P. Salt Mixture no. II, 4. The vitamin mixture supplied 2000 units of vitamin A, 222 units of vitamin D, 11.1 mg of α-tocopherol and the following in mg: ascorbic acid, 100; inositol, 11.1; choline chloride, 166.5; menadione, 5; *p*-aminobenzoic acid, 11.1; niacin, 10; pyridoxine hydrochloride, 2.22; riboflavin, 2.22; thiamine hydrochloride, 2.22; also 44 μg of biotin, 200 μg of folic acid, and 3 μg of vitamin B_{12} per 100 gm of diet.

At the end of 33 days, growth had ceased in both groups of animals. At this time each group of 21 rats was divided into 3 sub-groups, consisting of 7 rats each. These sub-groups received an oral supplement of calcium pantothenate solution given at levels of 0.5, 1.5 and 5.0 μg/gm of food. Calcium pantothenate was mixed into the food slurry daily. The rats were allowed to eat ad libitum but food consumption was measured.

After 26 to 28 days on these dietary regimes, the acetylating activities of the rats were determined. An isotonic saline solution, containing 6 mg of *p*-amino-

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[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association. (AFR 160-94.)

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland.

benzoic acid (PABA) was injected intraperitoneally, followed by a 21- to 24-hour collection of urine. The urine was analyzed for total and free PABA by the method of Bratton and Marshall ('39). Bound PABA was calculated by difference and expressed as per cent.

At the end of 4 weeks' repletion, all cold-exposed rats were transferred to the warm (25°C) room and allowed to eat ad libitum the same diets as before. Food consumption was measured and weights were recorded at 2- to 3-day intervals. This phase of the experiment continued for three weeks. Toward the end of the second week, acetylating activities were measured in these rats.

To examine further the effect of cold exposure upon acetylating activity, 12 rats ranging in weight from 300 to 350 gm were divided into two groups. One group was placed in the cold room at 5 ± 2°C, after a period of observation at 25°C, while the other remained at 25°C. Commercial chow[3] was fed ad libitum and the per cent acetylation of *p*-aminobenzoic acid was determined 4 and 7 days before, and 1, 4, 7, 14, 21, and 30 days after the beginning of cold exposure. To test the effect of sodium acetate upon acetylating activity, sodium acetate (30 mg/gm of food) was added to the food of both groups at the 30-day interval.

Weight changes and percentage acetylations were statistically evaluated by analyses of variance; food intake by a covariance analysis, in which the observed intakes were adjusted in terms of their regression on the initial weights of the rats; and weight and food intake by a covariance analysis in which final weight was adjusted for food intake and initial weight. The purpose of the latter analysis was to provide data approximately equivalent to a paired feeding experiment. Post-cold growth and food intake were evaluated by analyses of variance.

RESULTS AND DISCUSSION

Mean weight changes, adjusted food intakes, and acetylation percentages for the rats receiving the graded levels of calcium pantothenate are summarized in table 1.

Growth. Growth was somewhat depressed ($P < 0.01$) in the cold room during the repletion experiment. Cold, however, did not accentuate the effect of the pantothenate deficiency, for this growth depression occurred in animals receiving optimal amounts of calcium pantothenate and was fairly uniform at all levels of pantothenate supplementation. The uniformity of growth depression is shown by the lack of interaction between growth and level of pantothenate supplementation. Thus, as far as growth is concerned, rats apparently do not require a higher level of pantothenate in their diet when cold-exposed. Survival, however, was considerably less in the pantothenate-deficient cold-exposed rats—a fact already noted by Ershoff ('53). The behavior of the rats

[3] Purina Checkers. Ralston Purina Company, St. Louis.

TABLE 1

Weight, food intake and acetylating activity of cold-exposed rats
28-day experiment

Calcium pantothenate	Weight						Adjusted food intake[1]		Adjusted final weight[2]		Acetylation		
	Initial		Final		Δ								
	25°	5°	25°	5°	25°	5°	25°	5°	25°	5°	25°	5°	(25°)[3]
μg/gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	%	%	%
0.5	226	157	239	159	+ 13(7)	+ 2(3)[4]	261	438[5]	245	194	34	35	31
1.5	226	171	256	185	+ 30(7)	+14(6)	291	464	260	201	37	44	39
5.0	237	177	337	260	+100(7)	+83(6)	409	581	309	246	51	52	52

[1] $b = 0.939$; $r_{xz} = 0.63$, where x = initial weight and z = food intake.

[2] $b_1 = 0.718$; $b_2 = 0.215$; $R_{y.xz} = 0.89$; $r_{xz} = -0.32$; $r_{yz} = 0.18$; $r_{xy} = 0.79$, where x = initial weight, y = final weight and z = food intake.

[3] Post-cold animals.

[4] Number of survivors. In order to achieve the proportionality required for the analyses, items were removed randomly by two different schemes, followed by analysis of each set of adjusted data.

[5] Cold-induced increments are 177, 173, and 172 gm, respectively.

failing to survive in our experiment seemed to indicate that weight loss was not necessarily a precursor of death—in fact, death, in many cases, occurred in rats which had been maintaining their weights better than the average of their group.

There was a significant ($P < 0.01$) graded response of the final adjusted weights to increasing levels of calcium pantothenate. Failure to grow on the deficient levels is thus not completely dependent upon appetite failure. This is in agreement with the results of Voris et al. ('42), whose paired-feeding experiments showed that pantothenic acid has a growth-promoting effect not related to appetite. This effect assumes equal importance in both cold and warm rats, as shown by a lack of interaction between cold and level of dietary pantothenate.

Food intake. As noted before, food intakes were adjusted statistically for differences in initial weight of the animals. The values shown in table 1, therefore, would represent the most probable intakes of these animals had they had the same initial body weights. We observed significant ($P < 0.01$) anorexia associated with the decrease in the level of dietary pantothenate. What is more interesting, however, is the constancy of the cold-induced increment in food intake between the warm and cold rats, regardless of the degree of anorexia associated with the pantothenate deficiency. This same phenomenon has been observed previously in thiamine and riboflavin deficiencies (Vaughan and Vaughan, '57, '59) in cold-exposed animals and represents a type of appetite regulation which we are unable to explain. The cold rat is able to fulfill its needs for extra energy, while at the same time displaying a level of anorexia characteristic of a vitamin deficiency. This seems to us to imply that the control of appetite may be fractionated into components which respond characteristically to different stimuli.

Post-cold growth and food intake. The post-cold behavior of the experimental animals is summarized in table 2. An analysis of variance showed that the rats previously exposed to 5°C continued to eat at a significantly ($P < 0.01$) elevated rate. This effect persisted for the entire three weeks in the deficient rats (0.5 and 1.5 μg/gm levels), but disappeared after a week in those receiving 5.0 μg of calcium pantothenate per gram of food. At the same time, a significant ($P < 0.01$) degree of anorexia occurred as a function of decreasing levels of calcium pantothenate.

A rather striking growth response was noted: rats which had previously shown little or no growth, as a result of the pantothenate deficiency, now displayed a remarkable spurt in growth. Furthermore, the level of pantothenate in the diet appears to have no effect on the magnitude of this post-cold growth, as table 2 shows.

During this post-cold period, the deficient rats (0.5 and 1.5 μg/gm levels) which had been in the warm room for the whole experiment were beginning to show porphyrin deposits, wrist dermatitis and general roughness of the fur. These deficiency symptoms did not appear in the post-cold rats receiving the same levels.

Acetylation activity. Campbell et al.[4] have reported cold-induced increases in rat liver coenzyme A which persisted up to 24

[4] J. Campbell, G. R. Green, E. Schönbaum and H. Soco. 1958 Effect of exposure to cold on the coenzyme A content of liver tissue. Federation Proc., *17:* 22 (abstract).

TABLE 2

Post-cold weight gains and food intake

Calcium pantothenate	Food intake (*gm*)								Total Δ weight (3 weeks)	
	Week 0[1]		Week 1		Week 2		Week 3		25°	(25°)[2]
	25°	5°	25°	(25°)[2]	25°	(25°)[2]	25°	(25°)[2]		
μg/gm of food									*gm*	*gm*
0.5	67(7)[3]	100(3)[3]	71	82	56	69	62	88	0	+49
1.5	76(7)	107(6)	84	97	71	88	88	93	+13	+53
5.0	119(7)	152(6)	102	121	104	98	110	110	+28	+47

[1] Last week of cold exposure. [2] Post-cold animals. [3] Number of survivors.

days. Brumleve and Olson[5] also note similar increases. Thus, acetylating activity might be expected to increase during cold exposure.

Table 1 shows the results obtained by measuring the acetylation activities of the rats at the end of the 4-week repletion period. The deficient rats showed significantly ($P < 0.01$) lower percentages of bound *p*-aminobenzoic acid than did the rats receiving 5.0 μg/gm of food. Cold had no effect on percentage acetylation at any of the levels of dietary pantothenate—differences in means shown in table 1 are not significant. Removal of the cold-exposed rats to the 25° room also had no effect on acetylating activity (table 1). The level of calcium pantothenate seemed to be the only determinant of acetylating activity in this experiment, and this manifested itself only in a difference between the low (0.5 and 1.5 μg/gm) and high (5.0 μg/gm) dietary concentrations.

Results of further tests of the effect of cold on acetylating activity may be seen in table 3. Activity was measured at various time intervals in order to spot possible effects of short-term versus long-term exposures to cold. We were unable, by our methods, to detect *in vivo* any differences in acetylation between cold and warm rats at any of the times noted. Administration of acetate in the food also had no effect on acetylating activity.

TABLE 3

Acetylating activity of cold-exposed rats[1]

Days of exposure	Acetylation		Total bound PABA	
	25°	5°	25°	5°
	%	%	*mg*	*mg*
7	47	50[2]	1.14	1.28[2]
4	59	50[2]	1.38	1.30[2]
1	52	46	1.29	1.11
4	53	45	1.45	1.10
7	51	49	1.40	1.26
14	53	53	1.51	1.46
21[3]	65	60	0.94	0.80
30[4]	53	48	1.35	1.02

[1] Each number is the mean of 6 animals.

[2] Two pre-cold observation periods at 25°C.

[3] Three milligrams of *p*-aminobenzoic acid injected.

[4] Three per cent of sodium acetate in diet.

SUMMARY

Weight changes, food intakes and acetylating activities were measured in rats kept at 5° and 25°C while receiving graded levels of calcium pantothenate in their diet.

The cold-induced increment in food intake was uniform at all levels of dietary calcium pantothenate, and it appears that, for growth, rats do not require a higher level of calcium pantothenate in their diet when exposed to 5°C.

In vivo acetylating activity did not appear to be affected by cold exposure, although it was significantly lower in pantothenate-deficient rats.

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[5] S. J. Brumleve and R. D. Olson 1959 Metabolic factors in thermal regulation. Federation Proc., *18:* 19 (abstract).

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Comparative Feeding Value of Common Edible Fats as Measured by the Growth of Prematurely Weaned Pups, Guinea Pigs and Swine

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Economic competition between butter and its substitutes has stimulated prolific experimental activity directed toward a comparison of the nutritive properties of butterfat and the vegetable oils commonly employed in commercial margarines. Although it is generally agreed that young calves cannot tolerate filled milks in which the butterfat has been replaced by crude or refined plant oils (Gullickson and Fountaine, '39; Jacobson et al., '49; Jarvis and Waugh, '49; Barker et al., '52), attempts to duplicate these results in studies conducted with rats have led to considerable controversy. Data accumulated by Deuel and various associates ('43; '44a, b; '45a, b; '46a, b; '50), for example, have led these workers to conclude that butterfat and vegetable oils are of equal nutritive value. Their experimental findings have been corroborated by Zialcita and Mitchell ('44), Henry et al. ('45) and Lassen and Bacon ('49). On the other hand, considerable evidence exists to support the view that butterfat is nutritionally superior to oils of vegetable origin (Shantz et al., '40a, b, c; Geyer et al., '43; Parrish et al., '46).

However, Boutwell et al. ('43a, b; '45) have shown their ability to demonstrate superior growth in rats fed butterfat over those fed corn oil, to be dependent upon both the nature of the carbohydrate source and the level of B vitamins in the diet. These workers have also demonstrated an influence of weaning age on the reaction of rats to the source of dietary fat. In effect, a superior rate of gain of butterfat groups over corn oil groups could be shown in rats weaned at 14 days; but when rats weaned at 30 days were treated in like manner, the margin of difference between the two fats became negligible.

It is also noteworthy that the superior growth induced by butterfat rations as compared to corn oil rations in feeding trials conducted by Shantz et al. ('40a) was most evident during the initial three-week period of the test. However, when Deuel and Movitt ('45a) utilized rats weaned prematurely (14 days), all fats tested, namely butterfat, margarine, corn, cottonseed, peanut and soybean oils, appeared to support growth equally well. Zialcita and Mitchell ('44) have also reported failure to distinguish differences in the growth of rats weaned at 7 days and maintained on test diets containing butterfat or corn oil until 9 weeks of age.

In the experiment reported herein, young guinea pigs, dogs and swine weaned at three, 10 and 14 days of age, respectively, were utilized. Previous experience had shown these species to be capable of successful adaptation to the process of premature weaning and subsequent maintenance on artificial diets during their normal period of nursing. It was also believed that in an experiment conducted under such conditions the animals would be more critical of the nutritive qualities of dietary fat, particularly in comparison to butterfat, than animals carried on test during ages when they would not normally be consuming milk.

The species chosen as experimental subjects were considered to be representative of animals which would normally consume three distinctly different types of diets, one being an herbivore and the

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other two being omnivores of unlike dietary preference.

EXPERIMENTAL

During the course of the experiment, 108 puppies, 172 baby pigs and 266 young guinea pigs were used. The puppies and guinea pigs were placed in individual pens with perforated floors, whereas the baby pigs were housed, by experimental groups of 4, in pens fitted with an electrically heated bedding floor under a hover. In all cases, feed and water were available ad libitum throughout the experimental period, the duration of which was 4 weeks for guinea pigs and 6 weeks for the other two species.

The main components of the control diets fed to pups and guinea pigs consisted of dry cereal[4] and skim milk powder, plus appropriate vitamin and mineral supplements. Baby pigs allotted to control groups received a basal ration consisting of a mixture of common cereal grains plus skim milk powder, yeast, cane molasses, fishmeal, soybean oilmeal, vitamins and minerals. On analysis these control diets were found to contain ether extractable material to the extent of 0.1 to 1.0% (dogs), 1.0 to 2.0% (guinea pigs) and 1.5 to 2.5% (swine). To these dry diets were added 20% by weight of 15 various edible animal and vegetable fats and oils. Early work included diets containing 5 and 10% of fat by weight, but careful examination and analysis of data obtained during the first year showed the 5 and 10% levels to be intermediate between the control and the 20% level. In the interests of expediency, therefore, feeding at the 5 and 10% levels was abandoned after the first year. When fat was incorporated into dog and guinea pig diets, the level of skim milk powder was adjusted at the expense of Pablum so as to maintain the protein in the ration at a minimum of 20%. A similar adjustment was carried out in formulae of the baby pig diets, in which the protein level was approximately 27%. All rations included chromic oxide to function as an index substance in the determination of digestibility. Feed and feces collected during the final week of each test were analyzed for chromic oxide by the method of Bolin, King and Klosterman ('52) and for fat by ether extraction. The apparent digestibility of the ingested fats was then calculated from the chromic oxide:fat ratios in feed and feces.

The fats and oils to be tested were maintained at freezing temperatures and withdrawn only as needed. Fat-containing diets were mixed frequently so as to eliminate any possibility of rancid feed, and uneaten feed was replaced by fresh feed in the feeders daily. Of the fats used, only butter required special preparation. This was accomplished by siphoning off and filtering the lipid phase obtained by heating commercial butter to 60°C. The aqueous phase, designated as "butter residue" was also fed to 4 dogs and 4 guinea pigs. However, data obtained from these animals and from groups fed mixed tallow were not considered relevant to the problem under consideration and hence were omitted from figures 1, 2 and 3.

Weekly records of weight and feed intake were maintained on all species throughout the course of the experiment. As the 4 pigs in each group were housed together, it was impossible to obtain a direct measurement of their individual consumption of the ration. Consequently, at the conclusion of the test, individual feed intake of this species was estimated from the regression of group feed intake on group gain, the regression used being that calculated from the data of control groups.

RESULTS

With few exceptions the young dogs, pigs and guinea pigs were able to adjust satisfactorily to the experimental ration, the complete transition being accomplished within one week following the cessation of nursing. All three species accepted the fat-containing diets more readily than the control diets, but no initial preference was shown for any one fat over another. Pups exhibited the most reluctance to consume their control ration, presumably because of its extremely dry, powdery consistency. Although all animals were closely scrutinized throughout the test period for outward signs of impaired health, no evidence was found that diet exerted any abnormal or differential effect in this respect.

[4] Pablum.

TABLE 1

Average daily liveweight gains of prematurely weaned pups fed diets containing zero % (control) or 20% added fat[1]

Lot	Diet	Observed value	Value relative to average for butterfat as 100	Statistics	
		gm			
1	Control	62.5	87		
2	Control	54.3	75	No. of animals	108
3	Control	42.8	59	Lots	27
4	Control	53.8	75		
5	Control	58.3	81	*Analysis of variance*	
6	Control	51.8	72		D/F
7	Control	46.3	64	All causes	107
8	Butterfat	70.0	97	Groups	26
9	Butterfat	74.8	104	Remainder	81
10	Butterfat	75.5	105		
11	Butterfat	63.8	89	*Means*	
12	Butterfat	75.8	105	General	63
13	Soybean	65.8	91	Controls	53
14	Cottonseed	61.3	85	Butterfat	72
15	Corn	65.0	90		
16	Linseed	57.0	79	*Standard deviation*	
17	Rapeseed	54.8	76	gm	± 20
18	Coconut	68.0	94	%	± 32
19	Soybean (hyd.)	73.5	102		
20	Cottonseed (hyd.)	68.3	95	F values (P = 0.05)	
21	Coconut (hyd.)	64.3	89	Obs.	0.89
22	Lard	66.5	92	Nec.	1.63
23	Tallow (beef)	64.8	90		
24	Fish oil	49.8	69	Least sign. diff. for n = 4	
25	Fish oil (hyd.)	61.8	86	gm	± 28
26	Butter residue	61.8	86	%	± 45
27	Tallow (beef + pork)	78.3	109		

[1] Four pups per group.

The data contained in table 1 are intended to illustrate the manner in which the available information was analyzed. Calculations and statistical analyses comparable to those compiled in this sample table were carried out on average daily liveweight gains, average daily dry matter intake and average gains per 1000 digested Calories of all three species. The direct comparison between butterfat and all other sources of dietary fat in column 2 was derived by taking the average of the several butterfat lots in column 1 to be 100 (in this case 72 = 100) and converting each observed value to a figure relative to this basis.

The results obtained from this procedure were considered to be most expressive of the experimental data and are therefore charted in figures 1, 2 and 3. Each of these figures has been drawn to scale with the vertical midpoint equal to 100 and the outer perpendicular lines plotted on the basis of the appropriate least significant difference. Thus, values falling beyond the delineated boundaries can be considered significantly inferior or superior, depending upon the direction of the deviation, to the mean of all butterfat lots, namely to 100.

From a cursory inspection of the distribution of points in these three figures, it can be seen that the variability occurring between groups of like species fed identical diets (control or butterfat) was often as great as that occurring between groups of like species fed different diets. Analysis of variance and application of calculated values for least significant difference showed variation between the lowest and highest mean feed intake of the swine control groups, for example, of a degree sufficient to be statistically significant (P = 0.05). Similarly, 5 control

groups of guinea pigs ate significantly more than the remaining two, while statistically significant differences between gains of these 7 groups of guinea pigs were evident even when an adjustment for apparent digestibility and caloric value of the rations was made. Scattered significant differences were also found between the various groups of any one species fed butterfat, the species and criteria where these occurred being swine and average daily gain, guinea pigs and average daily intake and swine and adjusted daily gain.

With respect to the other fats tested, a statistically significant depression from the mean of all butterfat groups was observed in the growth rate of swine fed fish, rapeseed or coconut oil and of guinea pigs fed hydrogenated fish oil (fig. 1). Dogs appeared to gain equally well regardless of fat level or source. However, when butterfat and other fats were compared *en bloc*, there was 18, 10 and 14% more gain on the former by guinea pigs, swine and dogs respectively.

Statistical analyses of feed intake data failed to reveal any significant differences between the average daily consumption of the 25 groups of pups. Although one control group of swine and 5 control groups of guinea pigs maintained a daily

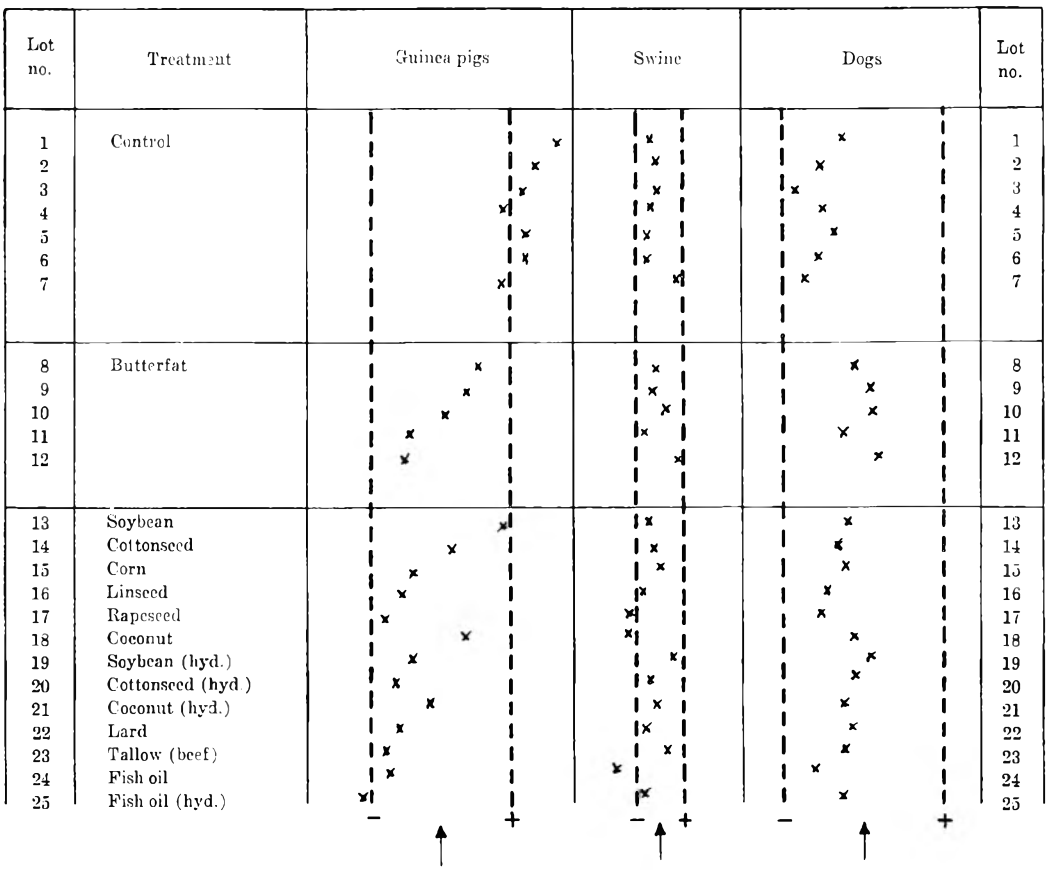

Fig. 1 Average daily liveweight gains of prematurely weaned dogs, swine and guinea pigs fed diets containing zero % (control) or 20% added fat (gains expressed relative to the average of all butterfat lots taken as 100).

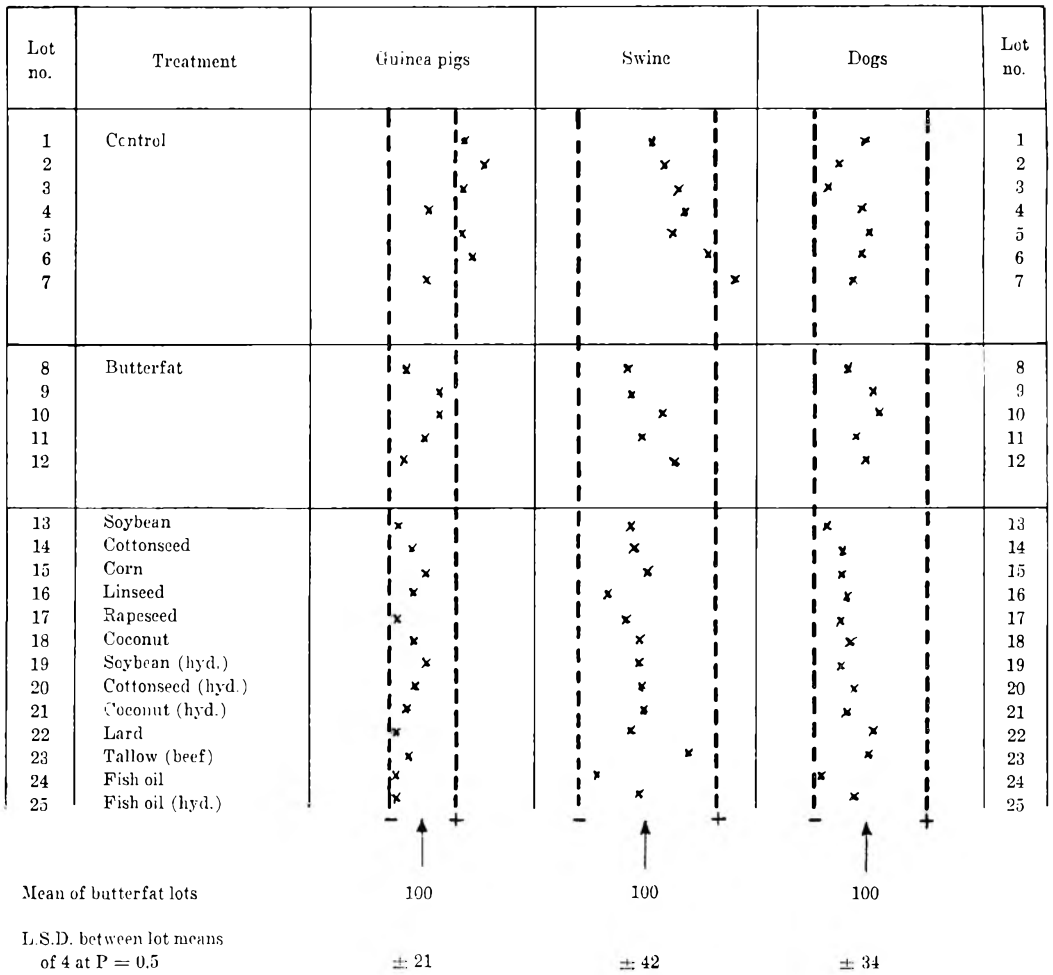

Fig. 2 Average daily dry matter intake of prematurely weaned dogs, swine and guinea pigs fed diets containing zero % (control) or 20% added fat (intake expressed relative to the average of all butterfat lots taken as 100).

feed intake significantly higher than that consumed, on the average, by butterfat groups, none of the test fats differed significantly from this butterfat average when intake of either of these two species was considered (fig. 2). However, when overall averages were computed for butterfat and for all other fats, it became apparent that guinea pigs, swine and dogs ate 11, 7 and 16% more, respectively, when their diets contained butterfat.

When the effect on gains of apparent digestibility and caloric content of the ration was taken into consideration by calculating average daily gain per 1000 digested Calories (fig. 3), butterfat, considered over all groups, was found to be 10% more efficient than other fats in the case of guinea pigs, 2% more efficient in the case of swine, and of like efficiency in the case of young pups. However, when lots of 4 animals were considered, only the guinea pigs fed hydrogenated fish oil showed a feed efficiency sufficiently reduced below that of butterfat groups to be of statistical significance ($P = 0.05$). Three control groups of guinea pigs plus the group fed soybean oil were apparently able to effect a more efficient conversion of digested Calories than groups fed but-

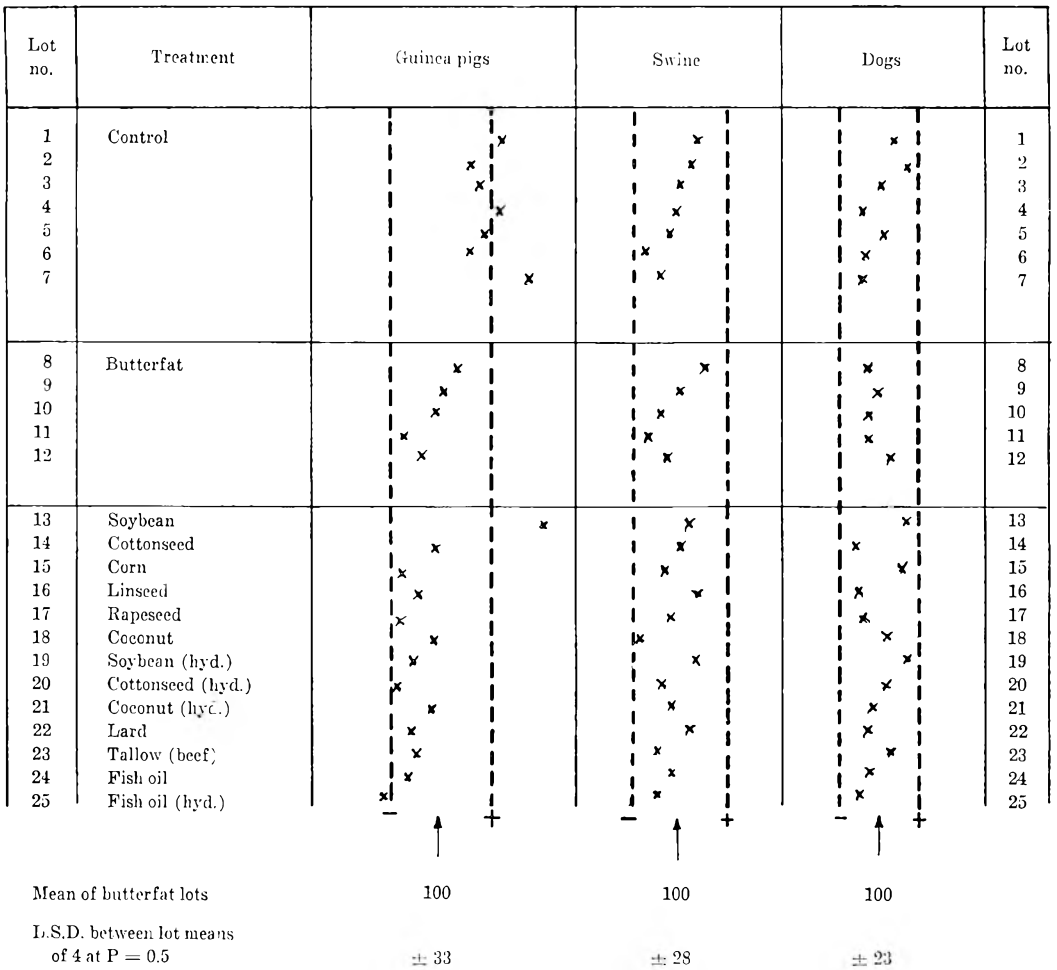

Fig. 3 Average gain per 1000 digested Calories made by prematurely weaned dogs, swine and guinea pigs fed diets containing zero % (control) or 20% added fat (adjusted gains expressed relative to the average of all butterfat lots taken as 100).

terfat in the ration. The depression of gains indicated in figure 1 for those groups of swine fed rapeseed, coconut or fish oil diets was no longer in evidence. Thus it would appear that this phenomenom resulted from reduced caloric intake and/or digestibility. An inspection of the original data revealed that the intake of all three groups was below the mean, with that of the fish and rapeseed oil groups being particularly low. Furthermore, it has been shown that the rat is able to utilize rapeseed oil only poorly (Deuel et al., '48a). Digestibility data obtained on the young pigs, dogs and guinea pigs used in this experiment are shown in table 2.

Both guinea pigs and swine appear to be similar to rats in their inability to efficiently utilize rapeseed oil in the diet. The apparent digestibility coefficient for this oil in the case of dogs, however, was 94%. Conversely, the latter species experienced considerable difficulty in digesting lard, which was well utilized by the swine and guinea pigs. The fish oil and hydrogenated fish oil were poorly digested by guinea pigs, the difference in digestibility between these fats and butter being significant ($P = 0.05$). When the digestibility data from pigs and guinea pigs were subjected to multiple correlation and partial regression analyses (Lloyd and Cramp-

TABLE 2

Digestibility of dry matter and ether extract of diets containing zero% (control) or 20% added fat and fed to young dogs, swine and guinea pigs

Lot		Guinea pigs		Swine		Dogs	
		D.M.	Ether extract	D.M.	Ether extract	D.M.	Ether extract
1	Control	90	—	84	—	94	—
2	Control	90	—	84	—	94	—
3	Control	88	—	85	—	93	—
4	Control	91	—	85	—	94	—
5	Control	90	—	89	—	95	—
6	Control	88	—	86	—	94	—
7	Control	91	—	85	—	94	—
8	Butterfat	83	94	82	79	93	96
9	Butterfat	83	94	88	99	94	99
10	Butterfat	86	95	88	95	96	99
11	Butterfat	79	95	87	96	94	97
12	Butterfat	78	97	86	92	95	98
13	Soybean	86	94	83	85	94	96
14	Cottonseed	85	95	87	98	96	99
15	Corn	75	92	90	96	95	98
16	Linseed	83	82	84	89	94	98
17	Rapeseed	84	72	84	78	94	94
18	Coconut	87	98	89	95	94	98
19	Soybean (hyd.)	73	90	88	99	96	98
20	Cottonseed (hyd.)	78	93	89	99	93	97
21	Coconut (hyd.)	84	98	89	99	94	99
22	Lard	80	94	83	90	94	79
23	Tallow (beef)	79	92	79	89	93	95
24	Fish oil	85	68	87	94	96	98
25	Fish oil (hyd.)	76	67	88	95	94	97
Mean		84	90	86	93	94	96
Standard deviation		5	9	3	7	1	4
L.S.D. between lot means of 4 at P = 0.05		15	25	7	18	3	12

ton, '57), an inverse relationship, significant at the 1% level, was found between the apparent digestibility of the fats and oils tested and the mean molecular weight of their constituent fatty acids. When these same two species were considered, it was further found that the influence of degree of saturation (as indicated by iodine number) on apparent digestibility was of minor importance. In pups weaned at 10 days of age, only 4% of the total variability in fat digestibility could be accounted for by the mean molecular weight of the fatty acids, whereas 2% of this total variation was ascribed to the degree of saturation of the dietary fat source.

DISCUSSION

The data compiled herein concerning liveweight change, feed efficiency (gain per 1000 digested Calories) and apparent digestibility of dry matter and fat appear to indicate that butterfat is not nutritionally superior to the other edible fats of animal and vegetable origin against which it was evaluated. This failure to differentiate between the various fat sources occurred despite the fact that the experimental animals were young guinea pigs, swine and dogs abruptly weaned at abnormally early ages. The importance which can be attached to the increased feed efficiency of guinea pigs fed soybean oil and the reduced feed efficiency for the same species fed hydrogenated fish oil in this experiment is questioned in view of the high variability occurring between groups on like diets and the failure of these differences to appear in dogs and swine. The inferior gains allowed in swine by diets containing coconut, fish and rapeseed oils (fig. 1) were not unexpected since Thomasson ('55; '56) ranked these oils 13th, 16th and 17th, respectively, in

a group of 18 tested for growth promoting value in rats. Deuel et al. ('48a, b) have also observed retarded growth in groups of rats receiving rapeseed oil. As in the case of our experiments, however, Thomasson failed to show a corresponding decrease in feed efficiency calculated as

$$\frac{\text{increase in weight in 6 weeks}}{\text{food calories in 6 weeks}} \times 100.$$

The fact that Deuel and co-workers were able to equate the lowered growth rate of the rats receiving rapeseed oil with that of butterfat and cottonseed oil groups by compensating for the relative indigestibility of the former oil is also compatible with our findings.

The trend toward increased voluntary consumption of rations containing butter, which was only revealed in our experimental series when the mean of all butterfat groups was compared with that of all fats other than butter, substantiates the earlier work of Deuel and Movitt ('44b). These workers have postulated that the preference exhibited by rats for rations containing butter is due to flavor, and were able to demonstrate experimentally an increased consumption of margarine or peanut oil diets to which 4 ppm of diacetyl were added. On the other hand, Boutwell et al. ('44) failed to find any increase in the voluntary intake of rats when their corn oil-lactose ration was supplemented with diacetyl. These authors, who differ from Deuel and his associates on many issues with respect to the comparative nutritive value of fats, have indicated their belief that appetite for, and consumption of, a given feed are directly influenced by its nutritive value (Boutwell et al., '41).

In general, the prolific accumulation of data contained in the literature, pertinent to the experiment reported herein, falls readily into two divisions, these opposing schools of thought being led by the above mentioned groups, Deuel et al., on the one hand and Shantz, Boutwell and co-workers on the other. Although early work by Shantz et al. ('40a) reported butterfat-filled skim milk to be more effective in promoting weight gains than liquid skim milk mixed with vegetable oils, this group later showed that these results could only be duplicated when lactose was the sole source of dietary carbohydrate (Boutwell et al., '43a, b). However, Deuel et al. ('43; '44a, b; '45a; '46a, b) were unable to demonstrate any differences between the nutritional properties of butterfat and numerous commonly used vegetable oils, as judged by weight change and diet efficiency, where the diets were composed solely of mineralized skim milk powder and vitamin-fortified fat. Later experiments wherein the carbohydrate was of mixed origin also failed to demonstrate any superiority of butterfat over cottonseed oil (Deuel et al., '48a, b) or any inferiority in growth or reproduction when butterfat was replaced by margarine fat over 25 generations of rats (Deuel et al., '50). These later results are in agreement with those of Boutwell et al. ('43b) who found gains on butterfat, corn oil, coconut oil, cottonseed oil, soybean oil, lard, commercial hydrogenated vegetable oil[5] and peanut oil to be essentially equal in the presence of mixed carbohydrate (48% of carbohydrate containing by parts: lactose, 3; sucrose, 15; dextrose, 5; starch, 15; and dextrin, 10). However, Boutwell and co-workers ('45), in a later attempt to reconcile the variability in results obtained by the various laboratories, varied both the dietary carbohydrate source and the level of B vitamins. Their findings indicated that, while at a medium (normal) level of B vitamin administration, butter was superior to corn oil when either sucrose, fructose-glucose, starch, dextrin, dextri-maltose or lactose constituted the source of carbohydrate, only butter groups ingesting lactose as their carbohydrate showed superior growth when a high level of B vitamins plus 1% of whole liver powder were incorporated into the feed mixture. No mixed carbohydrate comparable to that fed in their 1943 series was used.

In the feeding trials reported in this publication, the rations contained a mixed carbohydrate derived from skim milk powder and rice pablum (for dogs), skim milk powder and mixed cereal pablum (for guinea pigs) and skim milk powder and wheat, oat groats, cane molasses, soybean

[5] Crisco.

oilmeal and brewers' yeast (for swine). Our failure to show any superiority of butterfat over vegetable oils appears then to be compatible with the results obtained by Boutwell et al. ('43b) and Deuel and co-workers ('48a, b) when they fed a mixed carbohydrate to the test animals. However, as the source of dietary carbohydrate in the majority of experiments conducted by Deuel and his associates was lactose, a direct comparison of their results with those reported herein cannot be made. It should be noted that the experimental procedure of Deuel et al. in excluding results of both litter mates in cases where either sibling showed diarrhea, is questioned, the basis of disagreement being that in calves fed vegetable oils, scours have been reported as one of the first and most persistent symptoms of ill health (Gullickson and Fountaine, '39).

SUMMARY

In an experiment utilizing 108 puppies, 172 pigs and 266 guinea pigs weaned at 10, 14 and three days of age, respectively, butterfat was evaluated against a low-fat control diet and against 15 other edible animal and vegetable fats and oils fed as 20% by weight of the ration, the chief criteria being growth, voluntary feed intake, apparent digestibility and gain per 1000 digested Calories. Although some indications of a preference for rations containing butter were observed, no significant differences of importance could be established between any of the fats compared. These included corn, linseed, rapeseed, fish, soybean, cottonseed and coconut oils, the latter 4 oils in the hydrogenated form, lard, mixed tallow and beef tallow.

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Choline, Hepatic Fat and Insulin Polyphagia[1]

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The observation that reduction of dietary lipotropic requirement sufficient to prevent abnormal accumulation of stainable hepatic fat in animals accompanies a decrease in total caloric intake has been reported by several investigators (Best and Huntsman, '35; Mulford and Griffith, '42). Between the extremes of almost complete starvation and ad libitum food intake, the choline requirement of rats is proportional to the calories consumed (Best and Ridout, '38). However, little information is available concerning choline requirements of rats with caloric intakes exceeding ad libitum levels.

To investigate the effect of polyphagia on choline requirement, we gave injections of insulin twice daily to increase the food intake of young adult rats fed a diet previously shown to have been marginal in adequacy of choline content when consumed in normal quantity. Results indicate that insulin-induced polyphagia did not increase choline requirement, as determined by centrolobular hepatic lipid concentration, but resulted in periportal accumulation of lipid. The periportal lipid accumulation was not prevented by dietary supplement of choline.

EXPERIMENTAL

Two groups of 41 male, Wistar rats (84 to 130 gm initial weight) were fed ad libitum a basal hypolipotropic diet supplemented with either 0.50% or 0.05% of choline chloride. The basal diet was derived by slight modification from one previously used to study choline deficiency (Best, Hartroft, Lucas, and Ridout, '49). The constituents of this diet, with their proportions by per cent of weight, follow: vitamin-free casein, 10; gelatin, 5; zein, 3; L-cystine, 0.3; salts (Wesson, '32), 5; non-nutritive cellulose, 2; sucrose, 41.7; dextrin, 20; beef fat, 10; corn oil, 2; and vitamin mixture,[3] 1. In preliminary experiments, the lesser amount of choline chloride (0.05%) added to this diet and fed to rats of similar age, weight, sex, and strain was sufficient to prevent more than a minimal accumulation of stainable fat in centrolobular positions of their livers.

Twenty-eight animals in each group were injected subcutaneously twice daily with insulin,[4] beginning with one unit per injection and gradually increasing to a maximum of 16 daily for periods up to 9 weeks. Unmodified insulin was administered in the mornings, and NPH insulin in the evenings. In the beginning of the experiment, a number of animals died from overtreatment with insulin; 10 of the original 28 animals survived more than 5 weeks in the group receiving the small amount of choline (0.05%) and 11 of 28 in the group receiving the larger amount (0.50%). Thirteen control rats in each dietary group received subcutaneous injections of 0.9% aqueous sodium chloride twice daily. Only those rats maintained on their various regimens for at least 41 days were included in the results, because differences in weights of the insulin-treated and control animals were not apparent before that time.

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[2] Trainee in Experiment Pathology under U.S.P.H.S. Grant no. 2G-66.
[3] Each gram of the vitamin mixture contained in mg: thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 4.5; folic acid, 0.09; menadione, 2.25; *p*-aminobenzoic acid, 5.0; inositol, 5.0; α-tocopherol acetate, 5.0; ascorbic acid, 45.0; also vitamin A, 900 units; vitamin D, 100 units, biotin, 20 μg, and vitamin B_{12}, 1.35 μg.
[4] Lilly.

Dry ingredients of the diets were mixed thoroughly, and the supplements added and blended before the addition of fat. Quantities sufficient to feed the animals for about 10 days were prepared and stored in sealed, plastic containers at 4°C. These precautions prevented rancidity. Animals were housed in individual cages with wire bottoms and offered water ad libitum. Glass food containers were sterilized daily, and uneaten portions of the diet were discarded after weighing.

Autopsies were performed within 6 hours after death on rats dying between the 5th and 9th week of the experiment; in most instances they were autopsied in less than two hours. Postmortem changes did not interfere with histologic and histochemical studies. Blocks of tissues from all major viscera were fixed by immersion in formalin[5] or Bouin's solution and frozen sections prepared of the former blocks and paraffin sections of the latter. Formalin-fixed frozen sections (liver, kidney, heart) were stained to demonstrate abnormal fat (Wilson, '50), and the paraffin sections were stained by a variety of techniques to demonstrate beta cell granulation in the pancreas, connective tissue and reticulin fibers.

Sections of liver, stained for fat, were examined microscopically; the experimental histories of the animals from which the tissues had been obtained were unknown to the observers. Particular attention was given to recording the lobular distribution of fat within parenchymal cells. Amounts present in central and peripheral positions were recorded in terms of a scale with 7 points defined as follows:

Grade 0: stainable fat absent.

Grade 1: fat present in less than 50% of cells.

Grade 2: fat present in more than 50% of cells but less than 85%.

Grade 3: fat present in more than 85% of cells, but 50% or more of cytoplasm filled with fat in less than 15%.

Grade 4: 50% of cytoplasm of more than 15% but less than 50% of cells filled with fat.

Grade 5: more fat than in grade 4, but less than in grade 6.

Grade 6: more than 85% of cytoplasm in area under consideration filled with fat.

This type of quantification affords only approximate comparative indices, but as will be seen, the distributions of stainable fat in this experiment are of as great significance as the relative amounts.

[5] Four per cent of aqueous formaldehyde containing 100 gm $CaCl_2$ and 100 gm of $Co(NO_3)_2 \cdot 6H_2O$/l. The addition of cobalt seems to aid in the preservation of fat.

TABLE 1

Summary of data

Experiment regimen	Number of rats[1]	Mean values						
		Days on regimen	Daily food intake	Initial body weight	Final body weight	Hepatic weight	Hepatic fat[2]	
							Centrolobular	Periportal
			gm	*gm*	*gm*	*gm*		
Insulin, 0.05% choline chloride	10	57	20.4	112	392	19.0	1.6	3.5
Saline, 0.05% choline chloride	11	56	16.5	110	310	16.0	2.2	1.6
Insulin, 0.50% choline chloride	11	50	18.6	112	335	16.8	0.2	3.6
Saline, 0.50% choline chloride	11	52	16.0	111	315	16.1	1.6	1.0

[1] Surviving more than 5 weeks.

[2] Estimated microscopically, using progressive scale of zero to 6, the highest number corresponding to the highest content of fat.

RESULTS

These data (table 1) indicate that most animals receiving insulin for longer than 5 weeks ate more food and gained more weight than those given saline, even though every rat was offered more food daily than it consumed. Because of marked individual variation in food intakes and growth in the insulin-injected groups, differences in means of these variables between insulin-injected and saline-injected groups were not highly significant. The *t* test yielded confidence limits of 0.02 for weight gain and 0.10 for food intake in the 0.05% group. However, it is probable that the insulin injections resulted in increased weight gain in many animals. The results are biased in the direction of minimizing differences, because rats dying after overtreatment with insulin, after at least 5 weeks of treatment, were included in the analysis and probably lost weight shortly before dying. Food intake of many of these rats was low during the last day of life, but in no case did the daily food records suggest a terminal illness of longer than 24 hours. Two animals in the 0.05% choline chloride-supplemented group and 8 in the 0.50% group died spontaneously. All of the insulin-injected animals surviving until the termination of the experiment gained more weight than any animal not receiving insulin. Histologic examination of the organs of the animals dying spontaneously revealed no features to distinguish them from those killed. Thus, it was thought appropriate to include them in the results.

The animals in each of the 4 experimental groups had distinctive patterns of hepatic lipid distribution with little variation among members of any one group. The saline-injected group, receiving the 0.50% choline chloride diet, had the least stainable hepatic lipid, only small droplets being present in scattered parenchymal cells without definite orientation in the lobule (fig. 4). In the group of animals injected also with saline, but fed the diet containing the lesser amount (0.05%) of choline chloride, slightly more fat was present, particularly centrolobularly (fig. 2). A few centrolobular fatty cysts were present in one rat. This result had been predicted by the preliminary titration experiment designed to determine the amount of choline which would just permit this evidence of "border-line" lipotropic deficiency. In animals treated with insulin and fed the basal diet supplemented with 0.05% of choline chloride, fat was found centrolobularly but in no greater quantities than in the saline-treated controls. However, livers of these rats consistently showed accumulation of fat in parenchymal cells in the peripheries of the hepatic lobules adjacent to terminal portal triads. In these areas, fat occurred in amounts clearly exceeding the levels found centrolobularly in the same livers or in livers of the saline-treated animals (fig. 1). In 5 animals the cytoplasm of many periportal cells was nearly completely filled with fat in small and moderately large cytoplasmic droplets. Nuclei in these fatty cells usually were centrally located, and fatty cysts were absent (fig. 5). In the 4th group, the insulin-injected rats receiving the higher choline supplement (0.50%), stainable lipid was nearly completely absent from centrolobular hepatic cells. However, peripherally located parenchymal cells showed fat accumulation of type and degree indistinguishable from those of the insulin-treated group fed the lower choline supplement (fig. 3).

Using paraffin sections stained by Gomori's aldehyde fuchsin method, we could see that pancreatic beta cells of insulin-injected rats contained fewer specific granules (insulin or insulin-precursor[6]) than did beta cells of saline-injected controls, regardless of level of choline in the basal diet (figs. 6 and 7). Gross or microscopic changes in other viscera examined were absent, including heart, kidneys, and vessels of the rats receiving the smaller amount of dietary choline. Hearts and kidneys of the other animals were also examined grossly and microscopically, with no changes noted. Pathologic changes were not expected, even in the group receiving the low choline supplement, as even 0.05% of choline chloride under

[6] The degree of aldehyde fuchsin-positive granulation in pancreatic beta cells has been shown to correlate closely with content of extractible insulin (Wrenshall, Hartroft and Best, '54).

these conditions is sufficient to prevent lesions other than minimal hepatic fatty change. The production of renal and cardiovascular lesions requires greater relative degrees of lipotropic deficiency than those produced by this experiment (Griffith and Wade, '39). Weights of adrenal glands varied moderately, but rats treated with insulin, including both those which died spontaneously and those which were killed, had slightly larger adrenals than the rats treated with saline. Other organs were normal grossly.

DISCUSSION

No attempt was made to determine whether the greater body weight of the insulin-treated rats represented chiefly an increase in adipose tissue or an increase in growth (nitrogen retention). Salter and Best ('53) found marked body weight increases in hypophysectomized rats fed ad libitum and given daily insulin injections, but the weight gains were accompanied by only slight nitrogen retention. In an experiment in which hypophysectomized rats were pair-fed by gavage, Wagner and Scow ('57) found that when food intake was increased above ad libitum levels, the animals showed increased but still subnormal rates of growth. Addition of insulin to the regimen had no effect on nitrogen retention, body weight, organ weights or body composition of the force-fed rats. Therefore, under the conditions of our experiment, insulin appears to have acted as a growth stimulant by increasing appetite, and weight gains induced by it were apparently caused chiefly by accumulation of adipose tissue.

Pretreatment of rats with insulin has been shown to increase the rate of lipogenesis in liver and adipose tissue *in vitro* (Chernick and Chaikoff, '50; Hausberger, Milstein, and Rutman, '54). Our object, however, was not to determine the mechanism by which insulin increases growth, but to determine whether the greater caloric intake induced by insulin would increase the amount of choline required to prevent development of the centrolobular type of fatty liver in rats. The results clearly indicate that increased food intake in the insulin-treated rats did not increase their requirement for choline. Had it been increased, greater amounts of fat would have been found in centrolobular regions in the livers of the insulin-treated animals, fed the basal diet with 0.05% of choline, than in those of the saline-treated controls. In no instance was such an increase found. Biochemical determinations of total hepatic lipid would not have confirmed this finding. Indeed, such determinations would have proved misleading, because additional stainable fat *was* present in the livers of the insulin-injected rats, but only in periportal rather than centrolobular regions.

No available evidence exists that choline deficiency in the rat leads to deposition of fat in periportal regions, except when every other portion of the hepatic lobule is first filled with stainable lipid. Furthermore, had choline deficiency been responsible for the accumulation of stainable fat within periportal cells, it would have been prevented or at least decreased by the higher supplement of choline chloride. Reference to table 1 and figure 3 indicates that choline chloride at this higher level was unable to prevent the accumulation of periportal lipid in rats injected with insulin.

Centrolobular fat in rats, therefore, was not increased by injections of insulin and centrolobular fat present under the dietary conditions employed was prevented by increasing the choline supplement. But the choline supplement was completely ineffective in preventing the appearance of visible fat in periportal positions in the rats injected with insulin. Substitution of saline for insulin prevented the development of abnormal amounts of periportal fat. These data provide evidence that the pattern of lipid deposition in the rat can be varied widely by the experimental procedure employed. Factors which prevent fatty changes in the center of the lobule do not prevent fatty changes in the periphery and conversely. These data confirm earlier observations and provide more evidence of the functional distinctiveness of topographical divisions of the structural hepatic unit (Rappaport et al., '54).

Our unpublished observations confirm evidence presented by Sherlock et al. ('51,

'58) that the lipid in fatty livers of obese patients subjected to hepatic biopsy is frequently periportal. We do not know why obese people have fatty livers, but abnormally large amounts of lipid distributed periportally appear to be characteristic of obesity in man. Insulin-induced polyphagia has been observed in rats as well as in man. Further observations must therefore be made of animals overfed by other methods to insure that our results are associated with increased food intake rather than a direct effect of the insulin. Elsewhere (Meyer and Hartroft, '60) we are reporting experiments in which obesity, induced by hypothalamic electrocoagulation, has under similar dietary conditions produced much greater deposits of periportal fat than reported here, and which was not prevented by additions of generous supplements of dietary choline (0.50%). Therefore, at present, the increased consumption of food observed in these experiments seems to be the common factor in the production of periportal types of fatty livers.

Semistarvation can be induced in rats by feeding them a low-protein diet ad libitum. When subsequently offered a normal diet, the animals temporarily overeat and develop fatty livers, again of the periportal type (Best et al., '55). This is further evidence supporting the hypothesis that increased caloric intake is an important factor responsible for deposition of abnormal amounts of hepatic fat, and this fat appears to be deposited first in periportal parenchyma.

We cannot exclude the possibilty that the increased caloric intake observed in our experiments, as well as that cited above, may unmask some otherwise unapparent limiting factor in the diets employed. Experiments of Elvehjem and co-workers[7] and Best and co-workers ('55), in which dietary amino acid imbalance produced periportal hepatic fatty change, make it necessary to consider the possibility that such imbalance may be responsible. Elucidation of these problems awaits further investigation.

[7] Elvehjem, C. A. 1956 Amino acid imbalance. Federation Proc., *15:* 965 (abstract).

SUMMARY

To investigate the effect of polyphagia on per cent of dietary choline requirements, young adult, male, Wistar rats were fed ad libitum a diet marginally adequate in choline content and given injections of insulin twice daily. Control rats received saline. The rats treated with insulin became polyphagic and gained more weight than those treated with saline. The percentage of dietary choline required to prevent centrolobular hepatic fatty change was not altered in the polyphagic animals. But their livers showed accumulations of abnormal amounts of stainable lipid in periportal parenchymal cells. Addition of a supplement of 0.50% of choline chloride to the diet did not prevent the accumulation of periportal fat associated with insulin-injection and polyphagia, although the supplement resulted in lesser amounts of centrolobular fat in both insulin-treated and saline-treated animals. These results demonstrated that (1) insulin-treatment and associated polyphagia with acceleration of weight-gain in rats was not attended by accumulation of stainable fat in *centrolobular* hepatic parenchymal cells; (2) insulin-treatment and polyphagia resulted in an abnormal accumulation of fat in *periportal* regions; (3) the periportal fatty change was not affected by dietary choline supplements (0.50%); and (4) therefore, insulin-treatment and its associated effects did not increase the per cent of dietary choline requirement.

This experiment again demonstrates that the physiopathology of the liver varies from one portion of the lobule to another.

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PLATE 1

EXPLANATION OF FIGURES

Figs. 1 to 4 Frozen sections of livers stained for fat with Oil Red O and counterstained with hematoxylin and light green. Fat appears black. × 190.

1 Choline chloride supplement 0.05%, insulin-treated. Fat concentrated peripherally (lower left) with only small droplets centrolobularly (upper right).

2 Choline chloride supplement 0.05%, saline-treated. Small amounts of fat centrolobularly (top), virtually none peripherally (bottom).

3 Choline chloride supplement 0.50%, insulin-treated. Similar to figure 1 except for absence of centrolobular fat. Central vein in upper left corner, portal area in lower right.

4 Choline chloride 0.50%, saline-treated. Virtually no fat visible in any part of lobule Central vein top, portal area bottom.

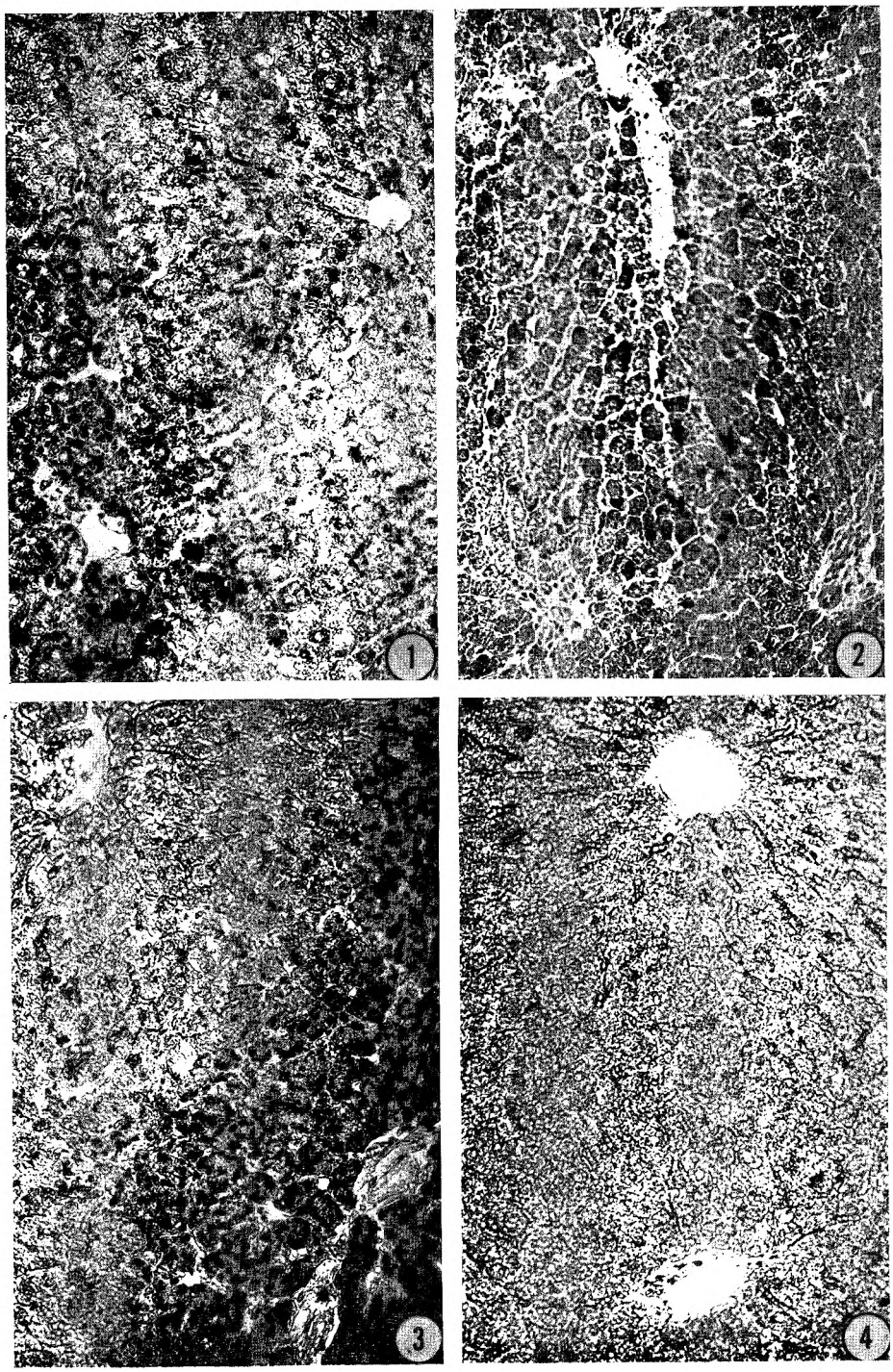
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PLATE 2

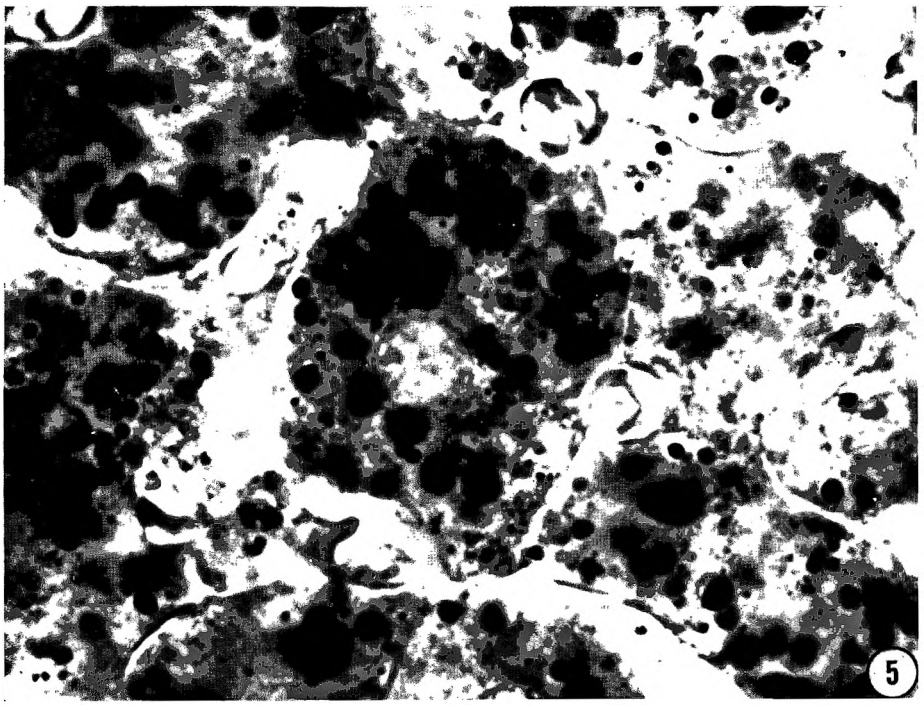
EXPLANATION OF FIGURES

5 Choline chloride supplement 0.05%, insulin-treated. Periphery of lobule of liver stained for fat with Oil Red O (fat appears black). × 1,400. Typical appearance of fat-laden periportal parenchymal cell of insulin-treated rat receiving either 0.05% or 0.50% choline chloride supplement.

6 to 7 Sections of pancreas stained by Gomori's aldehyde fuchsin method to show beta cell granules. × 650.

6 Choline chloride supplement 0.05%, insulin-treated. Granulation of beta cells sparse.

7 Choline chloride supplement 0.05%, saline-treated. Normal degree of beta cell granulation.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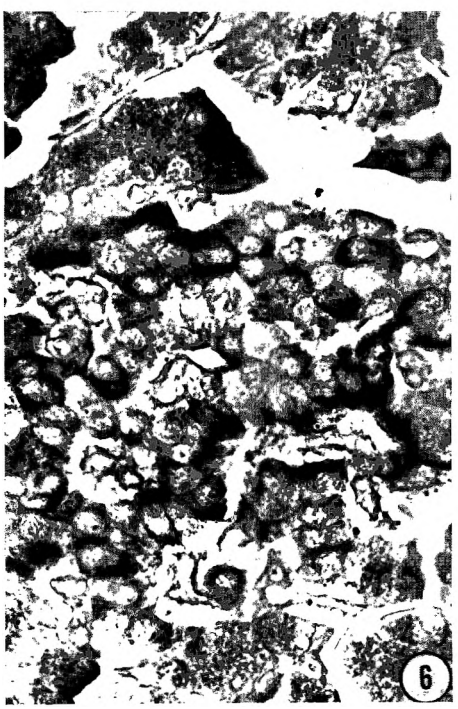
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An Effect of Dietary Sulfate on Selenium Poisoning in the Rat[1]

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Previous observations have shown that inorganic sulfate will counteract selenate toxicity in plants (Hurd-Karrer, '38; Weissman and Trelease, '55; Shrift, '54) and in microorganisms[3] (Fels and Cheldelin, '49; Postgate, '52) as measured by growth response. That selenate is in some cases a biological analogue of sulfate has become apparent, particularly in microbiological systems. For example, *Escherichia coli* under suitable circumstances can produce amino acids containing selenium in place of sulfur[4] and selenate, as well as molybdate and several other ions, appears to be an alternate substrate to sulfate for the yeast enzyme sulfurylase,[5] (Wilson and Bandurski, '58). Monty[3] observed that selenate, molybdate and tungstate were competitive inhibitors of the utilization of sulfate by *E. coli*, and attributed this effect to an inhibition of sulfurylase. With the recognition that sulfate tends to alleviate both the gross symptoms (Dick, '53, '56; Van Reen and Williams, '56) and metabolic changes (Dick, '56; Van Reen and Williams, '56; Mills et al., '58) due to excessive dietary intake of molybdenum in animals, it was deemed desirable to explore the possibility of an alleviation by sulfate of the symptoms of selenium toxicity as well.

EXPERIMENTAL

Young male rats of the Wistar strain[6] which had initial weights between 50 and 90 gm were maintained on a low sulfate basal diet and its modifications for a 4-week period to allow growth observations and for an 8-week period to allow liver observations in certain instances. The diet modifications included additions of 10 ppm of selenium as sodium selenite or sodium selenate and of 0.29, 0.58 and 0.87% of sulfate as sodium or potassium sulfate. Diet and tap water were offered ad libitum. The tap water contained 8.5 ppm of sulfate.

The percentage composition of the basal diet follows: vitamin-free casein,[7] 20; glucose, 70; salt mixture, 6; corn oil, 4. The following vitamin supplement was included in the diet (quantities in milligrams per kilogram of mixed diet) thiamine, 5; riboflavin, 8; niacin, 40; pyridoxin, 5; Ca pantothenate, 45; biotin, 0.4; vitamin B_{12}, 0.03; folic acid, 2; menadione, 5; inositol, 100; *p*-aminobenzoic acid, 100; α-tocopherol, 1500; vitamin A alcohol, 8; vitamin D concentrate, 750 I.U. The salt mixture had the following percentage composition: $CaCO_3$, 27; K_2HPO_4, 17; Na_2HPO_4, 12.4; $Ca_3(PO_4)_2$, 23.2; NaCl, 14.6; $MgCO_3$, 4.35; ferric citrate, 0.65; $MnCl_2$, 0.62; KI, 0.069; $ZnCO_3$, 0.033; cupric acetate, 0.075.

The liver observations were made by inspection and weight measurement. Liver fat was discarded as a criterion of damage

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[2] Fellow of the National Institutes of Health, on leave from Station Biochemistry, South Dakota State College, Brookings.

[3] K. J. Monty 1958 Inhibition by molybdate of sulfur metabolism in *Escherichia coli*. Federation Proc., *17*: 278 (abstract).

[4] T. W. Tuve and H. H. Williams 1957 Identification of selenomethionine in the proteins of *Escherichia coli* employing the chromatographic "fingerprint" method. J. Am. Chem. Soc., *79*: 5830 (communication to the editor).

[5] R. S. Bandurski, L. G. Wilson and C. L. Squires 1956 The mechanism of "active sulfate" formation. J. Am. Chem. Soc., *78*: 6408 (communication to the editor).

[6] Albino Farms, Red Bank, N. J.

[7] Obtained from Nutritional Biochemicals Corp.

with selenized animals, as the total liver fat was within the normal range with damaged livers in spite of apparent abnormal concentrations of fat on the periphery of the lobes.

RESULTS AND DISCUSSION

Table 1 shows the growth response obtained from selenium and sulfate additions to the diet. A large growth depression was obtained from the addition of 10 ppm of selenium as the selenite and selenate salts. The addition of sulfate as the sodium or potassium salts partially and progressively prevented the growth depression. The sulfate effect amounted to about 20% alleviation of the growth depression with 0.29% of sulfate and to about 40% alleviation with 0.58% of sulfate. A higher level of sulfate (0.87%) as potassium sulfate restored the growth even more significantly, but a growth depression was noted in the control group at this level. Thus, the data indicate a definite relationship between dietary sulfate level and the growth inhibition observed with selenium.

The liver observations made with the animals receiving selenium showed little effect of sulfate in preventing the liver damage except at the highest sulfate level (0.87%). At this level the livers had less damage and were slightly larger in size than those with the other groups. In general, the damage which varied from mild to severe with the different animals was similar to that described by Moxon ('37) and Trelease and Beath ('49) for chronic selenium poisoning.

The animals tolerated the seleniferous diet rather well as no mortality was evident for as long as 8 weeks and liver damage required longer than 4 weeks to develop to a moderate extent.

The present study does not establish the form of selenium which the sulfate counteracted. Since selenate and selenite are both subject to chemical change in the

TABLE 1

Effects of added sulfate on weight gain of rats receiving inorganic selenium

Diet	No. of animals	Sulfate added		Average weight gain[1] with standard error[2]
		Source	Level	
			%	gm
Experiment 1				
Basal	9	—	0	109 ± 8.8
	9	Na_2SO_4	0.29	117 ± 7.5
Basal + selenite[3]	9	—	0	40 ± 6.7
	10	Na_2SO_4	0.29	58 ± 5.7
Basal + selenate[3]	10	—	0	30 ± 3.3
	10	Na_2SO_4	0.29	52 ± 4.7
Experiment 2				
Basal + selenate[3]	7	—	0	28 ± 8.9
	7	Na_2SO_4	0.58	94 ± 7.0
Experiment 3				
Basal	7	—	0	141 ± 4.3
	7	K_2SO_4	0.29	137 ± 5.8
	8	K_2SO_4	0.58	136 ± 2.8
	6	K_2SO_4	0.87	119 ± 12.7
Basal + selenate[3]	8	—	0	56 ± 3.8
	8	K_2SO_4	0.29	69 ± 5.2
	8	K_2SO_4	0.58	89 ± 5.9
	8	K_2SO_4	0.87	94 ± 5.8

[1] Average weight gain was determined for a 28-day period.

[2] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

[3] Selenite and selenate were included at levels where selenium was 10 ppm of diet.

presence of oxidizable substances, the form of these substances following their addition to the diet may have been changed.

Nor does the present study provide information bearing upon the mechanism whereby sulfate alleviates the toxicity of seleniferous diets. It is possible that sulfate markedly affects the passage of selenium across membranes, as it has been postulated to do for molybdenum in animals (Dick, '56). Equally possible is that the ions of selenium and sulfur compete at the enzymatic level such as apparently occurs in micro-organisms. It is likely that a common mechanism underlies the alleviation by sulfate of the symptoms of selenate and molybdate toxicities.

The possibility that sulfate may be used as a control measure in selenium poisoning is evident. Linseed oil meal has been found quite effective as a preventive dietary treatment for selenium poisoning with rats (Halverson et al., '55) as have certain organic arsenicals with rats and pigs (Hendrick et al., '53; Wahlstrom et al., '55). These treatments are active against poisoning from selenite sources and toxic grain but whether sulfate will provide as broad protection is not known. Since the other treatments are not completely effective and because they have some other limitations, it is hoped that sulfate will prove useful for supportive treatment.

SUMMARY

Dietary sulfate was shown to restore partially the growth of selenized rats receiving a purified diet with selenium added as selenite or as selenate. Sulfate levels of 0.29, 0.58 and 0.87% as sodium or as potassium salts progressively relieved the growth inhibition due to selenium. Alleviations of greater than 40% were observed. Sulfate, however, did not substantially prevent liver degeneration due to selenium.

ACKNOWLEDGMENT

The authors are indebted to Mrs. Julia H. Phifer for her technical assistance.

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Magnesium Requirement of Guinea Pigs and Rats

EFFECT OF CALCIUM AND PHOSPHORUS AND SYMPTOMS OF MAGNESIUM DEFICIENCY[1]

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In 1936, Wulzen and Bahrs reported that guinea pigs fed a diet based chiefly on milk and cereal grains became stiff in the hind limbs, eventually losing power of locomotion; and that the syndrome could be prevented by feeding fresh kale or alfalfa. Later, these investigators ('41) reported that the first symptom of the deficiency was wrist stiffness, but that with time the muscles atrophied and deposits of calcium phosphate were found widely distributed in soft tissue. The reports of Dasler and Bauer ('49), of Smith et al. ('49), and our own unpublished observations, suggest that the soft tissue calcification and wrist-stiffness are separate syndromes. No observations of wrist stiffness will be reported here.

Hogan and coworkers ('50) observed that a high percentage of guinea pigs developed soft tissue deposits when they consumed a diet containing 0.8% of calcium and 0.9% of phosphorus. The incidence of deposits decreased when the phosphorus level was lowered to 0.5%. House and Hogan ('55) observed later that the addition of potassium and magnesium to a diet high in phosphorus prevented the occurrence of deposits as well as improving the rate of gain. O'Dell et al. ('56) showed that the ameliorative effect of these supplements was due in part to their effect on acid-base balance, inasmuch as the guinea pig does not excrete ammonia in the urine and cannot tolerate an acid diet. Presumably potassium played the major role in alleviating the deficit of cations in the diet.

The specific significance of dietary magnesium for the guinea pig has recently received attention[2] (Maynard et al., '58). It has long been recognized, as pointed out in the review by Duckworth ('39), that high levels of dietary calcium accentuate the symptoms of a magnesium deficiency. This observation has been confirmed recently by Hegsted et al. ('56) but an adequate explanation has not been found for the aggravating effect of high levels of dietary phosphorus on the calcinosis syndrome. The balance studies of O'Dell et al. ('57) showed that high levels of phosphorus decreased magnesium absorption, causing the guinea pigs to go into negative balance. These observations led to the suggestion that excess phosphorus may increase the magnesium requirement.

Our observations to be reported here, show that phosphorus as well as calcium increases the magnesium requirement of both guinea pigs and rats; that symptoms of magnesium deficiency in the guinea pig differ somewhat from those in the albino rat, but are similar to those observed in calves and the cotton rat.

EXPERIMENTAL

Male guinea pigs with an average weight of about 200 gm and male rats of the Wistar strain weighing about 75 gm, both reared in our laboratory colonies, were used. They were caged in groups of 4 on raised-screen floors. The experimental period for the guinea pigs was 12 weeks, at the end of which all surviving animals

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[2] B. L. O'Dell, E. R. Morris and W. O. Regan 1958 Effect of phosphorus on magnesium requirement. Federation Proc., *17*: 487 (abstract).

were killed, autopsied and examined for visible deposits of calcium phosphate in soft tissues. All animals which died during the experimental period were autopsied. The rats were under observation for 4 weeks. Feed and distilled water were supplied ad libitum.

The basal diet, used for both species, was essentially the same as that described by O'Dell et al. ('56). The major components in per cent composition were: acid-washed casein, 30.0; sucrose, 44.2; cellulose,[3] 15.0; soybean oil, 4.0; salts,[4] 4.0; potassium acetate, 2.7; and choline chloride, 0.1. In addition to the vitamin mixture[5] described, the diet contained 2.5 mg of aureomycin hydrochloride per kg. According to analysis, the basal diet contained 0.86% of calcium 0.45% of phosphorus and 0.005% of magnesium. Supplements of calcium were supplied as $CaCO_3$, magnesium as MgO and phosphorus as $NaH_2PO_4 \cdot H_2O$, all at the expense of sucrose.

Serum was prepared from blood obtained by cardiac puncture at the end of the 12-week growth period. Calcium was determined by the method of Clark and Collip as described by Hawk et al. ('54) and magnesium by the thiazole yellow method of Mitchell ('54).

RESULTS

A. Effect of dietary level of calcium and phosphorus on magnesium requirement. Three levels each of calcium and phosphorus were fed to guinea pigs according to the experimental design shown in table 1. When the level of dietary calcium was maintained at about 0.9% and the phosphorus increased from 0.4 to 1.7%, the rate of gain dropped precipitously in the absence of magnesium. The mortality rate rose to 100% and the incidence of visible deposits at the intermediate level was high. At the highest level of phosphorus, the animals did not survive long enough to develop visible deposits in soft tissue. The addition of 0.3% of magnesium to the diets entirely prevented deposits and virtually eliminated mortality. The rate of gain was markedly improved although the gain on the diet containing 1.7% of phosphorus was somewhat subnormal. This was probably due to a calcium deficiency resulting from the excessive intake of phosphate. When the diet contained 2.5% of calcium, 1.7% of phosphorus and added magnesium (last line of table 1), the rate of gain was near normal for a purified diet of this type.

In the absence of supplementary magnesium, the detrimental effect of phosphorus was in direct proportion to its concentration at all levels of calcium. No animal survived as long as 8 weeks when the diet contained 1.7% of phosphorus, and most survived less than 4 weeks. However, in the presence of adequate magnesium, excess phosphorus had little injurious effect. With an elevated level of phosphorus, for example 0.8%, and no supplementary magnesium, increasing levels of calcium depressed the rate of gain and increased mortality. Thus, it seems clear that the injurious effect of excessive phosphorus is not the result simply of an adverse calcium to phosphorus ratio. It is true that in the presence of adequate magnesium, the high calcium to phosphorus ratio improved the gain slightly; but in the absence of magnesium, phosphorus was detrimental in proportion to its concentration regardless of the calcium content.

As has been reported for other species, excess calcium accentuated symptoms of a magnesium deficiency. When dietary phosphorus level was maintained at 0.4% and calcium was increased from 0.9 to 2.5% without added magnesium, the average daily gain decreased from 3.0 to 1.9 gm. In the presence of magnesium, excess calcium was only slightly detrimental. Surprisingly enough, the diet containing an excessively high mineral content, 2.5% of calcium and 1.7% of phosphorus, supported a near maximal rate of

[3] Solka Floc., Brown Co., Berlin, N. H.

[4] The salts mixture which is a modification of the mix of Hubbell et al. ('37), supplied, as per cent of the diet: $CaCO_3$, 2.1; KH_2PO_4, 0.82; KCl, 0.44; NaCl, 0.28; $MnSO_4 \cdot H_2O$, 0.076; $FePO_4 \cdot 4H_2O$, 0.082; $CuSO_4 \cdot 5H_2O$, 0.006; NaF, 0.004; KI, 0.003; $ZnSO_4 \cdot 7H_2O$, 0.0025; $CoCl_2 \cdot 6H_2O$, 0.0003; $AlKSO_4 \cdot 12H_2O$, 0.0007.

[5] The biotin was kindly supplied by Hoffman-La Roche, Inc., Nutley, N. J.; the folacin and aureomycin by the Lederle Laboratories Division of American Cyanamid Co., Pearl River, N. Y.; and the other vitamins by Merck and Co., Rahway, N. J.

TABLE 1

Effect of dietary calcium and phosphorus levels on the requirement of guinea pigs for magnesium

Calculated dietary		No magnesium added				0.3% Magnesium added			
P	Ca	No.[1]	Daily gain[2]	Mortality[3]	Deposits[4]	No.	Daily gain[2]	Mortality[3]	Deposits[4]
%	%		*gm*	%	%		*gm*	%	%
0.4	0.9	14	3.0(11)[5]	50	29	22	5.7(19)	18	0
0.8	0.9	12	0.8(6)	100	83	8	6.0(8)	0	0
1.7	0.9	16	—(0)	100	0	12	3.7(12)	0	0
0.4	1.7	8	3.0(5)	100	0	8	6.0(8)	12	0
0.8	1.7	6	0.2(1)	100	83	6	5.8(6)	0	0
1.7	1.7	7	—(0)	100	57	7	4.7(7)	0	0
0.4	2.5	12	1.9(2)	100	8	7	5.0(7)	14	0
0.8	2.5	8	—(0)	100	25	12	4.8(12)	0	0
1.7	2.5	12	—(0)	100	33	12	5.0(12)	0	0

[1] Number of animals started.

[2] Daily gain for an 8-week period.

[3] Mortality during a 12-week period.

[4] Deposits visible upon autopsy at time of death or at end of 12 weeks.

[5] Numbers in parentheses indicate number of animals surviving at 8 weeks.

gain. We wish to emphasize, however, that, in the absence of magnesium, these calcium and phosphorus levels caused the earliest mortality and lowest rate of gain. The high levels did not increase the incidence of visible deposits, a result that was probably due to early mortality. Apparently, then, when adequate magnesium is available, a guinea pig can tolerate reasonably well, calcium to phosphorus ratios ranging from 0.5 to 6.0.

After excess phosphorus was observed to accentuate magnesium deficiency in the guinea pig, an attempt was made to confirm the results with rats. The data, summarized in table 2, are analogous to those obtained with the guinea pig although the effects are somewhat less marked. Increasing the calcium from 0.9 to 1.7% did not depress growth nor accentuate deficiency symptoms. On the other hand, when the diet contained 1.7% of phosphorus and no magnesium, 75% of the animals died within 4 weeks and the survivors gained at the rate of 4 gm per week. Supplementation with magnesium supported a near maximal growth rate. All of the magnesium-deficient animals showed hyperemia within 5 or 6 days and later developed skin lesions which were quite severe in animals on the high phosphorus diet. These animals were highly irritable and some died after a severe convulsion.

After it was apparent that the phosphorus intake of an animal has a direct influence on its magnesium requirement, it seemed worthwhile to make a quantitative estimation of this effect on the requirement. Guinea pigs were fed graded levels of magnesium with 0.9% of calcium and two phosphorus levels, 0.4 and 1.7%. Approximately 12 animals were started on each magnesium level and the results obtained are presented in figure 1. The average daily gains for a 12-week period are plotted against the logarithm of the dietary magnesium level. The mortality and incidence of visible deposits for each diet are shown below the data points. The rate of gain increased with increasing levels of magnesium up to a point and then leveled off. The point of intersection of the ascending line and the horizontal line is considered to be the minimal magnesium requirement for growth of the guinea pig. When the diets contained 0.4% of phosphorus the point of intersection was at about 80 mg of magnesium per 100 gm of diet. Furthermore, the diet which contained 90 mg % of magnesium completely prevented mortality and visible deposits.

As previously mentioned, the high level of phosphorus depressed the rate of gain in the presence of adequate magnesium. Although higher levels of calcium would no doubt improve the rate of gain, it would probably also increase the minimum magnesium requirement. The curves presented are believed to give a valid estimate of the effect of phosphorus on magnesium requirement. The minimal amount of magnesium required to support a maximal growth rate in the presence of 1.7% of phosphorus, as determined from the graph in figure 1, is about 240 mg %. Thus, the high level of phosphorus appears to increase the magnesium requirement about three-fold.

Another criterion of the magnesium requirement chosen for study was the magnesium content of the blood serum. The calcium and magnesium content of serum

TABLE 2

Effect of dietary calcium and phosphorus levels on the requirement of rats for magnesium[1]

Calculated dietary		No magnesium added		0.3% Magnesium added	
P	Ca	Weekly gain[2]	Mortality	Weekly gain[2]	Mortality
%	%	*gm*	%	*gm*	%
0.4	0.9	19	12	32	0
0.8	0.9	17	12	31	0
1.7	0.9	4	75	29	0
0.4	1.7	19	8	32	0

[1] Sixteen animals were used in each group except in the two groups on 1.7% calcium in which 12 rats were used.

[2] The weekly gain was for a 4-week period and was based on survivors only.

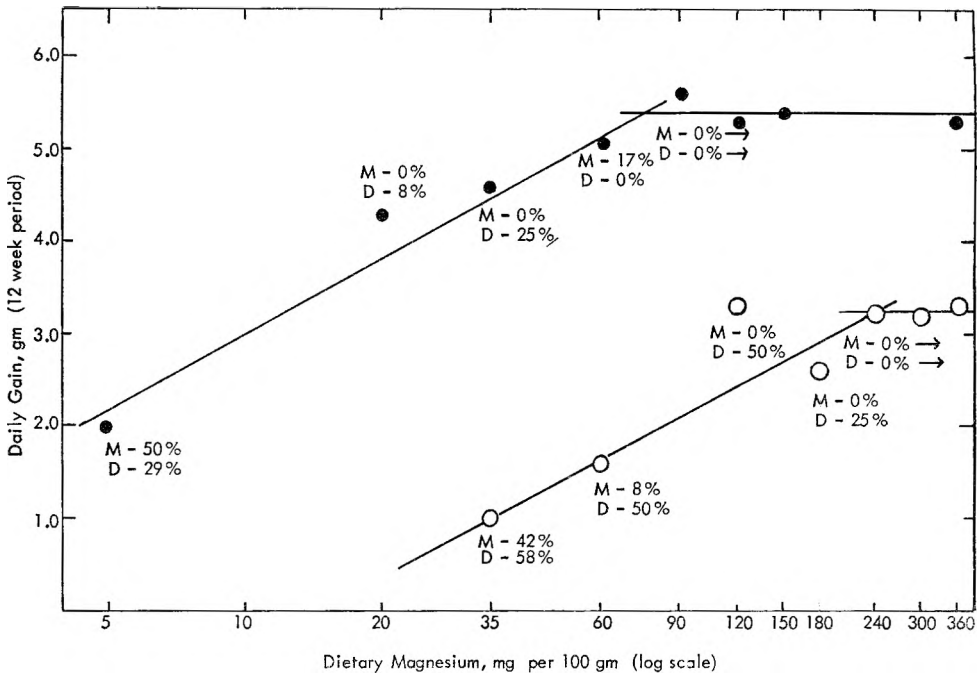

Fig. 1 Effect of dietary phosphorus level on the magnesium requirement of guinea pigs. Twelve animals were started on each diet and none survived on the basal diet containing 1.7% of phosphorus. The closed circles refer to 0.4% and the open circles to 1.7% of dietary phosphorus. Under the data points M refers to mortality and D to incidence of visible deposits upon autopsy.

from guinea pigs fed various levels of magnesium, 0.9% of calcium and the two levels of phosphorus are shown in table 3. Since no animals survived for 12 weeks when fed 1.7% of phosphorus and less than 35 mg% of magnesium, no serum values were obtained at the lower magnesium levels. Only at the 5 mg% level was the serum magnesium markedly below normal. However, a tendency was shown for it to rise gradually on the 0.4% phosphorus regime up to the 90 mg% level. This would tend to confirm the growth data that 90 mg% is the minimal magnesium requirement under the conditions imposed. Nevertheless, maximal serum magnesium (about 5 mg%) was not attained even at 180 mg% of dietary magnesium. In the case of the animals on 1.7% of phosphorus a maximal value was attained at a dietary level of 300 mg%. No explanation can be offered for the high value obtained for the 4 animals which received 120 mg% of magnesium, but this is probably because of the small sample size. Magnesium content of the diet had no effect on the calcium content of the serum.

B. Symptoms of a magnesium deficiency in the guinea pig. The deficiency symptoms in the guinea pig have not been clearly defined, although it seems probable that at least part of the syndrome described by Wulzen and Bahrs ('36) was the result of a magnesium deficiency. Maynard et al. ('58) described some of the symptoms of a low magnesium intake in the guinea pig. Under our laboratory conditions, the most striking symptoms are retarded growth and metastatic calcification. An identical calcinosis picture was described by Hogan et al. ('50). Gross damage occurs to the kidneys which become enlarged and grayish in color. Microscopically the kidneys and muscles exhibit marked degeneration and infiltration with mineral. The guinea pig does not show the typical symptoms of hyperemia, epidermal lesions and hyperirritability exhibited by the rat. Only occasionally have

TABLE 3

Effect of dietary magnesium on serum calcium and magnesium concentration[1] in guinea pigs

Dietary magnesium	0.4% Phosphorus		1.7% Phosphorus	
	Serum calcium	Serum magnesium	Serum calcium	Serum magnesium
mg/100 gm	*mg %*	*mg %*	*mg %*	*mg %*
5	11.0(11)[2]	2.1	—	—
20	10.6(7)	3.5	—	—
35	10.2(12)	3.7	11.0(5)	3.7
60	11.8(6)	3.9	10.9(6)	3.8
90	12.0(6)	4.5	—	—
120	11.2(7)	4.2	10.8(4)	5.5
150	12.5(4)	4.3	—	—
180	11.3(4)	4.2	10.7(4)	4.3
240	—	—	12.4(6)	4.6
300	—	—	12.1(6)	5.3
360	11.0(11)	4.9	11.6(12)	5.0

[1] Analyses were made at end of 12-week experimental period.
[2] Numbers in parentheses indicate the number of samples analyzed.

symptoms of nervous disorder been observed. In all of our experience, only once or twice have animals gone into a convulsive state after being handled.

In the severely deficient animal, abnormal gait and posture are the most obvious gross symptoms. This appears to be the result of a muscle stiffness or dysfunction. Figure 3 in plate 1 shows the typical stance of a magnesium-deficient guinea pig compared with a normal animal (fig. 2). Note that the hind limbs are not drawn up under the body in the normal manner. Such animals are reluctant to move, and in severe cases, cannot right themselves when placed on their sides. Administration of magnesium salts to such animals for a period of 7 to 10 days allows them to regain normal control of their limbs.

Another rather striking symptom of magnesium deficiency in the guinea pig is defective teeth, particularly noted in the incisors as erosion, darkening and decay. Finally, the incisors become so soft that they spontaneously break off at the gum line or can be removed easily with the fingers. Figure 5 shows severely damaged incisors which may be compared with a control in figure 4. Figure 6 shows a photograph of an eroded and broken incisor compared with a control. This syndrome does not occur in every animal fed a diet low in magnesium but a high percentage of the deficient animals which survive for three months or longer show some incisor damage. Except for the molars often becoming coated with a dark pigmented calculus, these are seldom affected. Figure 7 shows the lower mandibles from a deficient (upper) and a control animal. The deficient, damaged mandible also shows exostosis resulting from excessive growth of the roots of the molars. In addition, an overgrowth of molars appears typically so that in some cases the animals cannot eat well. Exostosis on the upper mandible commonly causes severely affected animals to appear "pop-eyed" because the eye is forced from its socket.

DISCUSSION

In a review by Duckworth ('39), he concluded, in part, that "A principal effect of magnesium deficiency appears to be a disturbance of normal calcium metabolism." We agree that a magnesium deficiency commonly causes deposition of calcium in soft tissues and may perhaps accelerate its removal or erosion from osseous and dental tissues. However, from recent observations it appears just as correct, if not more valid, to conclude that the principal effect of magnesium deficiency is a disturbance of phosphorus metabolism. Presumably, calcium is deposited in soft tissues as a phosphate. The serum calcium content is not changed by magnesium deficiency, but the serum phosphorus is elevated (O'Dell et al., '56; Maynard et al., '58). Although excess calcium accentuates the symptoms of magnesium

deficiency, the effect of excess phosphorus is more striking. When excess phosphorus is consumed, it is highly important that adequate magnesium be available to aid in its metabolism and to prevent its accumulation in the blood and soft tissues.

Magnesium deficiency symptoms in the rat have been adequately described by several investigators (Kruse, Orent and McCollum, '32; Tufts and Greenberg, '38). The syndrome includes: first, vasodilation, particularly evident in the ears, tail and feet; later, skin lesions and loss of hair; and finally, a nervous disorder which may end in convulsions and death. Magnesium deficiency in the calf (Moore et al., '36) and in the cotton rat (Constant and Phillips, '52) is shown most strikingly as a calcinosis. Deficiency symptoms in the guinea pig are quite similar to those observed in the latter species. Stiffness of the hind limbs observed in severely deficient animals is similar to the syndrome described by Wulzen and Bahrs ('36) but should not be confused with wrist-stiffness which these authors described later ('41), because this does not respond to magnesium.

It is significant, if somewhat anomalous, that the teeth of magnesium-deficient guinea pigs become eroded and weakened, presumably due to loss of mineral, whereas at the same time calcium phosphate accumulates in soft tissue. These facts point to the need for a better understanding of the function of magnesium in calcium and phosphorus metabolism.

SUMMARY

Weanling guinea pigs and rats were fed diets deficient in magnesium and of variable calcium and phosphorus content. The growth rate, incidence of visible calcium phosphate deposits, mortality and other symptoms of magnesium deficiency were noted.

Excessive intake of phosphorus was observed to accentuate the symptoms of magnesium deficiency. The injurious effect of phosphorus, when added to a diet low in magnesium, was more marked than that of calcium but the effect was largely eliminated by adequate magnesium. When the diet contained 0.9% of calcium and 0.4% of phosphorus the magnesium requirement was determined to be 80 mg per 100 gm of diet. When phosphorus was elevated to 1.7%, the requirement rose to 240 mg.

The symptoms of magnesium deficiency in the guinea pig include slow growth, soft-tissue calcification, stiffness in the hind limbs, exostosis of the mandibles, overgrowth of the molars, and erosion, softening and decay of the incisors.

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PLATE 1

EXPLANATION OF FIGURES

2 Guinea pig, 14 weeks old, reared on a stock colony diet. Note normal stance with hind limbs drawn well under body.

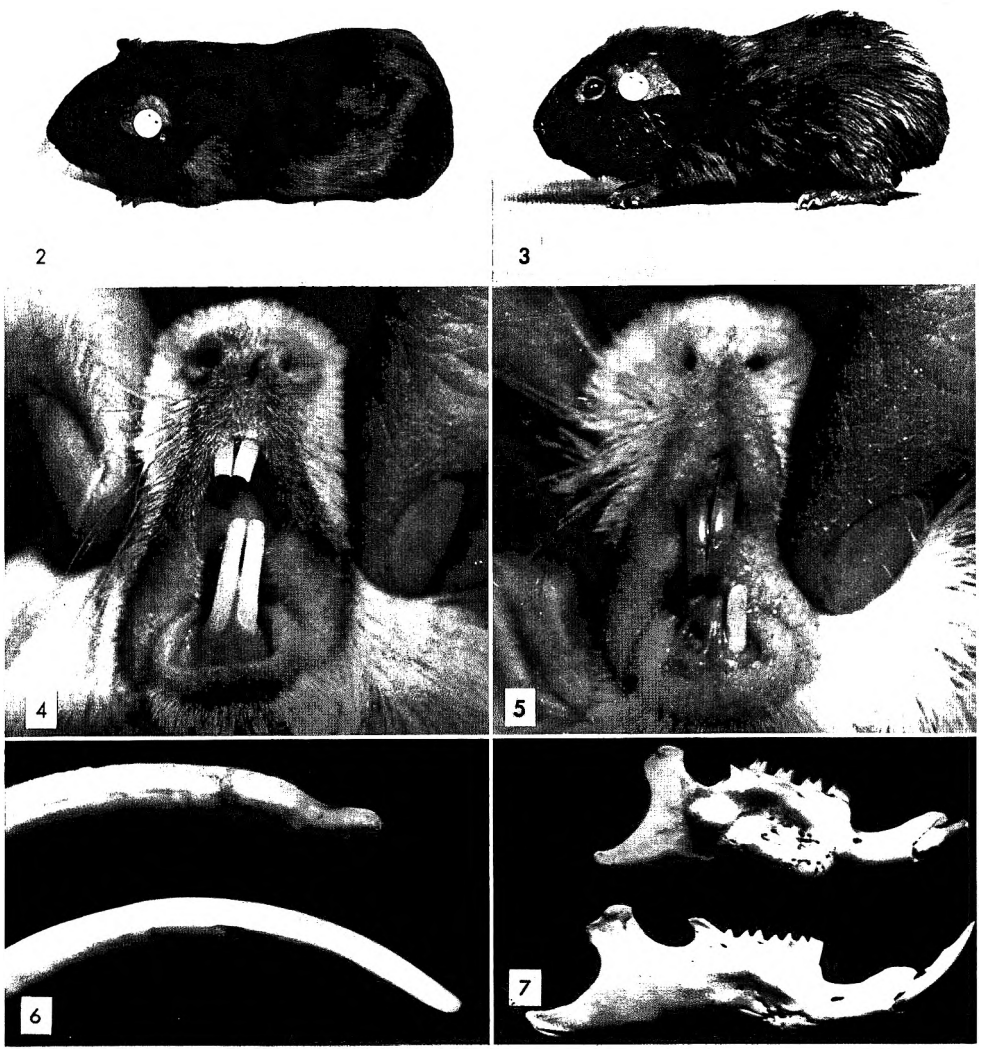
3 Magnesium-deficient guinea pig, 14 weeks old, receiving a diet which contained 0.9% of calcium, 1.7% of phosphorus and about 0.1% of magnesium. Note abnormal stance indicative of stiffness in hind limbs. If forced to move, the animals tended to hop like a rabbit. Treatment with magnesium allowed the animal to assume a normal stance.

4 Incisors of a control animal, 14 weeks old, receiving 0.9% of calcium, 0.4% of phosphorus and 0.3% of magnesium.

5 Incisors of a magnesium deficient animal, 28 weeks old, receiving 0.9% calcium, 0.4% of phosphorus and no supplemental magnesium. Note that incisors are dark in color, eroded and broken. Molars were covered with a brown pigmented calculus. At autopsy this animal showed a large number of visible deposits.

6 The upper incisor extracted from magnesium-deficient guinea pig, 14 weeks old which had received 0.9% of calcium, 0.4% of phosphorus and no supplemental magnesium. The control incisor in the lower photograph came from an animal which received 1.7% of calcium, 0.8% of phosphorus and 0.3% of magnesium.

7 Lower mandibles from a guinea pig, 14 weeks old. The one in the upper half of the photograph came from the same magnesium-deficient animal and the lower one from the control animal described in figure 6.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Evaluation of Protein in Foods

V. FACTORS INFLUENCING THE PROTEIN EFFICIENCY RATIO OF FOODS

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Derse ('58) and Chapman et al. ('59) have recently described methods for determination of the protein efficiency ratio of foods (PER) under standardized conditions. Despite the widespread use of this method, little is known about factors which influence PER. Barnes et al. ('45) reported, in confirmation of the original findings of Osborne et al. ('19), that PER values varied with the level of dietary protein. The maximal PER values for animal proteins were found at lower dietary levels than those for cereal proteins. Hegsted and Worcester ('47) and Sherwood and Weldon ('53) concluded that in studies on the relative value of various proteins, using growing rats fed diets of constant protein content ad libitum, there was no advantage to using PER in place of gain in body weight. Bender ('56) noted that PER was closely correlated with food intake, falling when food consumption was reduced. Sure ('55) observed that PER values found after 10 weeks on experiment were lower than those found after 4 weeks. The decrease was greater for dried whole egg and dried skim milk than for cereal proteins.

The present studies were conducted to determine the effects of protein level, duration of the experimental period and the sex of the rats used, on PER values found with casein or a mixture of plant proteins. The effects on PER values of differences between strains of rats were also tested.

EXPERIMENTAL

The rats used in the experiments reported herein were individually housed in screen bottom cages kept in an air conditioned room maintained at 74 to 76°F. Food and water were supplied ad libitum, records kept of the amount of food consumed by each rat, and the animals weighed individually at weekly intervals. Experiment 1 was 10 weeks in duration, whereas experiment 2 was 4 weeks in duration. At the end of the experiments, the animals were fasted for 24 hours, killed by ether anesthesia, exsanguinated and the livers removed and weighed. Also, the adrenal glands of the animals in experiment 1 were removed and weighed. Liver lipid levels were determined by the method of Folch et al. ('57) and were expressed on a fresh-weight basis.

In experiment 1, groups of 10 male and 10 female weanling rats (20 to 23 days old), of an inbred Wistar strain (strain A), received otherwise adequate diets containing 7, 10 or 15% of protein (N × 6.25) supplied by casein or by a mixture containing 85% of whole wheat flour and 15% of soybean flour. The basal protein-free diet used was that of Chapman et al. ('59), which contained in per cent: corn starch, 80; corn oil, 10; non-nutritive cellulose, 5; salts, 4; and vitamin mixture, 1. The protein-containing materials were added to this diet at the expense of cornstarch, to give the desired protein levels. Dietary fat levels were kept constant at 10% by adjustment of the amount of corn oil added.

Experiment 2 was conducted to determine PER values for casein, dried whole egg, the mixture of whole wheat and soybean flours, or whole wheat flour, using two additional strains of rats. The foods tested were added to the protein-free diet of Chapman et al., ('59) at the 10% protein level (N × 6.25), and fed to groups of 10 male weanling rats of WF strain (strain B), or the Charles River strain

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(strain C). As in experiment 1, the level of fat in the diet was kept constant at 10%. The PER values found after 4 weeks were compared with those obtained previously in this laboratory, using rats of strain A.

The data of the two experiments were subjected to appropriate statistical analysis by the methods outlined by Snedecor ('55).

RESULTS AND DISCUSSION

Data on weight gain, food consumption, and PER values found at 4 and 10 weeks with the male rats in experiment 1 are summarized in table 1. PER values obtained during the experiment are shown in figure 1. In 4 weeks, the animals which received 7, 10 or 15% casein protein (diets 1 to 3) gained 50, 80 and 105 gm respectively, whereas those which received comparable diets containing the plant protein (diets 4 to 6) had significantly reduced weight gains of 33, 56 and 88 gm, respectively ($P < 0.01$ by analysis of variance and application of Duncan's multiple range test). At 10 weeks, weight gain found with casein was still significantly greater than that found with comparable levels of plant protein.

With the exceptions of the values for 7 and 10% plant protein, PER values decreased significantly during the experiment. At 4 weeks, the value found with 10% casein protein (diet 2) was 2.75, whereas by 10 weeks, the value had dropped to 2.12. In contrast, the value for 10% plant protein (diet 5) dropped only slightly, from 2.09 at 4 weeks to 1.98 at 10 weeks. Sure ('55) also noted that PER values for plant proteins were less influenced by the length of the experimental period than were those for good quality animal proteins.

At 4 weeks, 7 and 10% casein protein gave comparable PER values of 2.82 and 2.75, respectively. By 10 weeks, however, the PER value for 10% casein protein was significantly less than that for 7% casein protein (2.12 vs. 2.38). The value for 15% casein protein was significantly less at both 4 and 10 weeks than those for the two lower levels of casein tested.

At 4 weeks, PER values with 10 or 15% plant protein (diets 5 and 6) were similar and significantly greater than those found with 7% plant protein (diet 4). By 10 weeks, however, the value for 15% plant protein (1.58) was significantly less than

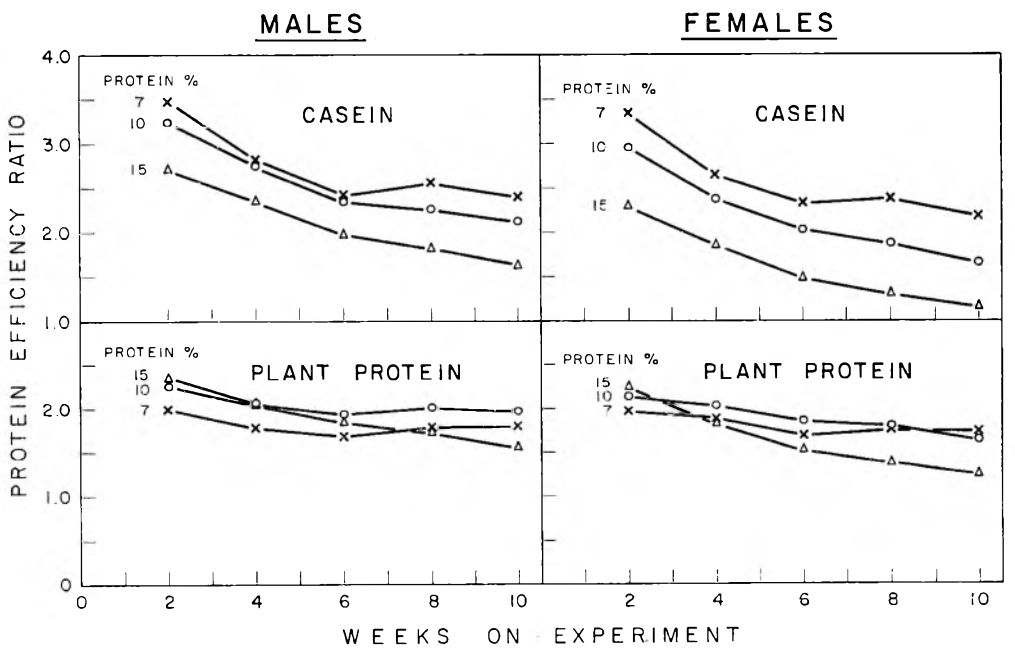

Fig. 1 PER values (gm gain/gm protein intake) for male and female weanling rats fed diets containing casein or plant protein for 10 weeks.

TABLE 1

Effect of quality and quantity of dietary protein on weight gains and protein efficiency ratios[1] found with growing male rats

	Casein			Plant protein		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Diet protein, %	7	10	15	7	10	15
Initial weight, gm	42	42	42	42	42	42
4 weeks						
Weight gain, gm	50 ± 6[2]	80 ± 8	105 ± 11	33 ± 2	56 ± 1	88 ± 5
Food consumption, gm	241	269	282	243	279	305
PER	2.82 ± 0.22	2.75 ± 0.23	2.37 ± 0.10	1.79 ± 0.07	2.09 ± 0.12	2.07 ± 0.06
10 weeks						
Weight gain, gm	110 ± 14	183 ± 16	211 ± 20	95 ± 13	155 ± 14	191 ± 11
Food consumption, gm	682	799	828	688	804	868
PER	2.38 ± 0.13	2.12 ± 0.12	1.62 ± 0.05	1.81 ± 0.08	1.98 ± 0.05	1.58 ± 0.04

[1] Grams gain in weight per gram of protein consumed.
[2] Standard deviation.

TABLE 2

Effect of quality and quantity of dietary protein on weight gains and protein efficiency ratios[1] found with growing female rats

	Casein			Plant protein		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Diet protein, %	7	10	15	7	10	15
Initial weight,gm	39	39	39	39	38	39
4 weeks						
Weight gain, gm	43 ± 6[2]	59 ± 9	67 ± 8	33 ± 6	49 ± 4	66 ± 6
Food consumption, gm	221	231	230	234	250	254
PER	2.65 ± 0.17	2.37 ± 0.16	1.86 ± 0.13	1.86 ± 0.21	2.02 ± 0.04	1.85 ± 0.08
10 weeks						
Weight gain, gm	98 ± 10	114 ± 12	114 ± 8	87 ± 7	106 ± 11	119 ± 13
Food consumption, gm	619	639	624	661	661	687
PER	2.17 ± 0.09	1.66 ± 0.09	1.17 ± 0.04	1.73 ± 0.06	1.65 ± 0.10	1.25 ± 0.07

[1] Grams gain in weight per gram of protein consumed.
[2] Standard deviation.

those for 10% plant protein (1.98) or 7% plant protein (1.81).

Data on weight gain, food consumption and protein efficiency values found at 4 and 10 weeks with the female rats in experiment 1 are summarized in table 2. Curves of PER values obtained during the experiment are shown in figure 1. As might be expected, weight gain was much lower with the females than with the males. In contrast to results with the males, in the females weight gain with 15% casein protein was not significantly greater at 4 weeks than with 15% plant protein, nor was weight gain with 7 and 10% casein protein significantly greater at 10 weeks than with comparable levels of plant protein.

Protein efficiency ratio values were also influenced by the sex of the test animal. At 4 weeks, the females utilized 15% casein protein much less efficiently than the males, probably, in part, because of their slower growth rate. With females PER values found for casein declined during the experiment, in agreement with results obtained in the males. Although the value obtained with the males for 10% plant protein remained relatively constant during the experiment (fig. 1), that obtained with the females dropped significantly from 2.02 at 4 weeks to 1.65 at 10 weeks. In further contrast to the findings with the males, 7% casein protein gave a significantly higher PER value at 4 weeks with the females than 10% casein protein. Furthermore, with the females, PER values found at 4 weeks with 7 or 15% plant protein were similar and lower than those found at the 10% level. At 10 weeks, 7 and 10% plant protein gave similar PER values with the females, whereas with the males, 10% plant protein gave a higher PER value than 7%.

The results of the studies showed that PER values varied not only with quality and quantity of the dietary protein, but also with the duration of the experiment and the sex of the test animal. These factors must be standardized if comparable results are to be obtained in different laboratories. Fixsen ('35) reported that a long experimental period was necessary for accurate determination of PER values. She concluded that short experiments gave higher values than long ones, and recommended that experiments be at least 60 days in duration. However, Mitchell ('24) pointed out that as "the composition of the gains put on by growing animals progressively changes with age . . . the best way of assuring the desired equality in the composition of gains . . . is to use animals of the same age, weight and previous treatment and to conduct the experiment for as short periods of time as may be required for an accurate measure of the actual gain in organized tissue." Thus, it would seem preferable to conduct PER experiments for shorter periods of time than, for example, the 10-week period reported by Sure ('57). Not only do differences between proteins tend to be obscured after 10 weeks, but also the order in which proteins are ranked as to quality may be markedly different at 10 than at 4 weeks. Furthermore, as Mitchell ('24) and Bender ('56) have pointed out, marked differences may exist in the composition of weight gains obtained in prolonged experiments with proteins of greatly differing nutritive value. In any case, the results of the present studies indicate that PER values determined under different conditions are not comparable.

The results presented herein show that female rats tend to give maximal PER values at lower dietary protein levels than males, suggesting a possible sex difference in protein requirement. Jones ('51) obtained evidence indicating that female rats had lower protein requirements than males, and Shelton et al. ('51) presented data suggesting that the lysine requirement was higher in male weanling pigs than in female.

Although Hegsted and Worcester ('47) and Sherwood and Weldon ('53) reported that calculation of PER was of no advantage over measurement of weight gain alone, the results of Chapman et al. ('59) and of the present studies indicate the existence, in most cases, of lower coefficients of variation for PER values than for corresponding weight gains.

The data on organ weights and liver lipid levels obtained in experiment 1 (table 3) showed that liver weight per gram of body weight was greater in females than in males, but was not influenced by the

TABLE 3

Organ weights and liver lipid levels in male and female rats fed diets containing various levels of protein supplied by casein or plant protein for 10 weeks

Diet no.	Diet protein	Males					Females				
		Liver		Adrenals		Liver lipids	Liver		Adrenals		Liver lipids
	%	*gm*	*% body wt.*	*mg*	*mg/100 gm body wt.*	%	*gm*	*% body wt.*	*mg*	*mg/100 gm body wt.*	%
Casein											
1	7	4.2	2.8 ± 0.3[1]	27.2	18.6 ± 3.3	6.30 ± 0.76	4.0	3.1 ± 0.3	33.9	26.3 ± 4.1	6.40 ± 0.97
2	10	5.5	2.6 ± 0.1	29.9	14.2 ± 0.8	6.77 ± 0.45	4.1	2.9 ± 0.2	37.4	26.0 ± 2.3	6.11 ± 0.65
3	15	6.3	2.6 ± 0.2	35.7	15.1 ± 1.3	7.48 ± 0.64	4.5	3.1 ± 0.4	38.9	27.0 ± 3.6	6.23 ± 1.07
Plant protein											
4	7	3.6	2.8 ± 0.3	20.2	16.1 ± 1.6	7.58 ± 0.91	3.8	3.3 ± 0.6	33.2	28.3 ± 4.4	6.37 ± 1.19
5	10	4.8	2.7 ± 0.3	28.6	15.8 ± 1.2	6.67 ± 2.87	4.0	3.0 ± 0.4	36.1	27.2 ± 4.1	5.55 ± 0.40
6	15	6.3	2.9 ± 0.2	34.8	16.2 ± 1.4	5.86 ± 0.29	4.5	3.1 ± 0.6	46.8	31.8 ± 3.0	5.57 ± 0.61

[1] Standard deviation.

quality or quantity of the dietary protein. Adrenal weights were larger, per unit of body weight, in the males fed diet 1, containing 7% of casein protein, than in those fed the other diets. The females fed 15% of plant protein (diet 6) had somewhat larger adrenal glands per unit of body weight than those fed the remaining diets. Liver lipid levels were not significantly influenced by the sex of the animals nor by the quality or quantity of dietary protein.

The results of experiment 2, in which PER values for 4 different foods were determined with rats of strains B and C, are presented in table 4, along with results obtained previously in this laboratory with rats of strain A. In 10 experiments, rats of strain A gave an average PER for casein of 2.59 (range 2.41 to 2.75), whereas, in experiment 2, rats of strains B and C gave PER values for casein of 3.35 and 3.18, respectively. The values found for whole egg, the wheat flour–soybean flour mixture, or whole wheat flour, were also significantly lower when strain A rats were used than when strain B or strain C rats were used. The apparent genetic effects on PER observed in the present study bring to mind the extensive work of L. S. Palmer et al. ('46) on genetic differences in food utilization by rats. Many other workers have also observed strain or breed differences in the nutritional requirements of animals (Hutt, '58).

Liver lipid levels found in experiment 2 did not differ significantly from those found previously in this laboratory with rats of strain A.

In the method for determination of PER outlined by Chapman et al. ('59), PER values obtained were corrected for differences between assays by multiplying the value found by the fraction 2.5/determined PER of reference standard casein. Application of this correction factor to the data obtained with rats of strains B and C in experiment 2 gave the following corrected PER values: whole egg, 2.99 and 3.14; the mixture of whole wheat and soybean flours, 2.00 and 2.01; whole wheat flour, 1.07 and 1.18, respectively. The corrected values for the plant proteins agree well with those found for strain A rats; whereas the values for egg protein appear to be over-corrected. These results suggest that variation in PER values found in different assays can be largely resolved by use of a standard method which contains a "built-in" reference standard.

Because of the many factors which influence the PER method for protein evaluation, it would appear that simplified *in vitro* procedures may be of value in routine screening of foods for protein quality. The pepsin-digest-residue amino acid index of Sheffner et al. ('56) and the simplified chemical score proposed by McLaughlan et al. ('59) are of special interest in this respect. Any *in vitro* method, however, must correlate closely with biological results, and it seems clear, as Friedman ('58) and others have pointed out, that such methods cannot at present take the place of a biological evaluation.

TABLE 4

Effect of strain of rat on protein efficiency ratios obtained with different foods

Food	PER values found with				
	Strain A	Strain B		Strain C	
		uncorrected	*corrected*[1]	*uncorrected*	*corrected*
Casein	2.59(10)[2]	3.35(1)	—	3.18(1)	—
Dried whole egg	3.44(9)	4.00(1)	2.99	4.00(1)	3.14
Wheat flour—soybean flour mixture	2.09(1)	2.64(1)	2.00	2.56(1)	2.01
Whole wheat flour	1.17(1)	1.44(1)	1.07	1.50(1)	1.18

[1] Corrected values were obtained by multiplying the determined PER value by the fraction 2.5/determined PER of reference standard casein.

[2] Figures within parentheses indicate number of experiments in which 10 male weanling rats received each food at the 10% protein level for 4 weeks.

SUMMARY

In studies on factors influencing the protein efficiency ratio (PER) of foods, groups of male and female weanling rats received otherwise adequate diets containing 7, 10 or 15% protein supplied by casein or by a mixture of plant proteins (whole wheat and soybean flours). PER values (grams of gain per gram of protein consumed) were calculated at weekly intervals for 10 weeks. Female rats tended to give maximal PER values at lower dietary protein levels than did males. PER values found with both sexes dropped as the experiment progressed, but the decline was much greater with the animals fed casein than with those fed the plant protein mixture. In both sexes, the protein level required for maximal PER tended to decline as the experiment progressed. Differences between casein and the plant protein mixture were greatest during the early stages of the experiment, in both sexes. In further studies, differences in PER values found with several strains of rats were largely eliminated by the use of casein as an internal standard. It is concluded that the factors studied must be standardized if comparable results are to be obtained in different laboratories.

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The Influence of Dietary Fats on Serum Cholesterol Levels in Cholesterol-Fed Chicks[1]

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Previous papers on the cholesterol and cholic acid fed rat have shown that the kind of dietary fat determines the serum cholesterol levels, other things being constant; also that the mean serum cholesterol level is correlated with the extent of the atheromatous lesions produced (Hegsted et al., '57a). From the study of numerous dietary fats and fat mixtures (Hegsted et al., '57b), as well as different fats fed at different levels (Hegsted et al., '59), it appears that in the rat both the fully saturated fatty acids and the essential fatty acid, linoleic acid, act synergistically to depress the serum cholesterol level. When the total fat content of the diet was held constant, the product obtained by multiplying the saturated fatty acid content by the linoleic acid content had a high negative correlation with the serum cholesterol level (Hegsted et al., '57b). When the amount, as well as the composition of the dietary fat is varied, the situation is much more complicated, since each fat does not affect the serum level proportionately at different dietary levels. Nevertheless, the linoleic-saturated acid product provides a parameter of an equation which fits the data on the serum cholesterol levels at least as well as other equations which were developed (Hegsted et al., '59).

The data from the rat studies may have some application to man. A mixture of equal amounts of coconut oil (mostly saturated fatty acids) and safflower oil (70% linoleic acid)—one of the most effective mixtures found in the rat studies—was as effective as safflower oil alone in lowering serum cholesterol in hypercholesterolemic men when fed as 40% of calories in a formula diet (Hashim et al., '59). On the other hand, some of the fats which promote the highest serum cholesterol values in rats are those very high in oleic acid, such as olive oil or a commercially prepared "triolein" which was 80% oleic acid. No data suggest that these fats are comparably hypercholesterogenic in man. On the contrary, olive oil may depress serum cholesterol levels in man (Ahrens et al., '57). Keys et al. ('57, '58) have concluded that monounsaturated fatty acids have a negligible effect on serum cholesterol.

This paper reports work upon the effect of different oils upon the serum cholesterol levels in chicks fed cholesterol. The relationship between fat composition and serum cholesterol level is quite distinct from that found in rats and appears to be similar to those reported in man by Keys et al. ('57) and Ahrens et al. ('57).

EXPERIMENTAL

The chicks, received from the hatchery as day-old cockerels, were fed a commercial starter mash for three to 7 days before being divided into comparable groups upon a weight basis, banded and given the experimental diets. All of the studies were terminated after three experiemntal weeks since our facilities do not readily permit work with larger birds. Groups of 6 chicks were housed together in cages with raised screen bottoms. The experimental diet, allowed ad libitum, had the following percentage composition: sucrose, 52.7; purified casein, 18; salt mixture (Hegsted et

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al., '41), 5; gelatin, 10; celluflour,[2] 3; $CaHPO_4$, 1; cystine, 0.3; choline, 0.3; and 10% of the appropriate oil or fat.[3] Fat soluble vitamins were added as 0.5 gm of halibut liver oil[4] and 50 mg of α-tocopherol. Thiamine, riboflavin, niacin, calcium pantothenate, pyridoxine, biotin, folic acid and menadione were added at levels previously described (Hegsted and Perry, '48).

The birds were bled from the wing vein and cholesterol determined by a microfluormetric method (Carpenter et al., '57).

RESULTS

In experiment 1, 12 groups of White Leghorn cockerels received diets containing 10% of 4 different fats, each with 0.2, 0.4 and 0.8% of dietary cholesterol. After bleeding chicks on the 12th and 14th days, the above values were averaged to give the two-week serum cholesterol level. Chicks were bled again on the 21st day when they were killed. The results are shown in table 1. One can see that, on the average, coconut oil is the most hypercholesterolemic oil and safflower oil the least. The response at different times also depends upon the dietary fat. At all levels of cholesterol, the safflower oil diet caused a decrease in the serum cholesterol between the second and third weeks, whereas the triolein and coconut oil diets resulted in a continued rise. At either two or three weeks, the serum cholesterol level is approximately proportional to the log of the cholesterol dose, but note that the relative position of the different oils is not the same at the different levels of cholesterol. Thus, one can conclude with respect to chicks, as has been found with rats (Hegsted et al., '59), that the serum cholesterol level is dependent not only upon the time, cholesterol level, and kind of dietary fat, but also upon the cholesterol-time-fat interactions. This must be borne in mind since it may limit the general application of any particular experimental setup, such as those used in the following experiments.

Table 2 records the results of a second experiment in which three breeds of cockerels were used, each of which was fed 4 kinds of dietary fat either with or without cholic acid in the diet. All diets contained 0.8% of cholesterol. The effects of the different fats are similar to those already recorded in table 1. Coconut oil again produced the highest serum levels. Safflower oil or equal mixtures of safflower and coconut gave lower and essentially equal serum cholesterol levels. Olive oil

[2] Celluflour. Obtained from Chicago Dietetic Supply House, Chicago.

[3] The authors wish to acknowledge the generous assistance of the Research and Development Department of the Proctor and Gamble Company, Cincinnati, which supplied the oils used and the data upon their composition.

[4] Haliver Oil.

TABLE 1

Serum cholesterol levels in relation to the amount of dietary cholesterol with different kinds of fat

Dietary fat	Dietary cholesterol	Serum cholesterol		
		Second week	Third week	Difference
	%	mg %	mg %	mg %
Triolein	0.2	287	412	+ 125
	0.4	370	523	+ 153
	0.8	444	621	+ 177
Coconut	0.2	301	330	+ 29
	0.4	605	662	+ 57
	0.8	877	1052	+ 175
Safflower	0.2	237	222	− 15
	0.4	335	297	− 38
	0.8	456	343	− 74
Coconut-safflower	0.2	238	278	+ 40
	0.4	428	370	− 50
	0.8	519	560	+ 41

TABLE 2

The influence of breed, dietary fat, and cholic acid on serum cholesterol[1]

	Cholic acid	Time	Serum cholesterol				
			Olive	Coconut	Safflower	Mixture	Mean
		weeks	*mg %*	*mg %*	*mg %*	*mg %*	
Leghorns	—	1	324	567	313	235	360
	—	2	378	604	289	217	372
	—	3	561	647	307	300	454
	Mean		421	606	304	256	395
	+[2]	1	382	714	343	325	441
	+	2	430	875	321	375	500
	+	3	518	984	324	423	562
	Mean		448	856	329	374	501
White Rocks	—	1	283	505	292	253	333
	—	2	306	468	255	232	315
	—	3	419	470	289	309	372
	Mean		335	481	279	248	340
	+[2]	1	326	551	301	293	368
	+	2	387	647	355	301	423
	+	3	478	889	417	327	528
	Mean		389	703	354	309	440
Rhode Island Reds	—	1	318	575	296	201	348
	—	2	345	638	263	180	357
	—	3	453	734	253	271	428
	Mean		372	650	271	232	378
	+[2]	1	422	673	333	335	441
	+	2	459	804	308	388	490
	+	3	566	866	383	418	558
	Mean		486	778	343	378	496
	Grand mean		409	679	313	300	

[1] All diets contained 0.8% of cholesterol.
[2] 0.15% of cholic acid.

(of a composition similar to the "triolein" used in the previous experiment) was intermediate. Differences in the response with time of feeding are evident, the less hypercholesterogenic fats permitting maximum serum cholesterol levels in a short time. The addition of cholic acid to the diet causes some rise in serum cholesterol level as expected, this effect being more pronounced with the more hypercholesterogenic fats, but its inclusion apparently does not change the general relationship of the different fats to the serum cholesterol level. The influence of breed of bird was not great, although the White Rock groups tended to have lower, and the Leghorns somewhat higher, serum cholesterol levels.

Table 3 records the composition of a number of fats and mixtures of fats fed to groups of White Leghorn cockerels in diets containing 0.8% of cholesterol. The serum cholesterol values shown are the means obtained from bleedings at one, two, and three weeks. The zero order correlation coefficients relating serum cholesterol to the three principal fatty acid components where y = serum cholesterol level, S = saturated fatty acid, M = monounsaturated fatty acids, and P = polyunsaturated fatty acids, are as follows:

r_{yS} cholesterol-saturated fatty acid	±0.567
r_{yM} cholesterol-monounsaturated fatty acid	−0.108
r_{yP} cholesterol-polyunsaturated fatty acid	−0.453

The coefficients of correlation between the fatty acid components themselves are r_{SM}, −0.454; r_{SP}, −0.438; and r_{MP}, −0.526. The multiple regression equation including the three variables is:

$$\text{Serum cholesterol} = 1.186\,S - 1.120\,M - 2.230\,P + 622.6 \quad (1)$$

TABLE 3

Serum cholesterol in relation to dietary fat

Experiment 3

Group	Dietary fat	Iodine no.	Fatty acid composition			Mean serum cholesterol
			Sat'd	Mono-unsat'd	Poly-unsat'd	
			%	%	%	mg %
1	10% Triolein	83	7.8	83.2	3.4	554
2	10% Olive	82	12.0	76.6	7.0	620
3	10% Cottonseed	107	31.1	12.5	52.6	585
4	10% Linseed	181	19.6	15.2	64.5	575
5	10% Corn	125	15.2	25.7	58.8	461
6	10% Safflower	145	13.0	7.8	78.5	531
7	10% Coconut	9	91.3	7.0	1.7	927
8	8% Coconut, 2% Corn	32	76.0	10.7	13.1	685
9	5% Coconut, 5% Corn	67	53.3	16.4	30.3	503
10	2% Coconut, 8% Corn	102	30.4	22.0	47.4	550
11	8% Coconut, 2% Safflower	36	75.6	7.2	17.1	477
12	5% Coconut, 5% Safflower	77	52.2	7.4	40.1	550
13	8% Coconut, 2% Safflower	118	26.7	7.6	63.1	448
14	10% Lard	66	37.4	49.1	13.2	588
15	10% Hydrogenated cottonseed	81	20.0	62.7	13.2	510
16	5% Lard, 5% Safflower	100	24.8	28.2	43.5	485
17	5% Lard, 5% Cottonseed	81	34.2	30.7	31.8	655
18	5% Lard, 5% Corn	90	25.9	36.8	33.8	428
19	5% Olive, 5% Coconut	46	50.0	41.6	4.0	576
20	5% Olive, 5% Safflower	113	12.1	42.0	40.8	459

with a multiple coefficient of correlation (R) of 0.61. This value is not much higher than the zero order coefficients, r_{yS} and r_{yP}, and hence, is not patricularly informative. In any event, this treatment is not appropriate. Since S + M + P = 100, the value for any one of the variables can be calculated from the other two, and all of the information is available from equations containing any two of the parameters. Each of the following regression equations fits the data as well as does equation 1.

$$\text{Serum cholesterol} = 2.12S - 1.21P \pm C_1 \quad (2)$$

$$\text{Serum cholesterol} = 3.04S - 0.89M \pm C_2 \quad (3)$$

$$\text{Serum cholesterol} = -3.37P + 2.26M \pm C_3 \quad (4)$$

An inspection of the various regression and correlation coefficients thus fails to give a clear picture of the relative roles of the different types of fatty acids. There is a clear suggestion, however, that the saturated fatty acids (S) tend to elevate the serum cholesterol, the polyunsaturated acids (P) lower it, and the monounsaturated acids (M) are relatively less important. The similarity of equation 2 with that derived from studies upon human subjects by Keys et al. ('57, '59), is worthy of note.

The data also revealed a reasonable correlation between the log serum cholesterol and the log of the iodine number, r = −0.62. The correlation coefficient is practically identical with the multiple coefficients of correlation given above. If two outlying values obtained from the 8% coconut–2% safflower oil and the 5% lard–5% corn oil groups are eliminated, the correlation coefficient becomes −0.72.

The next experiment was similar to the one just described except that oils were chosen which would give a more complete spread of the iodine values. The correlations obtained in the previously described experiment depend heavily upon the coconut oil group. Male Rhode Island Red chicks were used. The composition of the oils and the mean serum cholesterol values obtained by averaging the first, second,

TABLE 4

Serum cholesterol in relation to dietary fat

Experiment 4

Group	Dietary fat		Iodine no.	Fatty acid composition			Mean serum cholesterol
	Coconut oil	Other		Sat'd	Mono.	Poly.	
	%	%		%	%	%	mg %
1	10	—	9	9.13	0.70	0.17	511
2	9	1% Safflower	23	8.35	0.71	0.94	391
3	8	2% Safflower	36	7.56	0.72	1.72	444
4	5	5% Safflower	77	5.22	0.74	4.04	340
5	9	1% Olive	16	8.33	1.45	0.21	497
6	8	2% Olive	24	7.53	2.21	0.25	413
7	5	5% Olive	46	5.14	4.47	0.39	372
8	9	1% Corn	21	8.37	0.89	0.74	295
9	8	2% Corn	32	7.61	1.07	1.31	277
10	5	5% Corn	67	5.33	1.64	3.04	288
11	9	1% Cotton	19	8.49	0.81	0.70	299
12	8	2% Cotton	29	7.85	0.91	1.23	317
13	5	5% Cotton	60	5.94	1.24	2.82	248
14	—	10% Safflower	145	1.30	0.78	7.92	276
15	—	10% Olive	82	1.15	8.24	0.61	381
16	—	10% Corn	125	1.52	2.57	5.91	271
17	—	10% Cotton	111	2.75	1.77	5.48	308
18	—	10% Cocoa butter	39	6.00	3.59	0.41	329
19	—	10% Lard	67	3.74	4.92	1.34	450
20	—	10% Beef tallow	42	5.41	4.37	0.22	357
21	—	10% Butter	38	6.17	3.46	0.37	419
22	—	10% Hydrogenated oil	105	2.47	2.91	4.63	350
23	—	10% Hydrogenated oil	91	2.47	4.51	3.05	327
24	—	10% Hydrogenated oil	70	2.51	6.91	0.58	353

and third week values are shown in table 4.

The relationship between the serum cholesterol values and the iodine number of the dietary fats is shown in figure 1. When the data were plotted in this way, it was immediately apparent that the coconut-corn oil and coconut-cottonseed mixtures (groups 8 through 13, table 4) did not follow the general trend. The addition of only a small amount of corn or cottonseed oil to the coconut oil (groups 8 and 11, table 4) yielded values practically as low as corn and cottonseed oil. These results may be compared with the values obtained with safflower or olive oil mixed with coconut oil which fall progressively with increasing unsaturation. The results obtained in these groups 18 to 13 were thus plotted separately. For the remainder of the oils the coefficient of correlation is −0.82, a reasonably good fit.

Using the results upon the 18 oils which fit the regression line reasonably well, an attempt was made to determine whether the iodine number of the polyunsaturated fatty acid content was primarily responsible for the effect upon the serum cholesterol level. The three coefficients of correlation are:

log serum cholesterol − log iodine number, $r_{yI} = -0.82$

log serum cholesterol − % of polyunsaturated fatty acid, $r_{yP} = -0.74$

log iodine number − % of polyunsaturated acid, $r_{yP} = \pm 0.75$

The coefficients of partial correlation are: $r_{yI.P} = -0.60$ and $r_{yP.I} = -0.34$.

Thus, the iodine number may be of more significance than the polyunsaturated fatty acid content.[5]

[5] The various multiple regression equations examined for the previous experiment, as well as several others, have been studied. The various regression coefficients are of similar sign and magnitude of those present for that experiment. Since these yield correlation coefficients of similar size to that obtained with the iodine number, and are not decisive in determining the effect of the various fatty acid components, as has already been demonstrated for the previous experiment, they are not presented here.

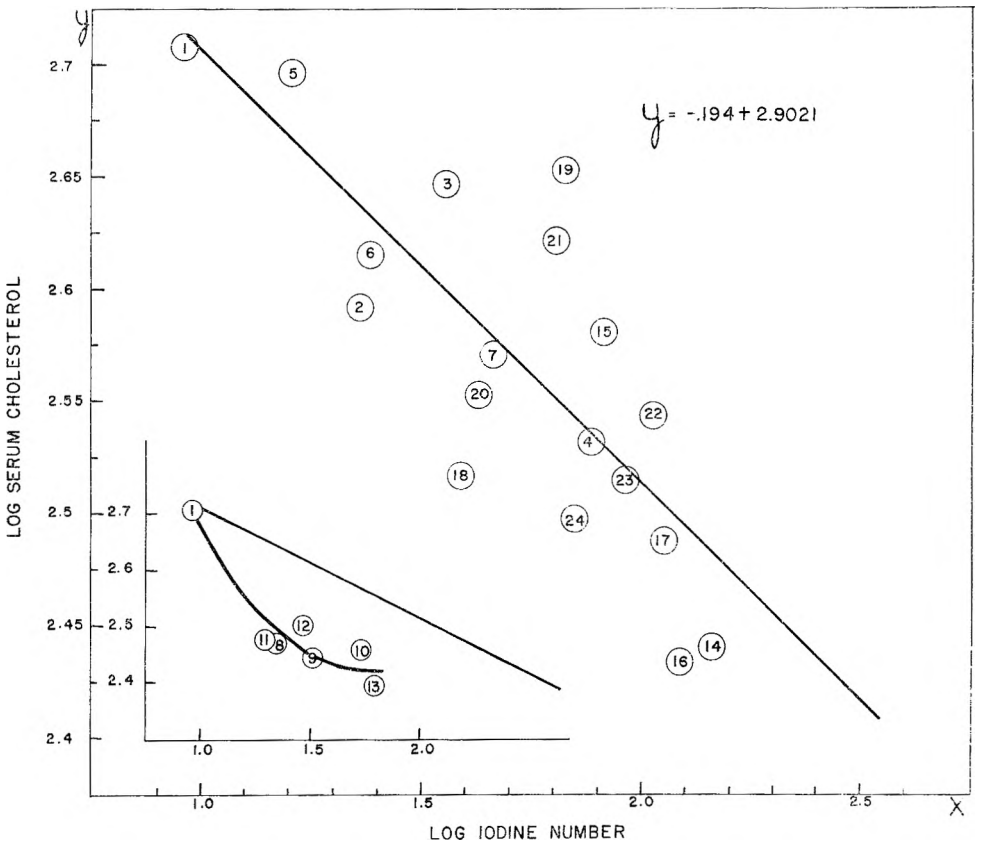

Fig. 1 Spot diagram showing the relationship between the iodine number of the dietary oils and the serum cholesterol levels of animals receiving these oils. The numbers refer to the groups listed in table 4. The data from groups 8 to 13 do not fit the general trend and are plotted separately in the insert.

DISCUSSION

The data presented clearly demonstrate a marked effect of the composition of the dietary fat upon the serum cholesterol levels in cholesterol-fed chicks. Within limits the effects of the different fats are reproducible. In several experiments in which coconut, olive, and safflower oil were used, their relative position was the same, with coconut oil being the most hypercholesterogenic and safflower oil the least. Experiments concerning 11 comparable groups are reported in tables 3 and 4. Two of these, experiments, those using 8% of coconut oil plus 2% of safflower oil, and 8% of coconut oil plus 2% of corn oil, gave results divergent from the other comparable groups. Results from the remaining 9 groups show a fairly good correlation ($r = \pm 0.85$). Insofar as we have been able to interpret the results, the two experiments yield similar conclusions with regard to the effects upon serum cholesterol values, although the absolute serum cholesterol values were generally higher in the first of the two experiments.

The experimental setup is far from ideal for an assay procedure. The serum cholesterol values do not stabilize over the experimental period and the mean values are derived from some groups in which the serum cholesterol value doubled during the experimental period. As would be expected, this variation, combined with a fairly large variation among individual chicks, gives rather large standard errors of the mean values reported. In most groups these ranged from 20 to 40 mg %.

Obvious ways to decrease these errors are possible, primarily by increasing the size of the experimental group and determining the serum cholesterol values at a single time period. However, the data are sufficient to demonstrate some of the important points, and the procedure used was dictated by the facilities available and the desire for a rapid assay.

The conclusions from these studies are contrary to those of Stamler, Pick and Katz ('59) who concluded that "unsaturated oils failed to suppress hypercholesterolemia and atherogenesis" in cholesterol-fed cockerels. These authors observed some significant differences in the serum cholesterol levels caused apparently by the kind of fat included in the rations. The difference in the experimental design, diets used, age of birds, and other factors, precludes anything more than speculation as to the reasons for the divergent conclusions of the two studies.

One aim of these studies is to develop an assay system which may yield results of significance in human nutrition. It is of interest, therefore, to compare the results with those reported upon human subjects. In a general sense, these results, like those reported in man and various animals, show agreement that fats high in saturated fatty acids elevate, while unsaturated fatty acids depress, the serum cholesterol values. However, in our experience (Hegsted et al., '57b) oils high in oleic acid (or other unsaturated nonessential fatty acids such as elostearic acid) elevate the serum cholesterol in rats. Keys et al. ('57, '58) conclude that the monounsaturated fatty acids have essentially no effect in man, whereas Ahrens et al. ('57) interpret their results in man to indicate that the ability to lower serum cholesterol values is more or less proportional to the over-all degree of unsaturation. As indicated, the results reported here with chicks are compatible with either the conclusion of Ahrens et al. or that of Keys et al. depending upon the interpretive approach. The results are not decisive in determining the relative activities of the mono- and polyunsaturated fatty acids in the chick, although we interpret the results as suggesting a closer relationship to degree of unsaturation (iodine number) than to an interaction between the saturated and polyunsaturated fatty acids.

Ahrens et al. ('58) have stated that one must distinguish between studies of the effect of the kind of fat and the amount of fat upon the serum cholesterol level. This distinction must be remembered in evaluating any set of data. On the other hand, any *satisfactory* interpretation of the role of fat upon serum cholesterol levels must relate *both* the amount and kind of fat. In studies upon rats (Hegsted et al., '59) in which both the amount and kind of fat were varied, the relative effects of different oils were influenced greatly by the amount included in the diet. The experiments reported here are similar to those of Ahrens et al. ('57) using men, in that a diet of constant composition was used, except for the dietary fat, the amount of fat being kept constant. The results are similar also to those obtained by Ahrens. The fact that the total fat intake was constant must be noted, since it may limit the general application of the findings. With both species the effect of the amount of fat under these conditions remains to be seen.

Several investigators (Beveridge et al., '58; Grande et al., '58) have presented evidence that corn oil has effects upon the serum cholesterol values which may not be explicable by its fatty acid composition. Similar evidence, not entirely consistent in different experiments, is apparent with the chick. Whether this effect is primarily dependent upon the sitosterol content remains to be explored.

SUMMARY

Studies upon cholesterol-fed chicks demonstrate that the kind of dietary fat has a pronounced influence upon the serum cholesterol level. Three breeds of chicks demonstrated only minor differences. The serum cholesterol levels appear to be proportional to the log of the dietary cholesterol, other things being equal.

The data demonstrate that fats high in saturated fatty acids promote hypercholesterolemia and that this effect is counteracted by unsaturated fatty acids. The

polyunsaturated acids (primarily linoleic acid in the fats studied) appear considerably more active than the monounsaturated acids but the data do not permit a clear-cut distinction between linoleic acid and unsaturation *per se* as the primary cholesterol lowering factor. Equally good correlation between serum cholesterol and iodine number (total unsaturation) and with multiple regression equations involving the different classes of fatty acids was found. The coefficients of parital correlation suggest that iodine number may be of more significance than the polyunsaturated fatty acid content.

The data also contain evidence that corn and cottonseed oil may contain factors which lower the serum cholesterol, which cannot be accounted for by the fatty acid composition.

It appears that the chick, fed cholesterol, may be a valuable model for studies in the area of dietary fat-serum cholesterol relationships and the findings may have application in human nutrition.

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kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
m^3	cubic meter	μμ	micromicron
	Volume		*Area*
cm^3	cubic centimeter	m^2	square meter
mm^3	cubic millimeter	cm^2	square centimeter
l	liter	mm^2	square millimeter
ml	milliliter		

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%	per cent	ppm	parts per million

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