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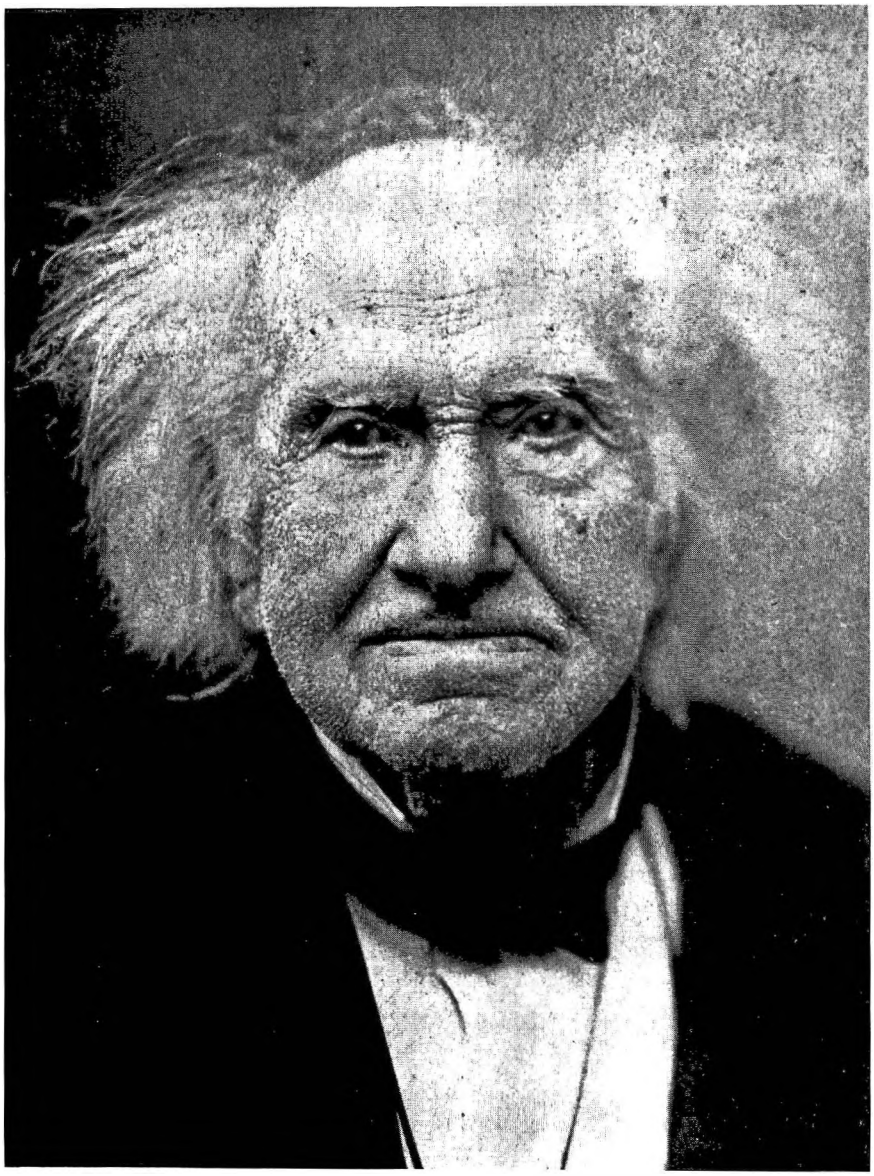

From a photograph taken in 1879

MICHEL EUGENE CHEVREUL

Michel Eugene Chevreul
— *A Biographical Sketch*

(August 31, 1786—April 19, 1889)

Michel Eugene Chevreul was a fascinating individual in many ways, not the least of which was that he lived a creative life until the age of 103. He was the chemist who first demonstrated that the sugar excreted by diabetics is glucose, and that fats can be fractionated into a saponifiable fraction containing fatty acids and an unsaponifiable fraction containing waxes and cholesterol. Chevreul was the inventor of the modern (stearic) candle, and also the author of a popular and lasting theory of colors. His life is a good illustration of the fact that the distinction between "pure" and "applied" research is at best flimsy and far less important than the recognition of *good* research.

Chevreul was born the 31st of August in 1786 in Angers, the capital of the central French province of Anjou. His ancestry was remarkable in that his forebears had been continuously interested in chemistry and biology for more than 200 years. As early as the XVIth century, one of his ancestors, Michel Chevreul, Squire of Bijou, a petty nobleman, had chosen the somewhat unusual profession, for one of his class, of apothecary. His son, Michel II, besides continuing in his father's tradition as an apothecary was a student of surgery (again, definitely not a gentleman's profession in those days) and, in spite of his marriage to the daughter of one of the king's lawyers, practiced as a master surgeon in a small town near Angers. Michel II's son, Michel III, became in his turn a surgeon and settled in Rochefort, a much more important city, and was succeeded there by his son Gilles I. Gilles in turn had two sons, both surgeons, Gilles II (who appeared to have had a more than ordinary interest in chemistry) and Michel IV (who also dabbled in metallurgy as a tinsmith). Michel's son, Michel V, the father of Michel Eugène, was an important figure in French XVIIIth century medicine. He became a Doctor of Medicine in Reims in 1777 and a Master of Surgery in Angers in 1778. His interest was in obstetrics, a somewhat unorthodox bend for a man of his training and qualifications at a time when obstetrics was almost entirely in the hands of midwives. He studied in Paris under Baudelocque, who started the first lying-in hospitals in France. He then started a course in obstetrics in Tours, and in 1782 published a treatise on midwifery which the local governor had printed and distributed to all midwives in the province. In 1784 Chevreul received the warrant of ordinary surgeon to Louis, Duke of Anjou (later Louis XVIII), for his castle at Angers and shortly after was made a corresponding member of the Royal Society of Medicine. More important, he wrote a project on the organization of courses for midwives (which the provincial governor asked him to put into effect). He was also the author of an impassioned pamphlet on the need for special institutions for foundlings. The great social movement which expressed itself in the French Revolution took the idea of a home for the "Nation's children" to heart as well. The first such institution was established in Angers, with M. Chevreul as the first director. He became a city and later a regional councillor as well, was elected Dean of the Angers Medical School in 1820, and died in 1845 at the age of 91.

Michel Eugène lived in Algiers until the age of 17, when, having completed his secondary education, he left Angers for Paris to study chemistry with Vauquelin. Vauquelin was one of the great early organic analysts. He was the first chemist to fractionate biological materials into proteins, fats, starches, sugars, and minerals such as lime, magnesium, silica and iron. Vauquelin was also the author of the ear-

liest balance study in nutrition. He analyzed hens to determine their mineral composition and conducted chemical balances on birds of similar size laying eggs, to determine where the inorganic matter of the eggs came from. He showed that, over a sufficient period of time, changes in the mineral composition of the hens were small and demonstrated that the amounts of various minerals in eggs were equal to the differences between minerals ingested in foods and those present in excreta. Vauquelin analyzed a large number of foodstuffs, showing in particular that there were differences in protein content between various beans and grains and also that plant juices and extracts contained many nitrogenous substances less complex than proteins. Together with Robiquet he isolated asparagine in 1806. He discovered the presence of magnesium in bones in 1801. Young Chevreul obviously could learn much from such a man. After working with Vauquelin in the latter's small private laboratory for a year, he moved with him to the Museum of Natural History, when Vauquelin, in 1804, was elected Professor of Applied Chemistry in that institution. The Museum of Natural History had been throughout the XVIIIth century the largest government-supported establishment in the Western World to be exclusively devoted to the study of the natural sciences, starting with botany. Shortly after this move, Chevreul was given the title of "preparator."

In 1806 Chevreul published his first work, "Chemical examination of bone fossils found in the department of Eure et Loir." In the next few years he published on a variety of topics, such as the effect of nitric acid on cork, the chemical analysis of various pigments (in particular, Guatemalan indigo), the spontaneous disposition of barium sulfite, the effect of nitric and nitrous acid on oxides, uric acid and others. He also separated sugar from the urine of a diabetic and showed that its crystalline form, its solubility in water and alcohol and its behavior on heating were similar in every way to the corresponding properties in cane sugar. In 1813 he was appointed a professor at the Lycee Charlemagne, then one of the best in France, and also presented his first communication on the subject of fats to the Academy of Sciences. In the course of the next 10 years he was to produce a series of publications in this field culminating in his 1823 treatise. These have become the basis for the modern knowledge of fats.

Typical of his later interests in applied as well as in the theoretical science, Chevreul's first communication dealt with the manufacture of soap from spermaceti, the wax-like substance which is obtained from the head of the sperm whale. He showed that by boiling this substance with caustic alkali he could produce soap (potassium palmitate) but that unlike what he observed with other fats, no glycerol was present in the water from which the soap was obtained. Instead he could separate a white crystalline material, insoluble in water but soluble in alcohol and ether which he called cetin. After recrystallizing, it melted at 49°C and came to be known as "cetyl alcohol."

Chevreul had actually started investigating fats in 1811 when Vauquelin gave him the task of examining the chemical nature of a sample of soap. Chevreul dissolved the soap in water and observed that on addition of hydrochloric acid, insoluble organic acids separated from the solution and formed a floating layer. He later showed that this layer was made up of a number of distinct fatty acids. Chevreul showed that when common fats were similarly heated, glycerol, a substance first discovered (and named) by Scheele in 1783, remained in solution while fatty acids separated. In 1814 Chevreul showed that lard contained essentially two main fats, one solid at room temperature which he called "stearine" and the second liquid, and which he called "elaine." Chevreul made soap from lard and potash and from the aqueous solution he crystallized potassium stearate which he called "mother of pearl." (Elaidic acid was later shown to be the isomer of oleic acid, the isomerization resulting from the mode of preparation). The water-insoluble organic material, of acid reaction to litmus, solid at room temperature, which was set free when lard or similar fats were dissolved in water and acidified with hydrochloric acid, he called "margarine" (from the Greek for mother-of-pearl). Incidentally, Chevreul

had previously defined an acid as a substance sour to the taste, capable of being attracted by positively electrified substances, capable of neutralizing basic substances, of reddening litmus and the color of violets, and of reddening hematine. Although they possessed all the other properties of acids, fatty acids did not taste sour. Chevreul considered them as acids anyway, a judgment vindicated by later advances in physical chemistry.

Chevreul isolated "hircin" from goat's fat. From this material he prepared "hircic" or "caproic" acid, an acid distinguished from stearic acid by a difference in melting points.

Chevreul introduced the use of alcohol-water mixtures to separate mixtures of soaps into various fractions. He also observed that when stearic acid was distilled destructively, liquid products of acid nature are formed, and an exceedingly irritating odor is developed. (This was later shown by Redtenbacher to be due to the formation of acrolein.)

Paper followed paper in which Chevreul described the isolation of one fatty acid after another, and in 1823 he published his "Chemical Investigations of Fats of Animal Origin" followed by "General Considerations of Organic Chemistry" which related in great detail and with great accuracy the methods followed in his investigations, in particular the use of saponification and of successive use of various solvents. In a related field Chevreul studied biliary fats and separated cholesterol (first obtained from gallstones by Poultier de la Salle) as a major component, the solubility and properties of which he described.

In 1824, Chevreul was appointed Director of the Dye Works of the Royal Manufactures of The Gobelins and Beauvais. While he acceded to this post because of his reputation as a chemist and of his interest in the chemical structure of dyes (which had started with his study of the nature of indigo), he promptly became interested in the optics of colors as well. The publication of his books on contrast of colors, on optical effects in dyed silks and other fabrics, and on dyestuffs span a period of 60 years, from 1829 to 1889. Chevreul made the important observation ("law of simultaneous contrasts") that "every color when placed beside another color is changed, appearing different from what it really is, and moreover equally modified the color with which it is in proximity." Chevreul applied this rule to a large number of combinations of colors and materials and summarized his findings in his 430-page, closely printed book (dedicated to his friend Berzelius) on "The principles of harmony and contrast of colors and their applications to the arts: including painting, interior decoration, tapestries, carpets, mosaics, colored glazing, paper-staining, calico printing, letter-press-printing, map-coloring, dress, landscape and flower gardening, etc." The British translator of the book into English, Charles Martel, wrote in his preface: "Our national deficiencies in the effective employment of colored materials, and in the applications of colors in various arts, have long been felt, but it was only during the Great Exhibition of 1851 that they were fully manifested. Our inferiority in this respect to most of our continental neighbors, especially the French (with whom the comparison was chiefly instituted), was then candidly admitted, and the cause of their superiority investigated. Now, this was attributed mainly to the teachings of M. Chevreul, who, under the authority of the French government, has delivered, alternately at Paris and Lyons, during the last twenty-five years, annual courses of lectures on the Contrast of Colors, before the manufacturers, artizans, and others interested in those cities. These lectures, greatly amplified, is what this volume consists of." Perusal of this book impresses the reader with the extraordinary curiosity and universality of interest of a man who could follow colors from the optics of light and visions to the chemistry of preparation and applications of dyestuffs to interior decorating including the analysis of schemes of colors of rugs, curtains, chairs, etc. His "Lessons on chemistry applied to dyeing" were also translated into German and English and for years constituted one of the most important textbooks in the textile industry. He published actively in this field until 1864. In 1865 also he published a history of medical prescription, in 1866 a history

of chemistry in four volumes, in 1878 a history of concepts of Matter and between 1856 and 1870 a number of books on the scientific method as well.

In 1825 Chevreul, together with Gay-Lussac, had taken a patent for the manufacture of the stearic acid candle, the candle still used today, which in short order replaced the old-fashioned candles made of tallow, bayberry wax, or other fats. He was awarded a 12,000 gold francs prize by the Society for the Advancement of Industry and a Special Medal of Honor by the 1855 International Exposition for this invention. In 1826 Chevreul succeeded Proust at the Academy of Sciences. In 1830 he succeeded Vauquelin as Professor of Applied Chemistry at the Museum. He became Director of the Museum in 1864 and remained in this position until 1879, serving in particular in this capacity during the Franco-Prussian War of 1870–71 when the Museum was systematically bombarded by the German troops. In 1875 he was awarded the Grand Cross of the Legion of Honor. He had received previously the Copley Medal of the Royal Society of which he was a foreign member.

Chevreul had married in 1818. (His son Henri, born in 1819 broke the family tradition in that he was the first in over two centuries not to espouse a scientific or medical profession. He became a judge and a noted antiquarian who published in a number of historical fields.) One of the curious aspects of Chevreul's later years was his active interest in (and acute skepticism about) spiritualism. In the 1850's, table-lifting and spiritual seances had become highly fashionable in the United States and the vogue had quickly migrated to Europe. Chevreul was appointed chairman of a committee of the Academy of Sciences to inform the public on the scientific interpretation of table-turning and other "psychic" phenomena. He investigated a great many spiritualist "experiments" and showed that most cases of table-turning were due to outright fraud while the others, as well as movements of the "divining rod" and the "exploring pendulum" were due to unconscious movement of the hand or body of the performers. His highly articulate and publicized utterances on the subject brought him into renewed contact with Faraday, who had called on Chevreul's laboratory twice in the past, in 1812 and 1845, but whose interests had of course differed in the intervening periods from those of Chevreul. Like Chevreul, Faraday considered it his duty as a scientist to disabuse the public mind of those false beliefs. Incidentally, as Jastrow rightly remarks, these two great men presented during that period a striking contrast in age of onset of senescence: Faraday, while only barely over 60, was prematurely aged, his memory failing almost completely and public action had become difficult for him; Chevreul at the same age was still extraordinarily active (and destined to live another forty years and more). Chevreul retired as Director of Museum in 1879 at the age of 93, as Director of the Dye Works of the Gobelins at age 97, but he continued as professor. At the age of 101, he announced that while heretofore he had organized his teaching so as to cover organic chemistry over a span of two years, he felt that in order to do justice to this enormously expanded field he had to completely revise his course and take three years to cover his subject. As a matter of fact he did complete the three academic years.

Few public functions were better attended than his 100th birthday celebration. People everywhere are fascinated by centenarians and here was one who was not only respected by scientists everywhere as a distinguished chemist, biochemist and physicist, but also known by the general public of all western countries as the inventor of the modern candle. On August the 31st, 1886, messages, delegates, honorary degrees (including one from Harvard) and many other distinctions poured into Paris from every corner of the world to honor the alert and durable investigator who still felt that "there was something to be learned every day in Science." Chevreul modestly disclaimed any particular merit save that of being "the oldest among students in France" and only in that sense, their Dean.

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SELECTED REFERENCES

Bouchard, Georges 1932 *Chevreul,* éditions de la Madeleine. Paris.

Grimal, P. 1958 *Chevreul* in Dictionnaire des Biographies. Presses Universitaires de France, 108 Blvd. Saint Germain, Paris.

Jastrow, J. 1937 Chevreul as psychologist. Scientific Monthly, *44:* 487.

Obituary of Chevreul and account of his funeral 1889 Nature, 39: 565.

Mc Collum, Elmer V. 1957 A History of Nutrition. Houghton Mifflin Company, Boston.

Prevost, M., et Roman d'Amat 1959 *Chevreul* in Dictionnaire de Biographie Française, vol. XLVII. Librairie Letouzey et Ane (publisher), 87 Blvd. Raspail.

Amino Acid Requirements for Maintenance in the Adult Rooster

IV. THE REQUIREMENTS FOR METHIONINE, CYSTINE, PHENYLALANINE, TYROSINE AND TRYPTOPHAN; THE ADEQUACY OF THE DETERMINED REQUIREMENTS[1]

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This paper extends the investigation of the maintenance requirements for the adult rooster not depleted of protein (Leveille and Fisher, '58; '59; '60) by presenting nitrogen balance results from which the needs for tryptophan and for methionine and phenylalanine in the presence and absence of cystine and tyrosine have been computed. In this, as well as in the previous studies of this series, the amino acid requirements were determined singly in the presence of a relative excess of some of the others. It was therefore a further purpose of the present investigation to evaluate an amino acid mixture, isonitrogenous with the starting mixture used in the determination of the requirements but containing only the maintenance requirement previously determined.

EXPERIMENTAL

White Leghorn roosters, 12 months old or older, were used in all studies. The procedures employed were the same as previously described (Leveille and Fisher, '59). The birds were maintained in metabolism cages in a temperature-regulated room (65° to 72°F) and received a stock mash diet when not on experiment. They were fed for a one-week standardization period a semipurified, pelleted diet containing 7.06% of whole-egg protein (for composition see table 1, Leveille and Fisher, '58). This diet, which was fed at 25 gm/kg of body weight/day supplied adequate amounts of nitrogen and energy (282 mg N and 90 Cal. of metabolizable energy/kg/day) to maintain body weight and positive nitrogen balance.

Following the standardization period, the birds received the test diets for an equilibration period of three days before 24-hour collections for nitrogen balance were begun for a three-day period. Fritz et al. ('36) have shown that this period is sufficient to overcome any effect of previous diet on nitrogen excretion in the adult rooster. The values given in the tables represent the average balances carried out on three consecutive days for each bird on each test diet. For a given series, the same birds were fed progressively lower levels of the amino acid under study.

All experimental diets were pelleted and the animals were trained to eat their daily allotment of 26 gm/kg of body weight within a single 30-minute period. The complete starting diet, shown in table 1, is the same as diet B previously described (Leveille and Fisher, '59).

After the balance studies had been completed from which requirements were computed for tryptophan, methionine, phenylalanine, cystine and tyrosine, an experiment was designed to evaluate the computed requirement levels for the maintenance of nitrogen balance. Two amino acid mixtures, both isonitrogenous with that of the complete starting diet, were compared. These two mixtures differed in their source of nonessential nitrogen

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[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station. Supported in part by a grant-in-aid from the National Science Foundation, and a fellowship to G. A. Leveille from Wirthmore Feeds Inc., Waltham, Massachusetts.

TABLE 1

Percentage composition of starting diet and of diets supplying the estimated maintenance requirement levels of adult roosters[1]

Ingredients[2]	Starting diet (complete)	Requirement diet (constant portion)
L-Arginine·HCl	0.56	0.55
L-Lysine·HCl (99%)	0.57	0.14
L-Tryosine	0.30	0.13
L-Tryptophan	0.09	0.07
L-Phenylalanine	0.39	0.10
L-Cystine	0.16	0.16
DL-Methionine	0.27	0.27
DL-Threonine	0.57	0.57
L-Leucine	0.64	0.48
DL-Isoleucine[3]	1.02	0.55
DL-Valine	0.97	0.23
L-Glutamic acid	1.72	—
Glycine	0.67	—
Dextrin	5.00	5.00
Glucose	17.25	17.25
Corn oil	12.00	12.00
Fiber	4.00	4.00
Minerals[4]	3.00	3.00
B-vitamin mix[4]	0.15	0.15
Choline chloride	0.24	0.24
Vitamin A, D and E mix[4]	0.15	0.15
Gelusil[5]	1.00	1.00
Starch	to 100	—
	Variable portion	
	Diet A	Diet B
L-Histidine HCl·H_2O		0.15
DL-Serine		0.83
DL-Alanine		0.63
L-Aspartic acid		1.13
L-Proline		0.54
L-Glutamic acid	1.73	1.62
Glycine	3.28	0.29
Starch	to 100	to 100

[1] These diets are calculated on the basis of feeding 26 gm/kg body weight/day and contain 280 mg N/26 gm.

[2] We gratefully acknowledge generous gifts of vitamins from the following concerns: Merck Sharp and Dohme, Rahway, N. J.; Pfizer and Company, Inc., Terre Haute, Ind. and Distillation Products Industries, Rochester, N. Y.

[3] D-Allo, L-isoleucine.

[4] Fisher and Johnson ('56).

[5] Warner-Chilcott, Morris Plains, New Jersey.

which was provided by glycine and glutamic acid plus a small amount of cystine, tyrosine and D-isomers in one case, and by all the nonessential amino acids in the other. The complete composition of these diets is shown together with that of the starting diet in table 1.

Since histidine had been shown nonessential for maintenance of nitrogen balance in the rooster during an 8-day experimental period, when no change in hemoglobin occurred either (Leveille and Fisher, '59), a separate group of 5 birds was given for two weeks the histidine-free amino acid mixture containing the maintenance requirements (diet A, table 1). At the end of the standardization period using whole-egg protein, and again after feeding the histidine-free diet for two weeks, hematocrit determinations were carried out as an indicator of hemoglobin concentration. The histidine-containing dipeptides, anserine and carnosine, were determined in breast muscle of 5 birds fed the whole-egg diet and in breast muscle from the 5 birds fed the histidine-free

diet for two weeks.[2] The dipeptides were extracted from breast muscle by homogenization with 10% trichloracetic acid (TCA), followed by removal of the TCA with petroleum ether and adjustment of pH to 2.2. A 2-ml portion of this solution, representing approximately 30 mg of original tissue per ml, was placed on a 50-cm column of the Spinco Amino Acid Analyzer.[3]

RESULTS

The nitrogen balance data from which the requirements for methionine, phenylalanine, cystine, tyrosine and tryptophan were computed are given in tables 2 to 5. The *maintenance requirement* was taken as before (Leveille and Fisher, '59) as the lowest level of amino acid that would maintain the nitrogen retention observed with the complete starting diet; and the

[2] We wish to thank Dr. L. E. Ousterhout, College Park, Maryland for suggesting the analysis of these compounds.

[3] The chromatographic analysis was carried out by Oxford Laboratories, Redwood City, California, according to the procedures of Spackman et al. ('58), and Moore et al. ('58a, b). The results are claimed to have a reproducible accuracy of 100 ± 3%.

TABLE 2

Nitrogen balance studies in the adult rooster, using graded levels of methionine in the presence and absence of dietary cystine[1]

Methionine		L-Cystine	Average body weight		Average nitrogen		
Form	Amount		Initial	6-day change	Intake	Excretion[2]	Balance
	mg/kg/day	*mg/kg/day*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
L	71	42	2444[3]	+38	308	259 ± 13	+49
L	136	0	2482	+13	305	243 ± 12	+62
L	70	0	2495	+ 9	303	273 ± 8	−30
L	35	0	2504	+10	302	309 ± 7	− 7
L	17	0	2508	−21	304	309 ± 11	− 5
	0	0	2487	−40	308	359 ± 13	−51
D	70	0	2442	+22	309	244 ± 4	+65
DL	71	42	2552[4]	+ 8	278	226 ± 5	+52
DL	37	42	2560	−70	281	251 ± 8	+30
DL	18	42	2490	−14	285	251 ± 8	+30
DL	8	42	2476	−26	288	298 ± 7	−10
	0	42	2450	−40	292	302 ± 7	−10

[1] All food offered was eaten.
[2] Mean value with its standard error.
[3] Four birds used in this trial.
[4] Three birds used in this trial.

TABLE 3

Nitrogen balance studies in the adult rooster using graded levels of L-*cystine*[1]

DL-Methionine	L-Cystine	Average body weight		Average nitrogen		
		Initial	6-day change	Intake	Excretion[2]	Balance
mg/kg/day	*mg/kg/day*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
71	42	2595[3]	+ 1	280	242 ± 5	+38
36	42	2596	+ 2	281	234 ± 8	+47
36	21	2672	+ 5	280	234 ± 6	+46
36	10	2601	− 3	280	266 ± 11	+14
36	5	2598	−16	281	245 ± 5	+36
36	0	2582	0	282	272 ± 8	+10
21	42	2535[4]	+ 8	281	265 ± 9	+16
21	21	2543	−23	282	309 ± 8	−27
21	10	2520	−37	285	325 ± 18	−40
21	5	2483	−37	290	315 ± 4	−25
21	0	2465	−35	294	319 ± 17	−25

[1] All food offered was eaten.
[2] Mean value with its standard error.
[3] Four birds used in this trial.
[4] Three birds used in this trial.

TABLE 4

Nitrogen balance studies in the adult rooster, using graded levels of L-*phenylalanine and* L-*tyrosine*

Consumption of food offered	L-Phenyl-alanine	L-Tyrosine	Average body weight		Average nitrogen		
			Initial	6-day change	Intake	Excretion[1]	Balance
%	*mg/kg/day*	*mg/kg/day*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
100	101	78	2457[2]	− 4	284	256 ± 10	+28
100	99	0	2453	−30	287	260 ± 10	+27
100	49	0	2423	−36	290	284 ± 15	+ 6
91	26	0	2387	−70	268	279 ± 11	−11
45	0	0	2317	−184	126	234 ± 12	−108
100	101	78	2435[3]	+15	281	253 ± 12	+28
100	52	78	2450	+ 9	280	250 ± 4	+30
100	26	78	2459	− 5	279	252 ± 9	+27
100	0	78	2455	+13	278	309 ± 12	−31
100	101	78	2382[4]	− 5	281	230 ± 9	+51
100	26	78	2387	−30	282	279 ± 15	+ 3
100	26	39	2357	−20	286	253 ± 11	+33
87	26	21	2337	−27	253	252 ± 18	− 1
64	26	0	2310	−100	193	235 ± 29	−42

[1] Mean value with its standard error.
[2] Three birds were used in this trial.
[3] Four birds used in this trial.
[4] Three birds used in this trial.

TABLE 5

Nitrogen balance studies in the adult rooster, using graded levels of L-*tryptophan*

Consumption of food offered	L-Trypto-phan	Average body weight		Average nitrogen		
		Initial	6-day change	Intake	Excretion[1]	Balance
%	*mg/kg/day*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
100	23	2528[2]	− 4	281	234 ± 8	+47
90	13	2524	−23	255	221 ± 5	+34
72	0	2501	−79	209	270 ± 7	−61

[1] Mean value with its standard error.
[2] Four birds used in this trial.

minimum maintenance level as the lowest concentration that would maintain nitrogen equilibrium. These values were estimated from the regression lines obtained by the method of least squares, as previously described (Leveille and Fisher, '60) by plotting nitrogen balance against the amino acid content of the diet (mg/26 gm of diet). The standard error of estimate for the regression lines was used arbitrarily to estimate a range for the maintenance requirement.

The body weight changes generally were less than 5% in either direction. As previously discussed (Leveille and Fisher, '59), small weight changes can be attributed to changes in water balance and to feather losses. Somewhat larger weight changes occurred with the lower levels of tryptophan, phenylalanine and tyrosine and were associated with food refusal; these losses probably reflect the decreased caloric intake.

From the values in table 2 the maintenance requirement for L-methionine in the absence of cystine was computed to be 90 mg/kg of body weight/day and the minimum maintenance level, 39 mg/kg of body weight/day. The range for the maintenance requirement was calculated to be 82 to 99 mg/kg/day. The data in table 2 suggest the full utilization of the D-isomer of methionine.

When cystine was present in adequate concentration, the maintenance requirement and the minimum maintenance level for DL-methionine were computed at 71 and 15 mg/kg body weight/day, respectively. Calculations based on the methionine requirement in the presence and absence of dietary cystine showed that cystine reduces the methionine requirement by approximately 20% at the maintenance requirement level and by 60% at the minimum maintenance level.

The results given in table 3 permit the computation of the minimum maintenance level for cystine which was found to be 37 mg/kg body weight/day. The maintenance requirement for cystine was not determined because the response to relatively large graded increments of either methionine or cystine was small and the technique therefore not sensitive enough for its determination. The slightly positive balance obtained with 36 mg of DL-methionine in the absence of cystine confirms the computed minimum maintenance level for methionine of 39 mg/kg/day and further corroborates the data in table 2 which suggested that the D-isomer of methionine is as well utilized as the L-form.

It is noteworthy that no food refusal occurred in any of the studies involving methionine or cystine, in contrast with such observations for tryptophan and phenylalanine.

Table 4 shows the nitrogen balance from which requirements for phenylalanine and tyrosine were computed. In the absence of tryrosine, the maintenance requirement for L-phenylalanine was 60 mg/kg/day with a range of 54 to 70 mg/kg/day. The minimum maintenance level was 38 mg/kg/day in the absence of tyrosine. In the presence of 78 mg/kg/day of L-tyrosine the L-phenylalanine maintenance requirement was computed to be 26 mg/kg/day and the minimum maintenance level, 13 mg/kg/day. Calculations show that tyrosine can spare about half the maintenance requirement and two thirds of the minimum maintenance level for phenylalanine.

The maintenance requirement for L-tyrosine in the presence of 26 mg/kg/day of L-phenylalanine (table 4) was computed to be 33 mg/kg/day. The minimum maintenance level for tryrosine was not determined.

Food refusal occurred in these studies with phenylalanine. In the presence of an excess of tyrosine, however, all the food offered was consumed even when the ration was completely devoid of phenylalanine (table 4).

From the nitrogen balance data of table 5 the maintenance requirement for tryptophan was computed at 19 mg/kg body weight/day and the minimum maintenance level at 7 mg/kg/day.

Table 6 gives a summary of the computed requirements from this as well as from the previous studies (Leveille and Fisher, '59; '60). The table also shows the amino acid levels supplied in the original complete starting diet which was patterned after whole-egg protein (Leveille and Fisher, '58). The computed maintenance requirement values for arginine, methionine and threonine were the same as those supplied by the starting diet.

Since the individual amino acid requirements were determined on rations containing an excess of most amino acids which were not under investigation, the adequacy of feeding only the requirement levels had to be determined. Table 7 shows the comparative nitrogen balance for (a) birds fed the original starting mixture, (b) birds given the computed maintenance requirements with glycine, glutamic acid and the D-isomers of a few acids supplying the nonessential nitrogen necessary to meet the total nitrogen requirement, and (c) for birds given the maintenance requirements with all nonessential amino acids supplying the additional nitrogen needed. The nitrogen retention of birds in the requirement-level experiment was lower than that of birds fed the starting mixture; and the birds receiving all of the nonessential amino acids were in better balance than those getting only glutamic acid and glycine. The adequacy of the determined maintenance requirements has been further upheld in approximately 20 additional roosters that have remained in positive nitrogen ballance (of the same magnitude as given in table 7) for feeding periods exceeding 4 weeks.

Still another group of 5 birds maintained positive nitrogen balance for a two-week period when receiving the histidine-free maintenance requirements with glycine and glutamic acid supplying the nonessential nitrogen. These birds were killed for anserine and carnosine analyses of breast muscle and compared with 5 birds that had been fed the stock diet, followed by the whole-egg diet for one week. Table 8 shows the hematocrit values for the two groups of birds as well as the concentra-

TABLE 6

Amino acid maintenance requirement and minimum maintenance level computed from nitrogen balance studies in the adult rooster

Amino acid[1]	Starting mixture[2]	Maintenance requirement[3]	Minimum maintenance level[3]
	mg/kg/day	*mg/kg/day*	*mg/kg/day*
Arginine	121	120	54
Histidine	37	0	0
Lysine	117	29	0
Leucine	165	124	54
Isoleucine	132	72	49
Valine	127	61	55
Threonine	74	74	55
Tryptophan	25	19	7
Methionine	71[4]	90[4]	39[4]
		71[5]	15[5]
Phenylalanine	102[6]	60[6]	38[6]
		26[7]	13[7]
Cystine	42	not determined	37[8]
Tyrosine	77	31[9]	not determined

[1] All amino acids expressed as the natural L-isomer; for the form actually fed see tables 1–5 as well as earlier papers in this series (Leveille and Fisher, '59; '60).
[2] Based on composition of whole-egg protein (Leveille and Fisher, '58).
[3] For definition see text.
[4] In the absence of dietary cystine.
[5] In the presence of 42 mg/kg/day of cystine.
[6] In the absence of dietary tyrosine.
[7] In the presence of 78 mg/kg/day of tyrosine.
[8] In the presence of 21 mg/kg/day of methionine.
[9] In the presence of 26 mg/kg/day of phenylalanine.

TABLE 7

Effect on nitrogen balance of giving the maintenance requirement of the essential amino acids, in the adult rooster

Amino acid mixture[1]	Average body weight		Average nitrogen		
	Initial	6-day change	Intake	Excretion[2]	Balance
	gm	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
Requirement—Diet A (glycine-glutamic)	2684[3]	+26	301	282±8	+19
Requirement—Diet B (all nonessential amino acids)	2710[3]	+10	299	272±8	+27
Original starting diet	2465[4]	+13	280	241±8	+39

[1] The essential amino acid portion of diets A and B (including a small amount of cystine, tyrosine and D-isomer nitrogen) contributed 115 mg N/kg/day. In ration A, glycine and glutamic acid contributed respectively 84 and 81 mg N/kg/day, while the same total amount of nonessential amino acid nitrogen in ration B was derived from histidine, serine, aspartic acid, alanine and proline in addition to glycine and glutamic acid.
[2] Mean value with its standard error.
[3] Seven birds were fed diet A for the first week and diet B the second week.
[4] Average of 62 birds that have been fed the starting diet.

tion for the histidine-containing dipeptides. The hematocrits showed no difference between groups but both anserine and carnosine levels were distinctly lower in the breast muscle of the birds fed the histidine-free maintenance amino acid mixture.

DISCUSSION

Although the individual amino acid requirements were computed from nitrogen balance data for relatively small numbers of birds over a short feeding period, the adequacy of a mixture of all the determined requirement levels has been confirmed in

TABLE 8

Values for hematocrit, anserine and carnosine from roosters fed whole-egg protein or amino acid diet devoid of histidine

Diet	Hematocrit	Breast muscle	
		Anserine	Carnosine
	% cells	*μmoles/gm wet tissue*	
Whole-egg protein	48 ± 0.6[1]	37.7	15.5
Histidine-free, amino acid maintenance diet			
End of 1st week	48 ± 0.6		
End of 2nd week	49 ± 2.7	22.9	11.1

[1] Standard error of the mean.

a total of 32 birds fed for varying periods, some exceeding 4 weeks.

In this as well as in a previous study of this series (Leveille and Fisher, '59) histidine was found to be not essential for maintenance of nitrogen equilibrium over relatively short-term feeding periods. Nasset and Gatewood ('54) have shown that a histidine deficiency in the adult rat was accompanied by a decrease in the level of hemoglobin, the catabolism of which may serve as a source of histidine. In our previous study, (Leveille and Fisher, '59) no change in hemoglobin was noted in birds fed the histidine-free diet for 8 days. In this study the hematocrit[4] was determined as a possible indicator of hemoglobin with no change between birds receiving histidine-free or the histidine-containing diets. On the other hand, there was a considerable decrease in anserine and carnosine of breast muscle of birds fed the histidine-free diet. While the lower values do not necessarily prove that they are abnormal or would decrease still further, they at least suggest that histidine may be necessary for prolonged feeding periods.

A high degree of correlation between the computed amino acid requirements and the amino acid composition of feather protein (Block and Bolling, '51) was noted. The linear correlation coefficient for the minimum maintenance level and the amino acid content of feather protein ($r = +0.86$) was statistically highly significant ($P < 0.01$). The degree of correlation for the minimum maintenance level was higher than that for the maintenance requirement ($r = 0.86$ vs $+0.51$ [$P < 0.05 > 0.02$]). This implies that the amino acid needs for feather synthesis represent a major portion of the *minimum maintenance level*, whereas requirements for other purposes and functions are satisfied to a greater extent at the *maintenance requirement level*. If the amino acid requirement for feather synthesis is constant, it might be expected that the requirement of animals depleted of their protein reserves to the endogenous state would show an even higher degree of correlation with the amino acid content of feathers.

SUMMARY

1. The maintenance requirements for tryptophan, phenylalanine, methionine, cystine and tyrosine were computed from nitrogen balance studies in adult roosters not depleted of protein.

2. A diet supplying the determined maintenance requirements maintained the birds in positive nitrogen balance.

3. Using a histidine-free diet which maintained positive nitrogen balance no changes in hematocrit levels were noted, but breast muscle concentration of anserine and carnosine decreased compared with control birds fed a histidine-containing ration.

4. A significant correlation was found to exist between the maintenance requirements and the amino acid content of feather protein.

[4] Direct hemoglobin determinations of chicken blood are not accurate because in this species the erythrocytes are nucleated. Although the relationship between hematocrit and hemoglobin is not precisely established, it was thought that the former would provide some indication of a sharp abnormality in the latter.

LITERATURE CITED

Block, R. J., and D. Bolling 1951 The Amino Acid Composition of Proteins and Foods. Charles C Thomas, Springfield, Illinois.

Fisher, H., and D. Johnson, Jr. 1956 The amino acid requirement of the laying hen. I. The development of a free amino acid diet for maintenance of egg production. J. Nutrition, *60:* 261.

Fritz, J. C., W. A. Hendricks and H. W. Titus 1936 The influence of previous feeding on the N excretion of fasting birds. Am. J. Physiol., *115:* 281.

Leveille, G. A., and H. Fisher 1958 The amino acid requirements for maintenance in the adult rooster. I. Nitrogen and energy requirements in normal and protein-depleted animals receiving whole egg protein and amino acid diets. J. Nutrition, 66: 441.

——— 1959 Amino acid requirements for maintenance in the adult rooster. II. The requirements for glutamic acid, histidine, lysine and arginine. Ibid., *69:* 289.

——— 1960 Amino acid requirements for maintenance in the adult rooster. III. The requirements for leucine, isoleucine, valine and threonine with reference also to the utilization of the D-isomers of valine, threonine and isoleucine. Ibid., *70:* 135.

Moore, S., D. H. Spackman and W. H. Stein 1958a Automatic recording apparatus for use in the chromatography of amino acids. Federation Proc., *17:* 1107.

——— 1958b Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem., *30:* 1185.

Nasset, E. S., and V. H. Gatewood 1954 Nitrogen balance and hemoglobin of adult rats fed amino acid diets low in L- and D-histidine. J. Nutrition, *53:* 163.

Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., *30:* 1190.

Further Aspects of Amino Acid Imbalance, with Special Reference to the High Arginine Requirement of Chicks Fed Casein Diets[1]

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In a previous report from this laboratory (Fisher et al., '60) it was shown that growth of chicks, like that of rats (Harper, '59), was depressed upon the addition of amino acid mixtures to rations deficient in an essential amino acid, if the same amino acid was also lacking from the added amino acid mixture. It was further observed that the depression occurred only when the diets provided all nutrients in excess of the maintenance requirements. A different type of imbalance was created when the nitrogen supplement added to the basal diet was not balanced for good growth, either in itself or in conjunction with the dietary protein, except for a single limiting amino acid. It was also suggested that the high arginine requirement of the chick fed casein diets might possibly be the result of an amino acid imbalance.

The present report extends the previous study to (1) observations on the effect of supplementing a high-quality protein with an amino acid mixture lacking several essential amino acids, (2) a study of the factorial supplementation of peanut protein simultaneously deficient in two amino acids (lysine and methionine), and (3) the elucidation of the high arginine requirement of the chick peculiar to casein-containing rations.

GENERAL PROCEDURE

Week-old Vantress cockerels were used in all three trials. Duplicate groups of 7 chicks per lot were assigned to each dietary treatment. For the first week the chicks were fed a standard starting ration, at the end of which time they were selected by weight for assignment to the treatment groups. Feed and water were provided ad libitum and the experimental diets fed for a 2-week period until the animals were three weeks old. The composition of the basal ration and that of the amino acid mixtures used as supplements are shown in table 1. The protein sources for the three experiments consisted of sesame meal, peanut meal and casein and isolated soybean protein,[2] respectively. Amino acid supplementation of these proteins was based on the calculated average analyses from the values given by Block and Weiss ('56). The amino acid composition of the isolated soybean protein was supplied by the manufacturer. Protein content was determined conventionally by nitrogen analysis × 6.25.

EXPERIMENTAL RESULTS

Trial 1. Based upon the indirect evidence that the addition of a mixture of nonessential amino acids to a lysine-deficient protein did *not* aggravate the lysine deficiency, it was concluded (Fisher et al., '60) that only combinations of protein and amino acids, well-balanced for growth except in the single limiting amino acid, would produce an imbalance resembling an exaggerated deficiency of the single limiting amino acid. In the first experiment of this series a mixture consisting of essential and nonessential amino acids lacking in several (4) essential amino acids and incapable of supporting growth, was added to diets containing graded levels of sesame protein properly supple-

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

TABLE 1

Composition of basal ration and amino acid mixtures

Basal ration		Amino acid mixture		
			Amount	
Ingredient	Amount	Amino acid	Incomplete amino acid mix[1]	Casein-isolated soybean protein Δ[2]
	%		*% of mixture*	*% of total diet*
Mineral mix[3]	4.94	DL-alanine		0.08
Corn oil	3.00	L-arginine·HCl	12.0	
Fiber	3.00	L-aspartic acid		0.37
B-vitamins[4]	0.15	L-cystine	3.0	
Vitamin A, D and E mix[4]	0.10	L-glutamic acid	25.0	1.48
Choline chloride	0.20	L-histidine HCl·H_2O		0.28
Protein[5]	Variable	L-leucine		0.90
Glucose	to 100	DL-isoleucine	12.0	0.68
		L-lysine·HCl (99%)		0.78
		DL-methionine	3.0	
		L-phenylalanine		0.41
		DL-phenylalanine	8.0	
		L-proline		2.08
		DL-serine		0.22
		DL-threonine		0.64
		L-tryptophan		0.15
		DL-tryptophan	3.0	
		L-tyrosine	6.0	0.75
		DL-valine	12.0	1.32
		glycine	16.0	
			100.0	10.14

[1] This mixture was used at the levels indicated in experiment 1, table 2.

[2] This mixture, representing the amino acid composition *differences* between 20% (N × 6.25) casein and 15% (N × 6.25) isolated soybean protein was used in experiment 3, table 4. The methionine and glycine content of the casein and isolated soybean protein rations was set at 0.7 and 0.5% of total diet. The amounts representing the differences for isoleucine, threonine and valine in the amino acid mixture were doubled since the racemic forms were used and a corresponding amount subtracted from the glutamic acid difference.

[3] For composition see Fisher et al. ('60).

[4] For composition see Fisher and Johnson ('56).

[5] The variable ingredients which were added at the expense of glucose consisted of sesame meal (N × 6.25 = 46.3%), peanut meal (N × 6.25 = 48.5%), isolated soybean protein (N × 6.25 = 82.5%) and crude casein (N × 6.25 = 82.9%). The amino acid contents as given in Block and Weiss ('56) were used in calculating the additions necessary for the various amino acid levels indicated in tables 2–4.

mented with lysine (at 7% of the protein level). The results, shown together with the complete design of the experiment (table 2), clearly demonstrate a severe growth depression due to the unbalanced amino acid mixture at the 11% protein level, and essentially no depression on the 28% protein diet. This type of imbalance differs from the amino acid imbalance characterized by an exaggerated single amino acid deficiency for the following reasons. In the case of an exaggerated single amino acid deficiency, the growth depression was equally great at low- or high-protein levels provided the amino acid supplement was added in the same proportion to the protein concentration (Fisher et al., '60). In this study, in which the same protein source was used, as well as the same levels of protein were compared, *no* growth depression occurred at the high-protein level, and a much more severe depression occurred at the low-protein concentration when the amino acid supplement was completely unbalanced in terms of several essential amino acids.

The slightly reduced growth rate of groups fed the amino acid-supplemented and unsupplemented 28% protein diets compared with that of those fed the un-

TABLE 2

The effect of protein level on the growth-depressing properties of an imbalanced mixture of amino acids

Dietary variables		3-week av. weight	Feed utilization
Sesame protein[1]	Amino acid mixture[2]		
% of diet	*% of diet*	*gm*	*gm gain/gm feed consumed*
11	—	237±11[3]	0.36
11	4	174±11	0.28
23	—	405±5	0.61
23	8.4	367±9	0.62
28	—	387±5	0.58
28	10.2	379±7	0.65

[1] Adequate lysine (7% of protein) was provided through supplementation with L-lysine·HCl (95%).

[2] For composition see table 1. The amounts added represent a constant percentage of the sesame protein (36%).

[3] Mean ± standard error. Each value represents the average of 14 chicks (2 lots of 7). Average starting weight for all groups was 98 gm.

supplemented 23% protein diet is probably only a reflection of the reduced caloric density of the former diets, since the increase in sesame meal to provide the higher protein level added considerable fiber and indigestible matter to the 28% protein rations.

Trial 2. According to the definitions recently set forth by Harper ('58), an amino acid imbalance (in contrast with unbalance or toxicity) is perhaps best exemplified by the growth depression which occurs when a small quantity of a second most limiting amino acid is added in the absence of a supplement of the primarily deficient acid. Peanut meal protein offered a good opportunity to investigate more fully certain aspects of this phenomenon, since this protein is deficient in both lysine and methionine but otherwise adequate for rapid growth of chicks. The peanut protein was supplied at a low-protein level of 12% and also at the 24% level. As shown in table 3, the two limiting amino acids, lysine and methionine, were added factorially at each protein level.

The results indicate different patterns of response to the amino acid supplementations at the two protein levels (table 3). At 12% of protein, methionine gave a greater growth response than lysine, and the double supplementation was no better than the methionine supplementation alone. On the 24% protein diets the amino acid response was reversed: methi-

TABLE 3

Effect on growth and feed utilization of supplementing peanut protein with its two limiting amino acids (lysine and methionine)

Dietary variables			3-week av. weight	Feed utilization
Peanut protein	Lysine	Methionine		
% of diet	*% of protein*	*% of protein*	*gm*	*gm gain/gm feed consumed*
12	4	0.8	104±4[1]	0.09
12	4	2.3	127±6	0.18
12	7	0.8	116±5	0.13
12	7	2.3	128±11	0.21
24	4	0.8	194±6	0.37
24	4	2.3	185±7	0.37
24	7	0.8	229±11	0.45
24	7	2.3	269±13	0.52

[1] Mean ± standard error. Each value represents the average of 14 chicks (2 lots of 7). Average starting weight for all groups was 88 gm.

onine alone gave no response, lysine produced a large growth response which was further accentuated by the double supplementation. The effects of amino acid supplementation on the 24% protein ration follow an expected pattern on the basis of previous reports (Harper, '58). However, the responses obtained with the 12% protein level in the present case do not permit an overall generalization of the phenomenon of imbalance without further stipulation. Such a stipulation was provided in the previous report from this laboratory (Fisher et al., '60), which suggested that an imbalance could be created only when all nutrients were provided at levels sufficiently above the maintenance requirement to permit growth. At the 12% protein level most of the protein is required for maintenance and consequently the relationships of the limiting amino acids toward meeting the maintenance requirement play an important role in interpreting the results. Viewed in this light, the methionine response and the lack of response to lysine at the 12% protein level are manifestations of the relatively high methionine maintenance requirement of the chicken and the comparatively small maintenance requirement for lysine (Leveille and Fisher, '60).[3]

Trial 3. The suggestion made previously (Fisher et al., '60) that the high arginine requirement of chicks fed on casein-containing rations might be due to an amino acid imbalance was tested in this experiment. Since casein protein, when conventionally expressed on a 16% nitrogen basis, supplies approximately 20% more amino acids than, for example, soybean protein, an imbalance might be created in terms of this excess quantity of amino acid which places a stress on the most limiting amino acid in casein, namely, arginine.

In this study, isolated soybean protein was used at the 15% protein level supplying 1.24% of L-arginine (free base). This is considered the optimum requirement for arginine of chicks fed a diet containing 20% of protein from sources other than casein (Snyder et al., '56). To the diet containing 15% of isolated soybean protein a mixture of amino acids was added, representing the difference between 20% of casein (N × 6.25) and the 15% of soybean protein. The complete design is shown in table 4, the amino acid mixture in table 1.

[3] From the data of Leveille and Fisher ('60) for the maintenance requirement of the chicken it can be calculated that chicks receiving the 12% protein diet containing 0.8% of methionine and 4% of lysine would require approximately 9.4 mg of methionine per day and actually consumed only 4.5 mg. By contrast, the lysine requirement of these chicks is only about 2.87 mg per day with an actual consumption of 8.2 mg per day. It is therefore not unexpected that methionine is the first limiting amino acid at this protein level and that no response was obtained to added lysine.

TABLE 4

Increase in the arginine requirement, using an isolated soybean protein ration, as a result of adding an amino acid mixture representing the difference in amino acid composition between 15% of isolated soybean and 20% of casein protein

Dietary variables			3-week av. weight	Feed utilization
Protein source and % of diet	Arginine[1]	Amino acid mixture[2]		
	% of diet	*% of diet*	*gm*	*gm gain/gm feed consumed*
Isolated soybean protein, 15%[3]	1.24	—	288 ± 8[4]	0.52
Isolated soybean protein, 15%	1.24	10.14	232 ± 15	0.46
Isolated soybean protein, 15%	1.60	10.14	301 ± 9	0.59
Casein protein, 20%	1.24	—	257 ± 11	0.47
Casein protein, 20%	1.60	—	275 ± 9	0.52

[1] Added as L-arginine·HCl.

[2] Represents the difference in amino acid composition between 15% of isolated soybean protein and 20% (N × 6.25) of casein protein.

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

[4] Mean ± standard error. Each value represents the average of 14 chicks (2 lots of 7). Average starting weight for all groups was 101 gm.

The results indicate that the addition of the amino acid mixture to the isolated soybean protein (thereby simulating 20% casein) depressed growth, perhaps by creating (or exaggerating) an arginine deficiency. This assumption is clearly warranted since an arginine supplementation resulting in 1.60% of total arginine (free base) not only overcame the growth depression obtained with the 1.24% ration but permitted utilization of the amino acid mixture, as exemplified by the superior growth of the amino acid-supplemented 1.6% arginine diet compared with the 15% soybean protein diet to which no amino acid mixture had been added.

DISCUSSION

The results of trial 1 (table 2) confirm the previous assertion (Fisher et al., '60) that an amino acid imbalance can be created which is different from one corrected by the addition of a small amount of a limiting amino acid. Trial 1 differs from the one in which a single amino acid deficiency is exaggerated in at least two respects: (1) a similar growth depression was also obtained through the addition of a mixture of nonessential amino acids (Fisher et al., '60) and (2) the imbalance using diets with a single amino acid deficiency was as great at a high-protein level as it was with low protein, when the amino acid supplement was added in the same proportion to the total protein. This was not the case in trial 1 where the imbalance was completely overcome at the high-protein level even though the amino acid supplement was given in the same proportion to total protein.

The second experiment, in which peanut protein deficient in lysine and methionine was studied, illustrates the importance of giving adequate consideration to the relative requirements of amino acids for maintenance or growth when dietary changes are made to approach either the maintenance requirement only or, simultaneously, the aggregate requirement for maintenance and optimal growth requirements. Thus, at the low-protein level (table 3), which supported primarily maintenance and little growth, the methionine requirement assumed greater importance than the lysine requirement, with the reverse true at the high-protein level which permitted rapid growth. These observations are supported by the proportionally greater maintenance requirements for methionine versus lysine and in turn a greater requirement for lysine than for methionine for rapid growth (Leveille and Fisher, '60; Klain et al., '60).

Applications may be made of these results in predicting the relative change in amino acid requirement (expressed as a percentage of the protein) with changes in the protein content of the ration. Since the methionine requirement for maintenance, relative to the other amino acids, is much higher than in the case of growth, the methionine requirement could be predicted to show a considerable decrease as protein level increased. The data of Griminger[4] for the methionine requirement at 10 and 20% protein levels show a reduction of 29% at the higher protein level than at the lower level. For lysine, the maintenance requirement is very low relative to other amino acids, whereas the requirement for rapid growth is relatively high. The lysine requirement would therefore not be expected to decrease as much as the methionine requirement when protein is increased to a higher level. This is confirmed by recent data from this laboratory which showed a 14% reduction in the lysine requirement as percentage of the protein as the protein level is increased from 11 to 23% (Fisher et al., '60), which is less than half the change registered for methionine.

Aside from providing a partial explanation for the long-perplexing problem of a high arginine requirement of chicks fed casein-containing diets (table 4), trial 3 touches upon other important considerations related to the problem of amino acid imbalance. In the first place, attention is drawn to the inadequacy of the term "imbalance" as related strictly to the addition of a nitrogen supplement to a ration containing an amino acid-deficient protein. It has been shown with casein that an inherent imbalance exists for the growing chicken in contrast with another protein, which became imbalanced when amino

[4] Griminger, P. 1955 Effects of certain factors on the amino acid requirements of the chick. Doctoral thesis, University of Illinois, Urbana.

acids were added to simulate the composition of casein. It seems therefore only a question of the base reference line whether to consider the isolated soybean protein plus amino acid mixture (to simulate casein) a case of amino acid imbalance, and the response to casein alone, an amino acid deficiency. Therefore, it appears to be primarily a question of semantics whether to apply the term imbalance, deficiency, or exaggerated deficiency to a condition as observed in trial 3.

The considerable difference in amino acid content of equal amounts of casein versus other proteins makes important the need to pay closer attention to the amino acid composition of isonitrogenous rations. Since it is common practice to compare diets on the basis of total nitrogen content rather than on total amino acid content, observed differences in the requirement for a single essential amino acid studied with various protein sources might well be related to differences in amino acid content of isonitrogenous rations. From the standpoint of amino acid balance, casein appears to be a poor choice as a standard for protein evaluations as long as so little attention is paid to amino acid content and the emphasis remains on nitrogen equality between rations.

SUMMARY

Three trials with week-old chicks were carried out to elucidate aspects of amino acid imbalance.

1. It was found that the addition of an amino acid mixture lacking in several essential amino acids to a balanced low-protein diet induced an imbalance which was gradually overcome at higher protein levels even when the amount of the amino acid mixture added remained constant at a percentage of the protein.

2. The factorial supplementation of peanut protein at 12 and 24% of the diet with its two limiting amino acids, methionine and lysine, resulted in improved growth at the 12% protein level only when methionine was added. No effect was observed when lysine was added at this protein level. At the 24% protein level, a slight growth depression occurred upon the addition of methionine alone, while a large response was obtained from lysine alone and the addition of both amino acids improved growth still further. This differential response at the two protein levels can be explained in terms of the relatively greater methionine requirement for maintenance at the low protein level versus a relatively higher lysine to methionine ratio required for rapid growth at the high protein level.

3. The high arginine requirement of chicks fed casein-containing diets may be explained in part by the relative excess of amino acids in casein protein when the latter is calculated to contain 16% of nitrogen. Supplementing 15% isolated soybean protein with the amino acids making up the difference between 20% casein protein ($N \times 6.5$) and the 15% soybean protein depressed growth using the latter diet which contained 1.24% arginine; this depression was overcome by adding more arginine.

4. The implications of these experiments in terms of amino acid imbalance are discussed.

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LITERATURE CITED

Block, R. J., and K. W. Weiss 1956 Amino Acid Handbook. Charles C Thomas, Springfield, Illinois.

Fisher, H., and D. Johnson, Jr. 1956 The amino acid requirement of the laying hen. I. The development of a free amino acid diet for maintenance of egg production. J. Nutrition, 60: 261.

Fisher, H., P. Griminger, G. A. Leveille and R. Shapiro 1960 Quantitative aspects of lysine deficiency and amino acid imbalance. J. Nutrition, 71: 213.

Harper, A. E. 1958 Balance and imbalance of amino acids. Ann. New York Acad. Sc., 69: 1025.

——— 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, 68: 405.

Klain, G. J., H. M. Scott and B. C. Johnson 1960 The amino acid requirement of the growing chick fed a crystalline amino acid diet. Poultry Sci., 39: 39.

Leveille, G. A., R. Shapiro and H. Fisher 1960 Amino acid requirements in the adult rooster. IV. The requirements for methionine, cystine, phenylalanine, tyrosine, and tryptophan; the adequacy of the determined requirements. J. Nutrition, 72: 8.

Synder, J. M., W. D. Morrison and H. M. Scott 1956 The arginine requirement of chicks fed purified and corn-soya diets. Poultry Sci., 35: 852.

Influence of Selenium, Antioxidants and Type of Yeast on Vitamin E Deficiency in the Adult Chicken[1,2]

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During the past decade, considerable interest has been shown in a comparison of vitamin E-deficient diets containing Torula and dried brewers' yeast as the major source of protein. Schwarz ('52) showed that feeding such a diet containing Torula yeast to rats produced necrotic liver degeneration which could be prevented by adding either vitamin E, cystine or Factor 3. Feeding a diet of similar composition to chicks (Scott et al., '55) caused the development of exudative diathesis, which was prevented by vitamin E or Factor 3, but not by cystine. Schwarz and Folts ('57) and Patterson et al. ('57) demonstrated that selenium could replace Factor 3 for both rats and chicks.

Studies were undertaken in our laboratory to determine the effect of vitamin E-deficient diets containing Torula or dried brewers' yeast on reproduction in the chicken. The present report shows that feeding a Torula yeast diet reduced hatchability and markedly increased mortality of offspring during the first week after hatching. Addition of vitamin E prevented both abnormalities, but substitution of brewers' yeast for Torula yeast prevented only the high incidence of early chick mortality. Antioxidants or selenium had little or no effect on either hatchability or chick mortality.

PROCEDURE

In this study 20 Single-Comb White Leghorn pullets per group were used, and housed in 4′ × 8′ cages with wire floors. Three White Leghorn cockerels were placed in each pen. Composition of the basal diet fed is presented in table 1. In one treatment the 3% of tallow was substituted for glucose. All other supplements were added to the diet.

TABLE 1
Composition of basal diet

Ingredients	%
Yeast (Torula or dried brewers')	37.5
Glucose	47.8
Calcium carbonate	4.5
Dicalcium phosphate	1.5
Sodium chloride, iodized	0.4
Gelatin	4.0
DL-Methionine	0.3
Mineral mixture[1]	2.0
Vitamin mixture[2]	2.0

[1] KCl, 0.2; $MnSO_4$, 0.019 and $ZnSO_4$, 0.001%; plus glucose.

[2] Vitamin A, 1000 I.U.; vitamin D_3, 100 I.U.; vitamin K (menadione bisulfate), 0.12 mg; thiamine·HCl, 0.25 mg; riboflavin, 0.5 mg; niacin, 2.5 mg; Ca pantothenate, 1.25 mg; folic acid, 0.125 mg; vitamin B_{12}, 1.25 μg; pyridoxine·HCl, 0.35 mg; and choline chloride, 100 mg/100 gm diet, plus glucose.

In all experiments, eggs were saved for 7 days before incubation. In experiment 1, only eggs laid during the second and 6th week of the experiment were incubated. In all other experiments, all eggs of sound shell laid by the hens were incubated. Eggs were candled at 7 days to determine fertility, and the percentage of hatchability was based on fertile eggs only. Dur-

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[2] A preliminary report of some of this work was presented before the American Institute of Nutrition, 21st Annual Meeting, 1957, Chicago.

ing the first experiment, a high mortality in chicks hatching from eggs from certain treatments was observed. Therefore, in this experiment and all others, chicks hatched following the various treatments were placed in battery brooders and given feed and water ad libitum. The feed was a stock diet consisting largely of yellow corn, soybean oil meal, fish meal and other ingredients commonly used in starting-diets for young chicks. Chicks which died in the hatching tray, as well as those that died later in batteries, were included in the determination of percentage of mortality.

RESULTS

In experiment 1, three pens of hens received each dietary treatment. Results reported are based on eggs laid during the 6th week (table 2). Eggs laid by hens fed the Torula yeast basal diet hatched at a lower rate and produced chicks with much lower viability than those laid by hens given the same diet plus vitamin E. Addition of vitamin E improved hatchability and reduced the percentage of mortality of chicks to a low level. Addition of an antioxidant, N,N′-diphenyl - *p*-phenylenediamine (DPPD) may have improved hatchability to some extent, but had no effect on viability.

In experiment 2, two pens of hens received each dietary treatment. The experiment was conducted for 5 weeks and all eggs laid were incubated. Feeding the Torula yeast basal diet without supplemental vitamin E again caused low hatchability of fertile eggs and viability of chicks (table 2). Again, addition of vitamin E markedly improved hatchability and reduced mortality.

It was observed in experiment 1 that rate of egg production was low for all birds fed the experimental diets, regardless of supplements. Because the diet had no source of supplemental fat, 3% of tallow was added as one of the treatments in experiment 2 to determine whether egg production rate could be improved, as well as to study the effect of this fat on hatchability and mortality of chicks. Addition of fat to the basal diet had no great effect on hatchability or chick viability, and did not improve rate of egg production.

Complete substitution of dried brewers' yeast for Torula yeast in the basal diet did not improve hatchability greatly, but markedly reduced mortality of chicks. Results from feeding a stock diet were comparable with those obtained by feeding the basal diet supplemented with vitamin E.

It was evident that the mortality of chicks from eggs laid by hens fed the Torula yeast basal diet deficient in vitamin E was extremely high. Many of the chicks died in the hatching trays before being removed from the incubator. Most of the others died within the first three or 4 days after hatching. Examination of some of the dead chicks showed the brains to be moist and edematous (encephalo-

TABLE 2

Effect of type of yeast, vitamin E, DPPD[1] and tallow on hatchability and viability of chicks

Diet	Experiment 1		Experiment 2	
	Fertile eggs hatched	Chick mortality	Fertile eggs hatched	Chick mortality
	%	%	%	%
Torula yeast basal	68.5	52.1(71)[2]	33.8	42.8(21)
Torula yeast basal + vitamin E (12 I.U./kg)	89.0	1.7(59)	72.4	1.1(90)
Torula yeast basal + DPPD (250 mg/kg)	81.4	53.3(60)	—	—
Torula yeast basal + tallow (3%)	—	—	41.8	46.7(30)
Brewers' yeast basal	—	—	41.5	10.7(56)
Stock	—	—	74.0	9.5(180)

[1] DPPD indicates N,N′-diphenyl-*p*-phenylene diamine.
[2] Number of chicks hatched indicated in parentheses.

malacia). Other characteristic gross lesions or abnormalities were not evident. Even though the chicks received a stock diet adequate in vitamin E, most of those that died had been too weak to consume much feed.

Experiment 3 was conducted to compare, in addition, diets containing Torula and dried brewers' yeast, with and without vitamin E and with and without antioxidant. The birds in two pens were fed each of the experimental diets and the experimental period extended for 14 weeks. Treatment and results are shown in table 3. No marked reduction in hatchability of fertile eggs was observed until the last 4 weeks of the experiment. This was probably caused by the large reserves of vitamin E in the birds used. Pullets were approximately 8 months old when placed on experiment and had been reared on range with abundant green plants. Hatchability was lower using the Torula yeast basal diet than with the brewers' yeast. However, a definite improvement in hatchability was obtained by supplementing both diets with vitamin E. Addition of the antioxidant, 2,6-ditertiary-butyl-*p*-cresol (BHT), to either basal diet failed to improve hatchability. Mortality of chicks increased progressively in those hatched from eggs laid by hens fed Torula yeast and Torula yeast supplemented with BHT. There was no increase in mortality when the diet was supplemented with vitamin E. On the other hand, no progressive increase in early chick mortality was observed among any of the treatments involving brewers' yeast. Therefore, a distinct difference in the expression of a vitamin E deficiency was evident in the adult chicken between birds fed diets containing Torula and brewers' yeast. In both cases, hatchability of fertile eggs was reduced, but only in the case of Torula yeast was early chick mortality increased.

During the course of these studies, results of experiments were reported demonstrating that selenium accounted for the marked differences observed with rats and chicks fed vitamin E-deficient diets containing Torula or brewers' yeast (Schwarz and Foltz, '57, and Patterson et al., '57). A 4th experiment was conducted, therefore, to determine the effect on hatchability and early chick mortality of adding selenium to a basal diet containing Torula yeast. One group received each experimental diet for the 13-week experiment. Results are presented in table 4.

Addition of selenium had no marked effect on hatchability and did not lower mortality level in chicks. Addition of vitamin E again markedly improved hatchability and reduced mortality in chicks. Brewers' yeast again failed to maintain

TABLE 3

Effect of type of yeast, vitamin E and BHT[1] on hatchability and chick viability (experiment 3)

Diet	Fertility 11–14 weeks	Fertile eggs hatched			Chick mortality		
		Weeks[2]			Weeks		
		4–6	7–10	11–14	4–6	7–10	11–14
	%	%	%	%	%	%	%
Torula yeast basal	81.7	82.7	83.6	54.8	12.6(223)[3]	17.0(294)	48.9(135)
Torula yeast basal + vitamin E (44 I.U./kg)	68.0	95.0	94.6	82.5	5.2(212)	5.1(297)	5.1(216)
Torula yeast basal + BHT (250 mg/kg)	76.8	85.9	84.6	56.5	13.4(172)	21.6(283)	34.9(166)
Brewers' yeast basal	86.5	81.2	91.9	77.4	11.9(185)	4.5(310)	7.0(271)
Brewers' yeast basal + vitamin E (44 I.U./kg)	79.6	92.4	92.7	84.9	6.1(230)	4.8(315)	5.4(297)
Brewers' yeast basal + BHT (250 mg/kg)	85.0	88.9	87.7	70.8	9.5(222)	6.0(317)	10.0(224)

[1] BHT indicates 2,6-ditertiary-butyl-*p*-cresol.
[2] Time hens were on experiment.
[3] Number of chicks hatched indicated in parentheses.

TABLE 4

Effect of selenium and vitamin E on egg production, hatchability and chick mortality (experiment 4)

Diet	Egg production	Fertile eggs hatched	Chick mortality
	%	%	%
Torula yeast basal	52.0	45.5	25.0(312)[1]
Torula yeast basal + selenium[2] (1 ppm)	47.8	50.4	29.1(278)
Torula yeast basal + vitamin E (44 I.U./kg)	41.6	72.8	9.5(401)
Brewers' yeast basal	26.3	47.7	10.3(146)

[1] Number of chicks hatched indicated in parentheses.
[2] Added as $(NH_4)_2SeO_4$.

high hatchability, but chick mortality remained at a level similar to that observed with the use of Torula yeast supplemented with vitamin E.

Rate of egg production is also presented for experiment 4. As in previous experiments, rate of egg production was low for all treatments. Addition of vitamin E had no beneficial effect. It was difficult to get an accurate expression of rate of egg production because birds fed these diets developed the habit of eating their eggs. Unless eggs were collected frequently, some would be broken by the hens and fall through the wire mesh floor. It appeared that the hens were attempting to obtain something to counteract an imbalance in their diet or overcome a deficiency, because after feed was changed to a stock diet, egg-eating ceased. Within three weeks after starting to receive a stock diet, egg production of all birds in the 4 pens in this experiment was over 60%. In experiment 4, rate of egg production for birds fed brewers' yeast was much less than that of those fed Torula yeast.

DISCUSSION

Results of experiments reported in this paper demonstrate a difference in the expression of a vitamin E deficiency between hens fed a diet containing Torula yeast as the major source of protein and those fed dried brewers' yeast. In the absence of supplemental vitamin E, hatchability of fertile eggs was markedly lowered with both diets, but high, early chick mortality was observed only in those hatched from hens fed the Torula yeast basal diet. In view of these results, it appears that vitamin E functions in the normal development of the chick embryo to the stage of hatching, but that vitamin E and some other nutrient (or antagonist) is involved in viability of chicks once they are hatched.

Recent work (Schwarz and Foltz, '57, and Patterson et al., '57) shows that an obvious difference between brewers' and Torula yeast is the selenium content. As shown in experiment 4, however, selenium had very little effect on hatchability and did not reduce mortality of offspring. It appears, therefore, that the difference in incidence of early chick mortality cannot be attributed to selenium content. It is also apparent that selenium cannot replace vitamin E as it functions in the development of normal embryos. One can argue that the level of selenium may not have been adequate to meet the needs of the laying hen. A level of 1 ppm, however, was equivalent to or greater than that contributed by brewers' yeast. Moxon and Poley ('38) reported that selenium from seleniferous wheat is readily transferred to the egg when fed to laying hens.

Studies on acceleration of vitamin E deficiency in chicks by Torula yeast have been reported recently by Bieri et al. ('58a, b) who found that the ash and lipid content of Torula yeast promoted exudative diathesis. Adding a combination of tall oil and a synthetic ash to a diet not containing Torula yeast was as effective in producing exudates as including Torula yeast in the diet. Prior to this work, Dam et al. ('57) had reported that samples of Torula yeast contained a higher level of

linoleic acid than previously reported. It is possible, therefore, that the effect of the difference between Torula and brewers' yeast observed in the hens is caused by the higher level of unsaturated fat present in Torula yeast, which, in turn, influences composition of egg yolks. Perhaps the inorganic constituents of Torula yeast had an influence, but quantity and composition of the ash of the two yeasts are not too different.

If the difference between the effect of the yeasts on early chick mortality was accounted for by presence of a higher level of unsaturated fat in Torula yeast, it is difficult to explain why two different antioxidants had no effect. In most studies with chicks, antioxidants are able to replace vitamin E in prevention of vitamin E-deficiency symptoms brought on by the presence of unsaturated fat in the diet. In previous studies in this laboratory with turkey hens, (Jensen et al., '56, and Jensen and McGinnis, '57), it was found that antioxidants (BHT and DPPD) were able to overcome part of the deleterious effect of unsaturated fat on hatchability. These birds were fed diets of natural ingredients, however, and it is probable that the antioxidants were functioning by preventing the oxidation of natural vitamin E in the diet. Diets used in the present study contained little or no vitamin E and, therefore, antioxidants would have been of no value from this standpoint. Apparently, DPPD and BHT were not transferred to the egg by the hen or, if transferred, were unable to function in place of vitamin E. Recently, Machlin et al. ('59), have shown that 1,2-dihydro-6-ethcxy 2,2,4-trimethylquinoline was more active than other antioxidants in the prevention of encephalomalacia and muscular degeneration in chicks. Furthermore, it was more active than α-tocopherol *in vitro* in the prevention of liver peroxide formation. Investigation of this antioxidant, as well as of higher levels of DPPD and BHT, and perhaps other antioxidants, should be undertaken before it is concluded that vitamin E *per se* is required for normal reproduction in the chicken.

Aside from a deficiency of vitamin E, it was apparent that purified diets containing Torula or brewers' yeast as a major source of protein were not adequate for optimum egg production in chickens. These diets apparently were deficient in a factor necessary for optimum egg weight (Jensen et al., '58) since egg weight was greatly increased when hens in experiment 4 received a stock diet consisting of natural ingredients. Results reported in this paper do not affect the use of Torula yeast as a food supplement because in the presence of adequate vitamin E, Torula yeast was comparable with brewers' yeast in all respects.

SUMMARY

Studies on the effect of feeding semipurified diets containing Torula or dried brewers' yeast as a major source of protein on the expression of vitamin E deficiency in Single-Comb White Leghorn hens are reported. Hatchability of fertile eggs was markedly lowered by feeding of both diets. Mortality of offspring was markedly increased by using Torula yeast, but not with brewers' yeast. Two antioxidants had no effect on chick viability. Addition of 1 ppm of selenium failed to improve either hatchability or chick viability.

LITERATURE CITED

Bieri, J. G., G. M. Briggs and C. J. Pollard 1958a The acceleration of vitamin E deficiency in the chick by Torula yeast. J. Nutrition, *64:* 113.

——— 1958b Acceleration of vitamin E deficiency by torula yeast. II. Effect of torula yeast ash and lipid. Proc. Soc. Exp. Biol. Med., *99:* 262.

Dam, H., G. K. Nielsen, I. Prange and E. Søndergaard 1957 The exudative diathesis produced by torula yeast. Experientia, *13:* 493.

Jensen, L. S., and J. McGinnis 1957 Studies on the vitamin E requirement of turkeys for reproduction. Poultry Sci., *36:* 1344.

Jensen, L. S., J. S. Carver and J. McGinnis 1956 Effects of vitamin E, and N,N′-diphenyl-paraphenylene diamine and fish liver oil on reproduction in turkeys. Proc. Soc. Exp. Biol. Med., *91:* 386.

Jensen, L. S., J. B. Allred, R. E. Fry and J. McGinnis 1958 Evidence for an unidentified factor necessary for maximum egg weight in chickens. J. Nutrition, *65:* 219.

Machlin, L. J., R. S. Gordon and K. H. Meisky 1959 The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken. Ibid., *67:* 333.

Moxon, A. L., and W. E. Poley 1938 The relation of selenium content of grains in the ration to the selenium content of poultry carcass and eggs. Poultry Sci., *17:* 77.

Patterson, E. L., R. Milstrey and E. L. R. Stokstad 1957 Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exp. Biol. Med., 95: 617.

Schwarz, K. 1952 A hitherto unrecognized factor against dietary necrotic liver degeneration in American yeast (Factor 3). Ibid., 78: 852.

Schwarz, K., and C. M. Foltz 1957 Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc., 79: 3297.

Scott, M. L., F. W. Hill, L. C. Norris, D. C. Dobson and T. S. Nelson 1955 Studies on vitamin E in poultry nutrition. J. Nutrition, 56: 387.

Studies of the Effect of Lysine on the Absorption of Radiocalcium and Radiostrontium by the Rat[1]

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The influence of amino acids and other organic compounds on the absorption of calcium 45 and strontium 89 from a single oral dose has been investigated by Wasserman et al. ('56). Certain amino acids, in particular L-lysine and L-arginine, were shown to markedly increase the absorption of these mineral elements. L-Lysine was also shown to promote the absorption of a single dose of calcium 45 by vitamin D-deficient rats (Wasserman et al., '57). The addition of lysine to either the diet or drinking water of rats has been shown by McClure ('52) to reduce markedly the incidence of dental caries. Since intraperitoneal injection of lysine was less effective, it is probable that this amino acid exerted its effect in the alimentary tract, possibly by increasing the absorption of calcium.

In view of the favorable effect of L-lysine on calcium and strontium absorption, it was of interest to obtain information concerning its mechanism of action, and examine the longer-term effects of lysine by supplementing both normal and calcium-deficient diets with various additions of L-lysine.

EXPERIMENTAL

For most of the single-dose studies Ca^{45} and Sr^{85} absorption was determined by administering orally 2 ml of the test dose to young male albino rats[3] (100 to 150 gm) that had been fasted for 24 hours. The animals were killed 24 hours later and the femurs removed for radioassay of accumulated Ca^{45} and Sr^{85}. The percentage of the dose of Ca^{45} or Sr^{85}, or both, taken up by one femur was then determined. Several previous reports (Wasserman et al., '56; Lengemann and Dobbins, '58; Lengemann et al., '59) have demonstrated the validity of using the accumulated Ca^{45} and Sr^{85} in the femur as an index of gastrointestinal absorption under properly defined conditions.

For the studies involving particular sections of the alimentary tract, the rats were anesthetized with ether, and 1 ml of the test dose was injected directly into the lumen of a ligated section of the tract. The animals were killed 5 hours after injection, and then one femur was taken for analysis.

Unless otherwise indicated, each milliliter of the dosing solution used in the gavage or ligation studies contained 5 mg of calcium chloride (1.8 mg Ca), 5 μc of Ca^{45}, and 0.5 μc of Sr^{85}, plus the substances being tested, and was adjusted to approximately pH 7.0.

The effect of supplementing an adequate diet for growing rats with additional lysine was studied over a 14-day balance trial with rats of about 80 gm, live weight, initially. The percentage composition of the basal diet was starch 48.5; vitamin-free casein, 15; sucrose, 20; vegetable oil, 10; mineral supplement, 4; tryptophan, 0.5; and a complete vitamin supplement, 2.[4] Three levels of L-lysine were added to this diet. Immediately prior to the actual trial, all rats were placed in individual cages and given the basal ration for one week. They were then divided into 4 groups, of 6

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[3] Albino Farms, Red Bank, New Jersey.
[4] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland.

rats each. One group received the basal diet and the other three groups were fed the diets containing 1, 2, and 4% of additional lysine. The drinking water supplied 40 μc of Ca^{45} and 20 μc of Sr^{85} per liter for the duration of the trial. All the rats were killed at the end of the 14-day trial and whole-body contents of stable Ca, Ca^{45}, and Sr^{85} determined on the ashed carcasses by standard procedures.

The absorption of Sr^{85} from a single feeding was studied by conditioning rats to rapidly consume their daily allowance of food. Four groups were then fasted overnight and the animals of each group given a meal (6 gm) of their respective diet containing 10 μc of Ca^{45} and 1 μc of Sr^{85}. The feed in the cups was removed from the rats after 4 hours, assayed, and corrections made for residual Sr^{85} where necessary. All the animals were killed 24 hours after feeding, and one femur was taken for Sr^{85} assay.

The effect of supplementing a low-calcium diet with additional lysine was studied by placing two groups of 6 rats in individual cages. One group was given a constant daily intake of a calcium-deficient diet (0.08% Ca) comprised in per cent of sucrose, 68; purified casein, 24; vegetable oil, 5; a calcium-free salt mixture, 3; and a complete vitamin fortification mixture. The rats in the other group were given an equal intake of this diet containing, in addition, 2.09% of L-lysine. For the duration of the 10-day trial 40 μc of Ca^{45} and 20 μc of Sr^{85} per liter were provided in the drinking water. The animals were then killed, and the deposition of Sr^{85} in one femur was determined.

The determinations of Sr^{85} in either liquid samples or femurs were made directly using a deep-well scintillation counter. For the estimation of Ca^{45}, the whole body or one femur was ashed at 600°C, the ash taken up in HCl, and Ca^{45} determined by the method described by Comar ('55). Stable Ca was determined gravimetrically as the oxalate precipitate.

In some of these studies, the Sr^{85} retention in the total rat was determined by the use of an improvised whole-body counter. A scintillation probe with a 2-inch NaI crystal was placed in a horizontal position and shielded with lead. The rats to be counted were placed in ventilated quart glass jars and counted at a distance of 16 inches; at this distance, movements of the rat within the jar would not appreciably affect the counting rate. The radioactivity in the rat was compared to a known standard.

RESULTS

The initial studies were designed to establish the principal site of action in the alimentary tract, and some of the conditions necessary for lysine to exert its effect of enhancing the absorption of calcium and strontium. The data of table 1 show the results of injecting test doses directly into different ligated sections of the alimentary tract. It is evident that, although most absorption of Sr^{85} and Ca^{45} took place in the duodenum and jejunum, the enhancing effect of lysine was most pronounced in the ileum. The increased absorptions of Sr^{85} and Ca^{45} in the ileum and colon were both significant at the 1% level of confidence. Relatively little effect occurred in the duodenum and jejunum.

In other experiments it was found that lysine, as was observed for lactose (Lengemann et al., '59), had to be in the same segment as the dose solution in order to

TABLE 1

The effect of lysine on the absorption of Sr^{85} and Ca^{45} from various ligated segments of the digestive tract[1]

Segment	Sr^{85} uptake by femur		Ca^{45} uptake by femur	
	No lysine	Lysine	No lysine	Lysine
	% dose/femur		*% dose/femur*	
Stomach	0.2 ± 0.07	0.2 ± 0.07	0.2 ± 0.03	0.3 ± 0.10
Duodenum	2.4 ± 0.12	3.0 ± 0.16	3.1 ± 0.12	2.9 ± 0.16
Jejunum	2.0 ± 0.18	2.6 ± 0.20	2.3 ± 0.23	3.1 ± 0.20
Ileum	0.6 ± 0.17	1.4 ± 0.10	1.0 ± 0.16	2.2 ± 0.23
Colon	0.6 ± 0.07	0.9 ± 0.05	1.3 ± 0.09	1.8 ± 0.11

[1] Each value represents the mean of 6 animals ± the standard error of the mean.

increase absorption. Also, the promotive effect of lysine on absorption of Sr^{85} from the ileum was discernible as early as 30 minutes after injection, and the deposition per femur was markedly increased after one hour.

The effect of pH on the action of lysine was studied by injecting test doses that ranged in pH from 5.3 to 10.7 directly into ligated ileal segments; the pH adjustments were made with NaOH. The depositions of Sr^{85} per femur indicated that the action of lysine was prevented once the pH of the solution was equal to or greater than 9.7 (the isoelectric point for lysine).

The results of the 14-day balance trial involving diets containing 4 levels of lysine are shown in table 2. It is apparent that supplementing the basal diet with lysine did not have any marked effect on the net retentions of stable Ca, Ca^{45}, or Sr^{85}. Whole-body counts of Sr^{85} in the rats, taken at two-day intervals throughout the trial, indicated that a similar situation existed at each stage of the experiment. It is of interest that the Sr^{85}/Ca^{45} ratio remained very close to 0.40 for each of the 4 treatments.

Since lysine had been shown to produce a marked increase in absorption of a single dose of Ca^{45} and Sr^{85} when the dose was administered to fasted rats, it seemed possible that lysine supplementation of a single meal given to fasted rats might also produce a response. The mean depositions of Sr^{85} per femur resulting from a single feeding of each of the diets used in the 14-day balance trial are shown in table 2. These results are in good agreement with those obtained in the balance trial and indicate that significant differences did not exist. In view of the balance trial, it can be assumed that absorption of Ca^{45} would also be only slightly affected by supplemental lysine.

The effect of supplementing a low-calcium diet with 2.09% of additional lysine on the absorption of Sr^{85} over a 10-day trial also indicated that extra lysine was ineffective under these conditions. The mean percentage dose of Sr^{85} per femur was 1.16 for the basal diet and 1.15 for the lysine-supplemented diet. Whole-body counts of Sr^{85}, taken at 4 and 7 days, showed that the lysine supplement did not at any period increase the retention of Sr^{85}. In view of this, it appeared likely that certain dietary conditions were interfering with the lysine response observed only when used alone.

The lack of effect of supplemental lysine in enhancing Ca^{45} and Sr^{85} absorption under conditions of chronic ingestion could be due to several reasons; among these are: (a) that the ratio of lysine to calcium is too low in the total diet to give a response; (b) that other substances in the diet inhibited the effect of lysine; (c) that the accumulative effect of the amino acids from digested protein in the intestinal tract produced a maximum response that could not be further increased by additional lysine. To test these possibilities, several experiments were performed. In the first experiment, the effect of different ratios of lysine to calcium was examined. The results, as shown in table 3, indicated that with a molar ratio of lysine to calcium of 13.9 there was a significant increase in Sr^{85} and Ca^{45} absorption. When the ratio of lysine to calcium was 1.39, there was no significant effect, but it is noteworthy that the

TABLE 2

The effect of supplementation with lysine on the whole-body net retentions of stable Ca, Ca^{45}, and Sr^{85} during a 14-day trial and the retentions of Sr^{85} from a single feeding[1]

Treatment	Balance trial			Single feeding Sr^{85}
	Stable Ca[2]	Ca^{45}	Sr^{85}	
	mg Ca	*% of dose*	*% of dose*	*% dose/femur*
Basal (estimated 1.3% lysine)	380 ± 23	63.2 ± 1.9	26.7 ± 1.2	1.09 ± 0.08
Basal + 1% lysine	383 ± 16	64.9 ± 1.8	24.6 ± 1.3	1.24 ± 0.11
Basal + 2% lysine	432 ± 11	69.6 ± 1.5	28.9 ± 1.5	1.23 ± 0.08
Basal + 4% lysine	396 ± 13	68.9 ± 1.7	27.9 ± 1.6	1.26 ± 0.05

[1] Each value represents the mean of 6 animals ± the standard error of the mean.

[2] Increases in body contents of stable Ca during the balance trial were calculated by using the mean Ca content per 100 gm body weight of three rats sacrificed at the start of the trial to compute the initial body contents of stable calcium.

TABLE 3

The absorption of Sr^{85} and Ca^{45} as affected by the levels of lysine and stable calcium in the dose[1]

Treatment	Molar ratio lysine:Ca	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}
		% dose/femur	*% dose/femur*	
10 mg $CaCl_2$ (3.6 mg Ca) + Sr^{85} + Ca^{45}	—	1.43 ± 0.07	2.28 ± 0.09	0.63
10 mg $CaCl_2$ + Sr^{85} + Ca^{45} + 0.125 mmole lysine	1.39	1.38 ± 0.12	2.35 ± 0.20	0.59
10 mg $CaCl_2$ + Sr^{85} + Ca^{45} + 1.25 mmole lysine	13.9	2.72 ± 0.32	4.13 ± 0.31	0.66
100 mg $CaCl_2$ + Sr^{85} + Ca^{45}	—	1.20 ± 0.05	1.72 ± 0.04	0.70
100 mg $CaCl_2$ + Sr^{85} + Ca^{45} + 1.25 mmole lysine	1.39	1.51 ± 0.17	2.38 ± 0.20	0.63

[1] Each value represents the mean of 6 animals ± the standard error of the mean.

TABLE 4

The influence of certain amino acids on the action of lysine in stimulating the absorption of Sr^{85} [1]

Treatment	Sr^{85} uptake by femur			
	No lysine	Percentage change from nonsupplemented group	Lysine	Percentage change from nonsupplemented group
	% dose/femur	*%*	*% dose/femur*	*%*
$CaCl_2$ + Sr^{85}	1.33 ± 0.09	0	2.37 ± 0.36	+ 78
$CaCl_2$ + Sr^{85} + arginine	3.24 ± 0.25	+144	3.22 ± 0.17	+142
$CaCl_2$ + Sr^{85} + glycine	1.47 ± 0.09	+ 11	3.00 ± 0.34	+126
$CaCl_2$ + Sr^{85} + glutamic acid	1.25 ± 0.06	− 6	1.89 ± 0.14	+ 42
$CaCl_2$ + Sr^{85} + histidine	—	—	2.15 ± 0.15	+ 62

[1] Each value represents the mean of 6 animals ± the standard error of the mean. The 2-ml dose of each animal contained 10 mg of $CaCl_2$ (3.6 mg Ca) and 1.0 mmole of each of the amino acids indicated (L-form).

average depositions of both Sr^{85} and Ca^{45} were appreciably greater when the higher absolute level of lysine was given. The results may be compared with earlier observations (Wasserman et al., '56) that a maximum response to lysine occurred at a lysine-to-calcium molar ratio of about three. Next, the influence of basic, neutral or acidic amino acids on lysine action was studied using arginine (basic), histidine (weakly basic), glycine (weakly acidic), and glutamic acid (acidic). The lysine plus test amino acid was given in a single oral dose; the data are presented in table 4. The presence of histidine or glutamic acid reduced the response normally produced by lysine. Due to a considerable variation in the results for the group receiving lysine only, however, the difference between this group and the group receiving lysine plus glutamic acid was not significant ($P > 0.05$). In a similar experiment with lactose instead of lysine, the presence of lactose alone was associated with a deposition of 3.24% of the dose of Sr^{85} per femur, compared with 1.83% for the control group ($CaCl_2$) and 2.13% for the lactose plus glutamic acid group. The presence of either arginine or glycine had little effect on the response to either lysine or lactose.

In view of the inhibitory effect of glutamic acid when present in equal molar concentration with lysine, a further single dose study was carried out in which the molar ratio of glutamic acid to lysine was raised from a 1:1 ratio to a 2:1 ratio in order to see whether this would cause a fur-

ther depression of the action of lysine. The effects of certain dietary components other than amino acids were also studied. It is apparent from the results shown in table 5 that doubling the level of glutamic acid did not increase its inhibiting effect although it is worth noting that the inhibition in this trial was significant at $P < 0.05$. Aspartic acid also showed a significant effect similar to that of glutamic acid. In view of these results, it was not surprising to find that the presence of casein also significantly depressed the response obtained with lysine alone. The marked reduction in lysine effect brought about by starch was somewhat unexpected, however, particularly as it did not apparently depress the action of lactose to the same extent, as indicated in another investigation.

In order to obtain more information concerning a possible chelation between glutamic acid and either calcium or lysine, a single-dose experiment with fasted rats was carried out to establish the molar ratio of glutamic acid to lysine necessary for glutamic acid to exert its maximum inhibiting effect. The treatments and depositions in one femur of an orally administered dose of Sr^{85} are given in table 6. The results indicate that there was an increasing inhibition until glutamic acid was equimolar with lysine. At this point the reduction was again significant at $P < 0.05$. The dose of calcium administered was 0.09 mmoles.

DISCUSSION

In view of the fact that lysine will markedly increase the deposition in the femur of a single dose of Ca^{45} and Sr^{85} when administered to fasted rats, it is of interest to consider why supplementation of a normal diet with additional lysine did not bring about a similar effect. It has been shown that a single oral dose containing 0.125 mmole of lysine (18.28 mg) will not affect the deposition of either Ca^{45} or Sr^{85} per femur when 10 mg of calcium chloride (3.6 mg Ca) are used as the level of car-

TABLE 5

The effect of certain dietary components on the action of lysine in stimulating the absorption of Sr^{85} [1]

Treatment	% dose of Sr^{85}/femur	Percentage change from nonsupplemented group
$CaCl_2 + Sr^{85}$	1.47 ± 0.14	0
$CaCl_2 + Sr^{85}$ + 1 mmole lysine	2.89 ± 0.11	+97
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 2 mmoles glutamic acid	2.10 ± 0.09	+43
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 1 mmole aspartic acid	2.08 ± 0.10	+41
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 36.6 mg NaCl[2]	2.39 ± 0.12	+63
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 36.6 mg sodium citrate	2.49 ± 0.12	+69
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 292 mg casein[2]	1.96 ± 0.13	+33
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 877 mg starch[2]	2.08 ± 0.19	+41
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 731 mg sucrose[2]	2.65 ± 0.15	+80
$CaCl_2 + Sr^{85}$ + 1 mmole lysine + 368 mg cottonseed oil[2,3]	2.86 ± 0.16	+95

[1] Each value represents the mean of 6 animals ± the standard error of the mean. The 2-ml dose of each animal contained 10 mg of $CaCl_2$ (0.09 millimole of Ca).

[2] The levels of these dietary components were devised to reproduce, as far as difficulties in solubility would permit, similar ratios of these nutrients to stable calcium as existed for the 14-day balance trial reported earlier.

[3] Wesson Oil.

TABLE 6

The absorption of Sr^{85} as influenced by the ratio of lysine to glutamic acid[1]

Treatment	% dose of Sr^{85}/femur	Percentage change from nonsupplemented group
$CaCl_2$ + Sr^{85}	1.34 ± 0.07	0
$CaCl_2$ + Sr^{85} + 1 mmole lysine	3.33 ± 0.14	+149
$CaCl_2$ + Sr^{85} + 1 mmole glutamic acid	1.89 ± 0.15	+ 41
$CaCl_2$ + Sr^{85} + 1 mmole lysine + 0.05 mmole glutamic acid	3.09 ± 0.11	+131
$CaCl_2$ + Sr^{85} + 1 mmole lysine + 0.1 mmole glutamic acid	3.03 ± 0.23	+126
$CaCl_2$ + Sr^{85} + 1 mmole lysine + 0.5 mmole glutamic acid	2.89 ± 0.16	+116
$CaCl_2$ + Sr^{85} + 1 mmole lysine + 1.0 mmole glutamic acid	2.44 ± 0.16	+ 82

[1] Each value represents the mean of 5–6 animals ± the standard error of the mean.

rier calcium. This constitutes a weight ratio of lysine to calcium (Ca) of 5 to 1, which may be compared with a calculated ratio of about 2 to 1 for the unsupplemented basal diet in the 14-day trial and one of about 8 to 1 in the ration supplemented with 4% of lysine. When the amounts of lysine and $CaCl_2$ were each increased by a factor of 10, thereby maintaining the same ratio of lysine to calcium, a 26% increase in Ca^{45} retention and a 38% increase in Sr^{85} retention were observed. The amounts of lysine (183 mg) and calcium (36 mg) given at this level may be compared with average daily intakes of about 120 mg of lysine and 70 mg of calcium by 100-gm rats. It seems, therefore, that because of the amount of lysine and ratio of lysine to calcium attainable in a normal diet, it would be possible to achieve only about a 30% increase in absorption of calcium and strontium. Even this increase could be achieved only if the total daily intake of lysine and calcium was present in the alimentary tract at the same time, and if there was no interference from other dietary factors.

In the particular situation where a low-calcium diet is being fed, however, the ratio of lysine to calcium will be at a maximal figure, and therefore conditions should be optimal for lysine to produce a response. The total daily intakes of lysine and calcium on the lysine-supplemented low-calcium diet were about 492 mg of lysine and less than 10 mg of calcium. This relatively large quantity of lysine and extremely wide ratio of lysine to calcium did not bring about any increase in Sr^{85} absorption, however, indicating that in a complex diet certain other dietary components must be interfering in some way with the action of lysine.

It therefore seems that, while lysine can markedly increase the absorption of calcium and strontium when administered alone to fasted rats, it is unable to do so in the presence of normal dietary constituents. It is possible, however, that increased calcium absorption by monogastric animals could be achieved by means of dosing with $CaCl_2$ plus lysine, if taken several hours before and after consumption of food.

The action of lysine alone in single-dose experiments with fasted rats appears to be very similar in many respects to that of lactose. The studies of Wasserman et al. ('56) and Lengemann et al. ('59) have shown that lactose produces an effect similar in magnitude to that of lysine, and that this effect is mainly through enhanced absorption of calcium and strontium from the ileum. Lengemann et al. ('59) also showed that the effect of lactose was apparent within 30 minutes following injection into the iluem, as observed with lysine in the present studies. The presence of

lactose, however, has been shown to increase markedly the absorption of calcium in balance studies with rats (Bergeim, '26), whereas supplementary lysine had practically no effect in the trials reported in this paper. This difference may have been due to the necessity of using a much lower percentage of lysine in the diet (maximum 5.3%) than that of lactose in the studies of Bergeim (25 and 50%). It has been shown in the present single-dose studies that the presence of equimolar amounts of either of the acidic amino acids, glutamic and aspartic, will prevent most of the action of lysine. Since both glutamic and aspartic acids are usually present in casein to at least as high a degree as lysine, it was thought that casein would itself largely inhibit the action of lysine. This has been shown to be the case. Therefore, the presence of glutamic and aspartic acids in casein would seem to be the main reason that no effect was observed in the balance study with a low-calcium diet. Glutamic acid has also been shown to be effective in largely preventing the action of lactose. Since the treatment doses were administered at about pH 7.0, the effect of pH *per se* in these studies was not important, but it may be of significance that lysine must exist in the cationic form and glutamic acid in the anionic form under the pH conditions of the intestine. Thus, glutamic acid could conceivably reduce the effectiveness of lysine by a form of chelation, either with the calcium or with the lysine. Chelation with the calcium could also explain its effect on lactose. It is of interest to consider the effect of a strong chelating agent, sodium ethylenediaminetetraacetate (NaEDTA) on the effect of lactose, as reported by Lengemann et al. ('59). These workers found that NaEDTA inhibited the effect of lactose but did not decrease the absorption of Sr^{85} in the absence of lactose. They also showed that giving an equimolar amount of NaEDTA with Ca did not alter the effect produced by lactose but that, as the molar quantity of NaEDTA was further increased, there was increasing suppression of the lactose effect. The action of lactose was prevented completely by 0.16 mmoles of NaEDTA. Similarly in the present studies, glutamic acid had practically no inhibiting effect on the action of lysine when it was present in approximately equimolar concentration with the calcium. When the molar quantity of glutamic acid was 11 times that of calcium, however, it suppressed approximately half of the action of lysine. The lower effectiveness of glutamic acid as compared to NaEDTA was probably due to the fact that it is a much weaker chelating agent.

Possible mechanisms for the action of lysine have been discussed by Wasserman et al. ('56). Although present knowledge does not permit definite statements to be made, it is of interest to consider a possible mode of action. As the action of lysine is prevented when the pH of the dosing solution is equal to or greater than the isoelectric point for lysine, it seems that its mechanism of action is probably related to its presence in the cationic form at pH less than 9.7. In this state, however, it is most unlikely that any form of direct complexing with calcium and strontium could occur, so a carrier mechanism would seem to be unlikely. It is possible that lysine acts in single-dose studies by a direct action on the gut wall.

SUMMARY

1. The action of lysine in increasing the absorption of a single dose of Ca^{45} and Sr^{85} by fasted rats was most pronounced in the ileum. It was shown that lysine will exert an effect only if present in the same ligated segment as the dose of Ca^{45} and Sr^{85}, and if the pH of the dosing solution is lower than 9.7 (the isoelectric point for lysine). Its effect upon absorption from the ileum was evident within one hour after dosing.

2. The supplementation of a basal diet containing sufficient lysine to meet the needs of the rat for this essential amino acid with three different levels of lysine did not significantly increase the net retentions of stable calcium, Ca^{45}, or Sr^{85} during a 14-day balance period. Similarly, an addition of 2.09% of lysine to a low-calcium diet did not influence the absorption of Sr^{85}. Possible reasons for the inability of lysine supplementation to increase calcium and strontium absorption in balance trials, as compared with the marked increases obtained in single dose studies with fasted rats, have been investigated.

3. In single, oral dose studies, it was found that 1.25 mmole of lysine will almost double the deposition of Ca^{45} and Sr^{85} in the femur when only 10 mg of $Cacl_2$ are present in the dose. The increase was only 26% in the case of Sr^{85}, and 38% in the case of Ca^{45}, however, when the level of $CaCl_2$ in the dose was raised to 100 mg.

4. The presence of either glutamic or aspartic acid markedly reduced the enhancement of absorption of Sr^{85} and Ca^{45} produced by lysine. Glutamic acid was also shown to have a similar suppressing effect on the action of lactose. A possible chelation between the glutamic acid and calcium has been postulated to account for this suppression. The presence of casein and starch also largely suppressed the action of lysine.

LITERATURE CITED

Bergeim, O. 1926 Intestinal chemistry. V. Carbohydrates and calcium and phosphorus absorption. J. Biol. Chem., *70:* 35.

Comar, C. L. 1955 Radioisotopes in Biology and Agriculture, Principles and Practice. McGraw-Hill Book Co., Inc., New York.

Harrison, H. E., and H. C. Harrison 1951 Studies with radiocalcium: The intestinal absorption of calcium. J. Biol. Chem., *188:* 83.

Lengemann, F. W., and J. W. Dobbins 1958 The role of bile in calcium absorption. J. Nutrition, *66:* 45.

Lengemann, F. W., R. H. Wasserman and C. L. Comar 1959 Studies on the enhancement of radiocalcium and radiostrontium absorption by lactose in the rat. Ibid., *68:* 443.

McClure, F. J. 1952 Dental caries in rats fed a diet containing processed cereal foods and a low content of refined sugar. Science, *116:* 229.

Wasserman, R. H., C. L. Comar and M. M. Nold 1956 The influence of amino acids and other organic compounds on the gastrointestinal absorption of calcium 45 and strontium 89 in the rat. J. Nutrition, *59:* 371.

Wasserman, R. H., C. L. Comar, J. C. Schooley and F. W. Lengemann 1957 Interrelated effects of L-lysine and other dietary factors on the gastrointestinal absorption of calcium 45 in the rat and chick. Ibid., *62:* 367.

The Prevention of Experimental Myopathies by Various Chlorides[1]

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Earlier investigations have shown that, in the rat, acute overdosage with $NaClO_4$ elicits motor disturbance characterized by rather persistent, tonic extensor cramps. Predominantly mineralocorticoid steroids —such as methylchlorocortisol (Me-Cl-COL) and desoxycorticosterone (DOC)— greatly facilitate these motor disturbances whereas glucocorticoids (e.g., cortisol and triamcinolone) prevent their occurrence (Bajusz and Selye, '59a; Selye and Bajusz, '59b). Furthermore, chronic treatment with Me-Cl-COL plus $NaClO_4$ produces a severe muscular degeneration resembling the juvenile type of muscular dystrophy: there is spotty myolysis and interstitial edema followed by inflammation around isolated necrotic fibers (Bajusz and Selye, '59b). These lesions in the skeletal musculature are usually accompanied by cardiac necrosis, and interestingly, both types of pathologic changes can be prevented by the administration of chlorides, especially KCl and $MgCl_2$ (Selye, '59).

We now wish to report on experiments indicating that—like Me-Cl-COL—a dietary deficiency in potassium or magnesium predisposes the musculature to the toxic effects of $NaClO_4$ and that, here again, various chlorides exert a prophylactic effect. The importance of chlorides for the maintenance of normal muscular function and structure is further supported by the additional observation that an electrolyte-steroid-induced, acute paralytic condition of the rabbit can also be prevented with KCl or $MgCl_2$.

METHODS

Influence of electrolytes upon the myopathy induced by K- or Mg-deficient diets in the rat

Female Sprague-Dawley rats (170), having a mean initial body weight of 45 gm (range: 40 to 54 gm), were subdivided into 17 equal groups and treated as indicated in table 1. The rats of group 1 were fed a commercial fox chow[2] and served as controls showing the effect of $NaClO_4$ in a normal diet. The animals of groups 2 to 9 were kept on a potassium-deficient diet, and those of groups 10 to 17 on a magnesium-deficient diet for 5 days prior to as well as during treatment with electrolytes.[3] The rats were allowed to drink distilled water ad libitum.

The percentage composition of the K-deficient diet was sucrose, 70.65; vitamin-free test casein,[4] 18.30; butter fat (salt-free), 5.00; vitamin supplement (see below), 1.05; and salt mixture (see below), 5.00. Vitamin supplement (gm/100 pound of diet): biotin, 0.0140; Ca pantothenate, 1.1340; folic acid, 0.1132; menadione, 0.2268; nicotinic acid, 3.1748; pyridoxine ·HCl, 3.1748; riboflavin, 0.2720; thiamine ·HCl, 0.2720; vitamin B_{12}, 0.0020; α-tocopherol, 0.9200; vitamin A conc., 200,000 I.U./gm, 0.0800; vitamin D conc., 400,000 I.U./gm, 0.9200. Salt mixture (gm/100 pound of diet): ammonium alum (potassium-free), 0.183; calcium carbonate, 905.840; calcium phosphate monobasic, 230.000; cobaltous chloride, 1.830; cupric sulfate, 1.830; ferric ammonium citrate, 36.600; magnesium carbonate, 64.500; magnesium sulfate anhydrous, 68.900; manganese sulfate, 1.830; sodium chloride,

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[2] Purina Fox Chow, Ralston Purina Company, Canada.
[3] These two diets supplied by General Biochemicals, Inc., Chagrin Falls, Ohio.
[4] Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

507.000; sodium fluoride, 0.183; zinc oxide, 18.300.

The composition of the Mg-deficient diet differed from the K-deficient ration only in that the salt mixture (gm/100 pound of diet) consisted of calcium carbonate, 905.840; calcium phosphate monobasic, 266.440; cupric sulfate, 0.920; ferric ammonium citrate, 84.520; manganese sulfate, 12.080; potassium iodide, 2.400; potassium phosphate dibasic, 226.440; sodium chloride, 507.200; zinc carbonate, 0.760.

After feeding the above diets for 5 days, treatment with various electrolytes was initiated in all groups except 2 and 0. The individual dose of $NaClO_4$ was .5 mM, that of the chlorides or their mixtures, 0.25 mEq. All electrolytes were of a "reagent" degree of purity[5] and were given in 2 ml of water, by stomach tube, twice daily.

Muscular cramps were noted soon after the administration of $NaClO_4$, but we appraised the rats daily one hour following the second gavage (that is, at 5 P.M.). The "flick test" was used for this purpose, because it proved to be highly effective in revealing a latent tendency for muscular cramps. This test involves placing the rat on a flat surface (such as a table) and giving it a flick in the sacral region with the index finger. Normal rats either show no response to this slight irritation or take a few steps forward, while animals sensitized with $NaClO_4$ make spastic extensor movements with their hind limbs (Selye, '59; Selye and Bajusz, '59b; Bajusz and Selye, '59a). The results of this test were expressed in an arbitrary scale: zero, no spastic response; 1, brief extensor cramp in hind limbs; 2, prolonged extensor cramp in hind limbs; 3, spreading of cramp to the whole body, with characteristic hunching of the back, and caudal extension of forelimbs. The mean readings, as determined on the second day following the initiation of treatment, are listed in table 1. It should be noted that the muscular response was most intense on the first two days of $NaClO_4$ administration and then gradually decreased even in the control animals (groups 1, 3 and 11); apparently, there is increasing adaptation to this effect of perchlorate.

The experiment was terminated on the 5th day (groups 2 to 9) or 7th day (groups 1, 10 to 17) after the initiation of electrolyte administration. In all instances, immediately after autopsy the triceps surae muscles were dissected, fixed in Susa solution and embedded in paraffin. The sections were stained with hematoxylin-phloxine or with PAS, for morphologic studies. The grade of the histologic lesions (fragmentation of muscle fibers, their abnormally increased stainability and/or necrosis; see figs. 1 and 3) was assessed in terms of an arbitrary scale of zero to 3: zero, no lesion; 1, just detectable lesion; 2, moderate lesion; 3, most severe type of lesion. Since the protection afforded by the chlorides against necrosis did not parallel that exerted against the development of all other abnormalities, the means of these readings are listed separately in table 1.

Electrolyte-steroid interaction in an acute paralytic condition of the rabbit

Female Belgian albino rabbits,[6] weighing 870 to 1230 gm, were arranged in groups having a mean body weight of 1050 gm. The animals were fed the standard Rockland rabbit ration and received tap water throughout the period of observation. All treatments, as indicated in table 2, were initiated on the first day and continued until the termination of the experiment.

Methylchlorocortisol (2α-methyl-9α-hydrocortisone acetate)[7] was given as a microcrystal suspension, 100 μg in 0.1 ml of water per 100 gm of body weight, once daily, subcutaneously. Dihydrotachysterol (DHT),[8] (25 μg per 100 gm of body weight), was administered as a microcrystal suspension, twice daily, in a mixture with the electrolytes. Monobasic sodium phosphate, NaH_2PO_4[9] was given in the amount of 1 mM per 100 gm of body

[5] Fisher Scientific Company.

[6] Purchased from the R. Robidoux Farm, St. Constant, Quebec.

[7] The authors gratefully acknowledge generous supplies of 2α-methyl-9α-chlorocortisol from The Upjohn Company.

[8] Calcamin. Generously supplied by Dr. A. Wander, Société Anonyme, Bern, Switzerland.

[9] See footnote 5.

weight, potassium chloride, KCl[10] and magnesium chloride, $MgCl_2 \cdot 6H_2O$[11] in a concentration of 0.5 mM per 100 gm, all twice daily. The electrolyte and DHT mixture was always given in 2 ml of water per 100 gm by stomach tube; the controls in group 1 received a corresponding volume of distilled water by stomach tube.

The experiment was terminated on the 5th day by killing the surviving animals with chloroform. However, by that time, several of the rabbits had died, exhibiting total paralysis of the extremities and cervical muscles. On the day of autopsy, the severity of the muscular paralysis was expressed in an arbitrary scale of zero to 3. Table 2 shows the mean grade of this reading as well as the incidence (percentage of positive response within the groups) for all animals (including those that died before the termination of the experiment) and the percentile mortality. In all instances, at autopsy the triceps surae muscles and the hearts were dissected, fixed in Susa solution and the paraffin embedded sections stained with hematoxylin-phloxine for subsequent histologic studies.

RESULTS AND DISCUSSION

The results of the first experiment, summarized in table 1, indicate that motor disturbances on the second day of electrolyte administration were not very severe among the controls treated with $NaClO_4$ alone (group 1) and that both potassium deficiency (group 3) and magnesium deficiency (group 11) greatly sensitized the rats to this effect of perchlorate. The aggravation was, in fact, much more pronounced than the figures of the table indicate, because, in the animals fed deficient diets, the muscular contractions were not only more severe but of much longer duration. Even 4 hours after the $NaClO_4$ administration, the "flick test" was still clearly positive in these groups, whereas it was already completely negative by the second hour in all the controls fed the normal laboratory diet. On the other hand, it is also clear that the conditioning effect of the deficient diets was abolished by the concurrent administration of various chlorides (groups 4 to 8

[10] See footnote 5.
[11] Merck and Company, Limited.

TABLE 1

Influence of electrolytes upon the myopathy induced in the rat by K- or Mg-deficient diets

Group	Treatment	Skeletal muscles				
		Cramps grade (0–3)	Necroses		Other histologic lesions	
			Grade (0–3)	Incidence	Grade (0–3)	Incidence
				%		%
1	$NaClO_4$	1.3 ± 0.26[1]	0.1 ± 0.10	10	0	0
2	K-deficient diet	0	0	0	0	0
3	+ $NaClO_4$	2.8 ± 0.25	2.6 ± 0.18	100	2.2 ± 0.24	100
4	+ $NaClO_4$ + KCl	0.1 ± 0.10	0	0	0.1 ± 0.10	10
5	+ $NaClO_4$ + $MgCl_2$	0.1 ± 0.10	0.2 ± 0.15	10	1.8 ± 0.21	100
6	+ $NaClO_4$ + NaCl	1.3 ± 0.32	1.8 ± 0.27	70	1.2 ± 0.26	80
7	+ $NaClO_4$ + $CaCl_2$	0.7 ± 0.17	0.8 ± 0.23	60	1.0 ± 0.10	80
8	+ $NaClO_4$ + NH_4Cl	0.4 ± 0.15	0.1 ± 0.10	10	0.2 ± 0.10	20
9	+ $NaClO_4$ + $MgCl_2$ + NaCl + $CaCl_2$ + NH_4Cl	0.5 ± 0.18	0.2 ± 0.15	20	0.7 ± 0.27	50
10	Mg-deficient diet	0	0	0	0	0
11	+ $NaClO_4$	2.1 ± 0.26	1.9 ± 0.25	100	1.6 ± 0.17	90
12	+ $NaClO_4$ + KCl	0.2 ± 0.15	0.1 ± 0.10	10	0.1 ± 0.10	10
13	+ $NaClO_4$ + $MgCl_2$	0.4 ± 0.17	0	0	0.1 ± 0.10	10
14	+ $NaClO_4$ + NaCl	0.3 ± 0.18	0.6 ± 0.17	50	0.2 ± 0.10	20
15	+ $NaClO_4$ + $CaCl_2$	0.4 ± 0.20	0.3 ± 0.18	20	0.7 ± 0.23	40
16	+ $NaClO_4$ + NH_4Cl	0.3 ± 0.22	0	0	0.4 ± 0.18	30
17	+ $NaClO_4$ + KCl + NaCl + $CaCl_2$ + NH_4Cl	0.4 ± 0.23	0.1 ± 0.10	10	1.0 ± 0.25	70

[1] Standard error.

and 12 to 16) or even by a mixture of these salts that contained an equivalent amount of the anion (groups 9 and 17). With the exception of NaCl (group 6), all the chlorides significantly reduced the occurrence of the muscular cramps to below the level normally seen in the controls (group 1). In this respect, $MgCl_2$ or NH_4Cl was at least as effective in the potassium-deficient animals as KCl or the chloride mixture was in the rats fed the magnesium-deficient diet.

During further treatment with electrolytes the severity of the muscular cramps gradually decreased, even in those groups receiving $NaClO_4$ alone. It would appear, therefore, that the animals developed a resistance to this action of the perchlorate. However, after autopsy, marked histologic abnormalities were observed in the triceps surae muscle. In the early stage, these consisted of abnormally increased stainability, fragmentation, and loss of cross-striation of the muscle fibers (fig. 1). The more pronounced lesions were characterized by myolysis and interstitial edema; sometimes, inflammatory processes were seen around isolated necrotic fibers, often with infiltration by polymorphonuclear leucocytes and monocytes of the disintegrated areas (fig. 3). When treated with $NaClO_4$, the animals deficient in K and Mg developed the most severe structural lesions (groups 3 and 11); here again, concurrent administration of chlorides reduced the occurrence of histologic changes (groups 4 to 9, 11 to 17). It is significant that these abnormalities were seen neither in the rats receiving the deficient diets alone (groups 2 and 10) nor in those fed the commercial fox chow and treated with perchlorate (group 1). On the other hand, in some animals (groups 5, 6, and 17), the protective effect of the chlorides was somewhat more evident against the necroses than against the less-pronounced prenecrotic changes.

The principal outcome of these experiments is that both potassium and magnesium deficiency enhanced the susceptibility of the rat to the myotoxic actions of perchlorate, whereas an excess of chloride decreased the effect. The protective action exerted by the various salts is independent of the cations to which the chloride is attached.

It is interesting that in all these experiments the skeletal-muscle lesions were modified by electrolytes in the same manner as the cardiac necroses that also develop under these experimental conditions and have been described in detail elsewhere (Bajusz and Selye, '59c). With respect to the pathogenesis of both types of lesion, it would be difficult to decide which among the various factors is actually the fundamental "primary pathogen" and which is the modifying, or "conditioning factor" responsible for the aggravation. Dietary deficiency of potassium or magnesium can, in itself, result in similar muscle lesions if the rats are fed the diet for a much longer period than in the present experiment[12] (Schrader et al., '37; Follis, '42; '58; Selye and Bajusz, '58). On the other hand, long-term administration of $NaClO_4$ also produces muscular necroses, at least in corticoid-conditioned animals (Selye, '59). Furthermore, both in man and in experimental animals, similar necroses can occur under diverse conditions (Ellis, '56). Presumably, this type of lesion is not characteristic of any one specific pathogen but represents, rather, a basic reaction of muscle to injury. Virtually nothing is known of the factors that determine whether necrosis will affect skeletal and/or cardiac muscles in any given set of pathogenic circumstances. It is noteworthy, however, that, under our present experimental conditions, both the cardiac and the skeletal muscle necroses were prevented by the administration of various chlorides. It should be noted, furthermore, that similar protection was observed in previous experiments, using corticoid-conditioned animals fed a normal diet, when $NaClO_4$ was administered subcutaneously, while the various chlorides were given *per os* (Selye, '59). This shows that the results obtained in the present study cannot be due to *in vitro* chemical reactions in the gavage mixture.

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[12] Greenberg, D. M., C. E. Anderson and E. V. Tufts 1936 Pathological changes in the tissues of rats reared on diets low in magnesium. J. Biol. Chem., *114:* xliii (abstract).

The results of the second experiment, performed on rabbits, are summarized in table 2. As seen from the figures, combined treatment with Me-Cl-COL plus DHT resulted in only a slight degree of muscular paralysis and only in 30% of the control animals (group 1). The muscular tonus in this group was definitely low, but actual paralysis was rarely observed; when present, it consisted only of the so-called "head-drop" symptom due to weakness of cervical muscles. However, a severe motor disturbance, which progressed to complete paralysis and 75% mortality, resulted if NaH_2PO_4 was administered simultaneously with the steroids (group 2). The first symptoms of the paralytic seizures were already obvious on the second or third days of the experiment; in most instances, this condition progressed rapidly so that within a few hours all skeletal muscles became paralyzed and death ensued. In these rabbits, various degrees of infarct-like cardiac necroses were seen at autopsy; however, histologic studies of the skeletal muscle revealed only occasional small necrotic foci and no other structural abnormalities. On the other hand, the severe paralytic condition normally induced by steroids plus NaH_2PO_4 was significantly inhibited by KCl (group 3) or $MgCl_2$ (group 4). In these two groups, the prophylactic effect of the chlorides is evidenced by the reduced mortality rate and the observation that neither cardiac nor voluntary muscle necroses occurred.

It was previously established that overdosage with mineralocorticoid hormones (e.g., DOC, aldosterone, Me-Cl-COL), especially in combination with NaCl, produces muscular paralysis in various animal species[13] (Selye, '43; Selye and Hall, '43; Gross and Schmidt, '58). This type of paralysis is accompanied by a decrease of the potassium and increase of the sodium content of the muscle cells. It has generally been considered to be mainly if not purely a muscular defect due to disturbances in the extra- and intracellular electrolyte components (Selye and Hall '43; Gross and Schmidt, '58; for review, Selye, '50; Selye et al., '51–55/56). Furthermore, since the administration of mineralocorticoids can also alter chloride excretion, the possible pathogenic role of variations in chloride metabolism in this respect has also been stressed (Selye and Hall, '43). The paralytic seizures produced with mineralocorticoid overdosage in animals resemble those occurring in familial periodic paralysis in man. In fact, the data of Conn and his colleagues ('57) indicate that this clinical condition is associated with intermittent aldosteronism and is beneficially influenced by the administration of potassium salts.

It was also shown that a synergism exists between corticoids and some steroids of the vitamin D group (e.g., DHT) with respect to their ability to produce cardiac necroses (Selye, '58; Selye and Bajusz, '59a). Subsequent preliminary investigations showed that this is also true of certain myotoxic effects of these two essentially different groups of steroids. The present experiments were designed to examine the effect of various electrolytes on the myopathy induced by combined treatment with a corticoid and a vitamin D derivative. In the light of

[13] Selye, H., and C. E. Hall 1943 The pathology of desoxycorticosterone overdosage in various species. Federation Proc., 2: 44 (abstract).

TABLE 2

Electrolyte-steroid interaction in an acute paralytic condition of the rabbit

Group	Number of rabbits	Treatment[1]	Muscular paralysis		Mortality
			Grade (0–3)	Incidence	
				%	%
1	6	None	0.8±0.28	30	0
2	16	NaH_2PO_4	2.8±0.13	100	75
3	14	NaH_2PO_4+KCl	0	0	0
4	15	NaH_2PO_4+$MgCl_2$	0.3±0.12	7	7

[1] In addition, the rabbits in all groups were treated with methylchlorocortisol plus dihydrotachysterol, described in the text.

our previous observations, it was not unexpected to find that NaH_2PO_4 greatly aggravates the steroid-induced paralytic condition whereas KCl prevents it. The similar protective effect afforded by $MgCl_2$ is more difficult to understand. Further studies will be necessary to establish whether Mg plays an active role in prevention or whether the chlorides of other cations act similarly. The results of previous experiments in which NaCl actually aggravated the myotoxic effect of DOC (Selye, '43; Selye and Hall, '43) do not exclude the possible prophylactic value of chloride; they may merely indicate that the amount of the anion was insufficient to counteract the sensitizing action of sodium.

SUMMARY

Rats fed a potassium- or magnesium-deficient diet for a short period became very sensitive to the myotoxic effect of $NaClO_4$. In such animals, the perchlorate elicits particularly severe motor disturbances and marked degenerative changes in the skeletal muscles. The occurrence of all these pathologic manifestations was inhibited by the concurrent administration of various chlorides (e.g., KCl, $MgCl_2$, NaCl, NH_4Cl, $CaCl_2$) alone or in combination. In this respect, KCl and $MgCl_2$ were equally effective in both K- and Mg-deficient animals.

Another type of neuromuscular disorder was elicited in rabbits with methylchlorocortisol plus dihydrotachysterol. When NaH_2PO_4 was given in addition to these steroids, the motor disturbance was greatly aggravated and a generalized muscular weakness developed that rapidly progressed to total paralysis and death. These acute paralytic seizures could also be prevented by the oral administration of KCl or $MgCl_2$.

LITERATURE CITED

Bajusz, E., and H. Selye 1959a Effect of various hormones upon the muscular contractions induced by sodium perchlorate. Acta Pharmacol. Toxicol., *15:* 235.

——— 1959b An experimental type of muscular dystrophy and role of chlorides in its prevention. Proc. Canad. Fed. Biol. Soc., p. 5.

——— 1959c Conditioning factors for cardiac necroses. Tr. N. Y. Acad. Sci., *21:* 659.

Conn, J. W., L. H. Louis, S. S. Fajans, D. H. P. Streeten and R. D. Johnson 1957 Intermittent aldosteronism in periodic paralysis. Dependence of attacks on retention of sodium and failure to induce attacks by restriction of dietary sodium. Lancet, *1:* 802.

Ellis, J. T. 1956 Necrosis and regeneration of skeletal muscles in cortisone-treated rabbits. Am. J. Path., *32:* 993.

Follis, R. H., Jr. 1942 Myocardial necroses in rats on a potassium low diet prevented by thiamine deficiency. Bull. Johns Hopkins Hosp., *71:* 235.

——— 1958 Deficiency Disease. Functional and structural changes in mammalia which result from exogenous or endogenous lack of one or more essential nutrients. Charles C Thomas, Springfield, Illinois.

Gross, F., and H. Schmidt 1958 Aldosterone overdosage in the rabbit. Acta Endocrinol., *28:* 467.

Schrader, G. A., C. O. Prickett and W. D. Salmon 1937 Symptomatology and pathology of potassium and magnesium deficiencies in the rat. J. Nutrition, *14:* 85.

Selye, H. 1943 Nephrosclerosis and tissue edema after desoxycorticosterone treatment. Anat. Rec., *85:* 49.

——— 1950 Stress. The Physiology and Pathology of Exposure to Systemic Stress. Acta, Inc., Montreal.

——— 1958 The Chemical Prevention of Cardiac Necroses. The Ronald Press Co., New York.

——— 1959 Wechselwirkungen zwischen Stress, Elektrolyten und Steroiden beim Entstehen verschiedener Kardiopathien und Myopathien. Endokrinologie, *38:* 195.

Selye, H., and E. Bajusz 1958 Provocation and prevention of potassium deficiency by various ions. Proc. Soc. Exp. Biol. Med., *98:* 580.

——— 1959a Interactions between electrolytes, corticoids and dihydrotachysterol (DHT) in the production of cardiovascular disease and nephrocalcinosis. Lab. Invest., *8:* 1499.

——— 1959b Effect of various stressors upon the muscular contraction induced by NaH_2PO_4 and $NaClO_4$. Am. J. Physiol., *196:* 681.

Selye, H., and C. E. Hall 1943 Pathologic changes induced in various species by overdosage with desoxycorticosterone. Arch. Path., *36:* 19.

Selye, H., A. Horava and G. Heuser 1951–55/56 Annual Reports on Stress, vol. 1–5. Acta, Inc., Montreal.

PLATE

PLATE 1

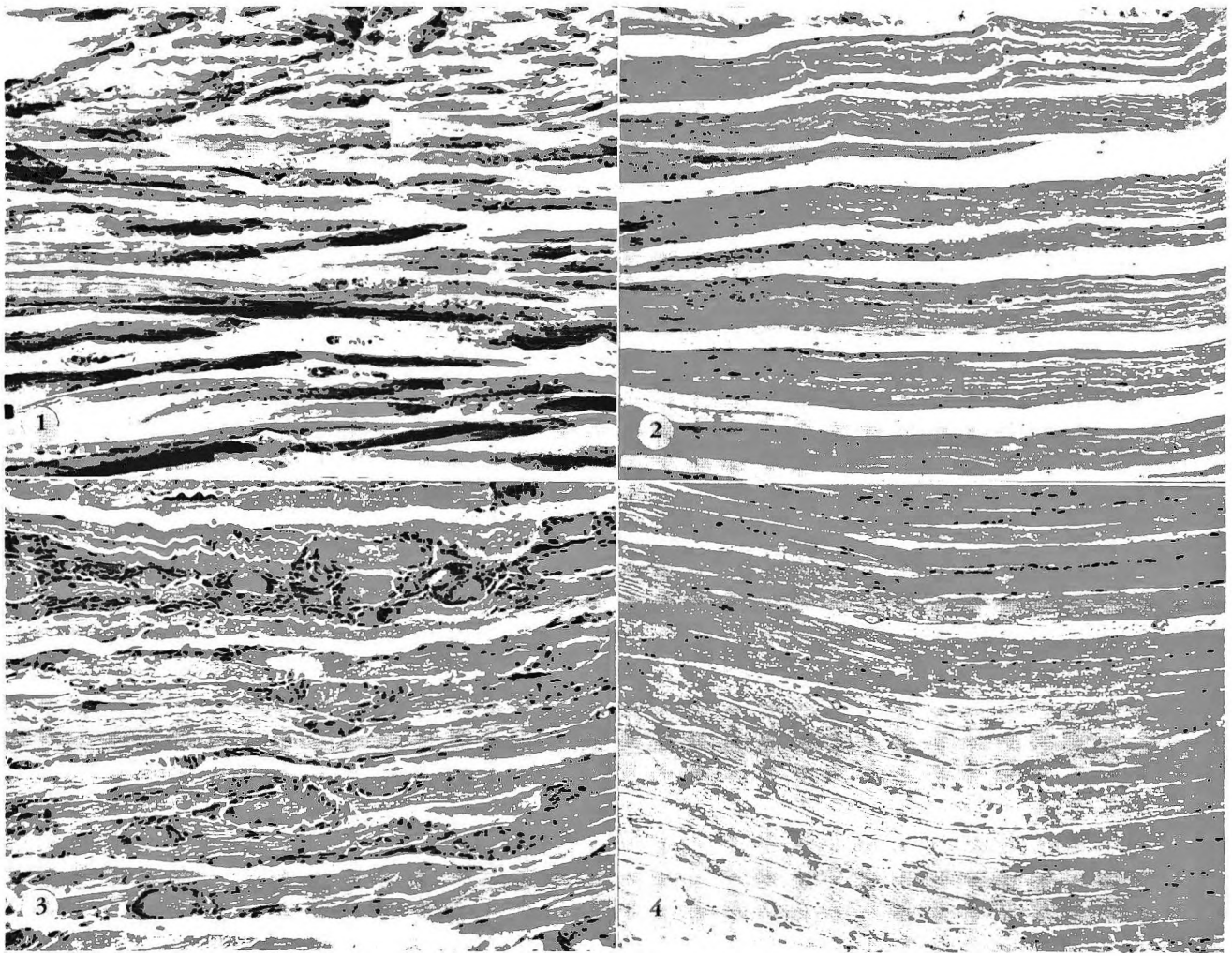
EXPLANATION OF FIGURES

1 Longitudinal section of the triceps surae of a rat fed a potassium-deficient diet and treated with $NaClO_4$. Note fragmentation and increased stainability of the muscle fibers. Hematoxylin-phloxine. × 160.

2 Prevention of the prenecrotic changes (shown in fig. 1) by the oral administration of $MgCl_2$. This animal was fed the potassium-deficient diet for the same period as that shown in the previous picture and was treated with equal amounts of perchlorate. The concurrent administration of $MgCl_2$ prevented the development of structural abnormalities. Hematoxylin-phloxine. × 160.

3 Fiber necroses with phagocytic processes in the triceps surae of a rat treated with $NaClO_4$, sensitized with a magnesium-deficient diet. PAS. × 160.

4 The pronounced structural changes shown in figure 3 are prevented by additional treatment with KCl. PAS. × 160.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Aberrant Iron Metabolism and the "Cotton-Fur" Abnormality in Mink [1,2]

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The "cotton-fur" (CF) abnormality in mink fed Pacific hake and whiting has been described (Stout and associates, '60). In addition to previously listed symptoms of reduced growth and achromotrichia, presence of anemia in CF animals has been noted (Adair and Davis, '57; Helgebostad and Martinsons, '58). The observation that the development of CF could be prevented by cooking the fish studied (Stout et al., '60) suggested that an induced rather than a natural dietary deficiency was the basis of this anomaly. Other work showed considerable protection against a similar type of achromotrichia in fox pups by feeding before graying was well advanced, a supplementary mixture of synthetic B-vitamins; these included thiamine, riboflavin, nicotinic acid, *p*-aminobenzoic acid, inositol, pyridoxine, biotin, pantothenic acid, folic acid, choline and vitamin B_{12}, or substances rich in these factors, such as raw cod roe, animal liver and dried brewers' yeast (Helgebostad and Ender, '51). No protection was evident when the diet of young foxes was supplemented with rice starch, glucose, Fe, Cu, Co, Zn and Mn, vitamins A, D, E, K, thiamine, pantothenic acid and vitamin C (Ender and Helgebostad, '47).

At various times achromotrichia has been linked nutritionally with deficiencies of pantothenic acid, *p*-aminobenzoic acid, folic acid, biotin, choline, cystine and lysine, and copper and zinc (Frost, '48). Concomitant with achromotrichia, anemia has been reported to result from deficiencies of pantothenic acid (McCall et al., '46), copper (Sjollema, '38), lysine and folic acid, (Klain et al., '57).

Previous observations at this station[3] indicated that folic acid, vitamin B_{12}, thiamine (injected singly or in combination), folinic acid or a crude liver extract had no perceptible effect on restoration of blood hemoglobin and hematocrit levels in CF mink. However, intramuscular injection of organic iron restored essentially normal hemoglobin and hematocrit levels to CF mink. This latter observation has also been reported in Norway (Helgebostad and Martinsons, '58).

Results of blood studies on CF mink in relation to normal mink are reported here. Also in an attempt to identify the nutritional factor(s) involved, effects of supplementing a CF-genic ration with (1) B-complex vitamins, (2) lysine plus tyrosine, (3) copper, and (4) iron, on pigmentation, growth, mortality rate and blood formation were measured. Further, results concerning the effect of oral iron supplementation on anemia of CF mink are presented.

EXPERIMENTAL AND RESULTS

Standard dark mink (100) selected in part from litters of females which were previously "cottons" and known to be susceptible to the CF condition were fed a CF-genic ration having the following percentage composition: horsemeat, 7; mixed rockfish, 10; turbot, 15; Pacific hake, 50; and a supplement, 18.[4] Similar mink (61), although randomly selected, were used for comparison, and fed a ration,

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[1] Technical Paper 1311, Oregon Agricultural Experiment Station.

[2] This study was supported by a grant from the Mink Farmers' Research Foundation, Milwaukee.

[3] Unpublished data, Stout, F. M. et al., 1957, 1958.

[4] Percentage composition: wheat germ, 25; alfalfa meal, 13; skim-milk powder, 8; meatmeal, 18; soybean oil meal, 18; rolled oat groats, 18.

known to support optimum growth and furring, of this percentage composition: horsemeat, 8; tripe, 10; beef liver, 3; mixed rockfish, 25; turbot, 25; mixed sole, 20; and a supplement, 9.[5] The animals were housed and fed as indicated previously (Stout et al., '60).

Mink fed the CF-genic ration were divided into groups of 10 to 20 animals; each group received one of the following supplements: (1) B-complex vitamins, (2) lysine and tyrosine, (3) copper or (4) iron. A fifth group, serving as a negative control, was not given a supplement. B-complex vitamins were injected intraperitoneally at weekly intervals for 14 weeks into male mink of group 1 in the following milligram amounts: thiamine, 2.7; riboflavin, 4.9; pyridoxine, 2.7; niacin, 4.9; pantothenic acid, 19.7; choline, 49; inositol, 70; *p*-aminobenzoic acid, 2.8; folic acid, 0.5; vitamin B_{12}, 0.5; and biotin, 0.5. Females received one half of this dosage. Lysine and tyrosine were supplied daily to mink in group 2 in the feed as 1.8 gm of L-lysine and 0.7 gm of L-tyrosine. Copper glycinate was injected subcutaneously into mink in group 3 in doses of 27 mg either monthly or bimonthly. Iron as ferric hydroxide[6] was injected intramuscularly at two levels: 50 mg biweekly for a total of 350 mg to half of group 4, or 50 mg monthly for a total of 200 mg to the remainder.

Blood data were obtained by the following methods. Three milliliters of blood were withdrawn from anesthetized mink by cardiac puncture and immediately transferred to oxalated tubes. Hemoglobin values were determined using a Spencer Hemoglobinometer, model 1000.[7] Erythrocyte counting was accomplished by methods outlined by Wintrobe ('46) using isotonic saline as a diluent. Hematocrit values were obtained following Wintrobe's method ('46); however, centrifugation was made at 2400 rpm for one hour.

[5] Percentage composition: wheat germ, 25; alfalfa meal, 12.4; skim-milk powder, 8.2; meat-meal, 16.5; soybean oil meal, 16.5; rolled oat groats, 16.5; brewers' yeast, 4.2; Fortafeed 249-C (American Cyanamid Co.), 0.4; terramycin (TM-10, Chas. Pfizer Co.), 0.25; DL-methionine, 0.05.

[6] Armidexan, Armour Veterinary Laboratories, Chicago.

[7] American Optical Company.

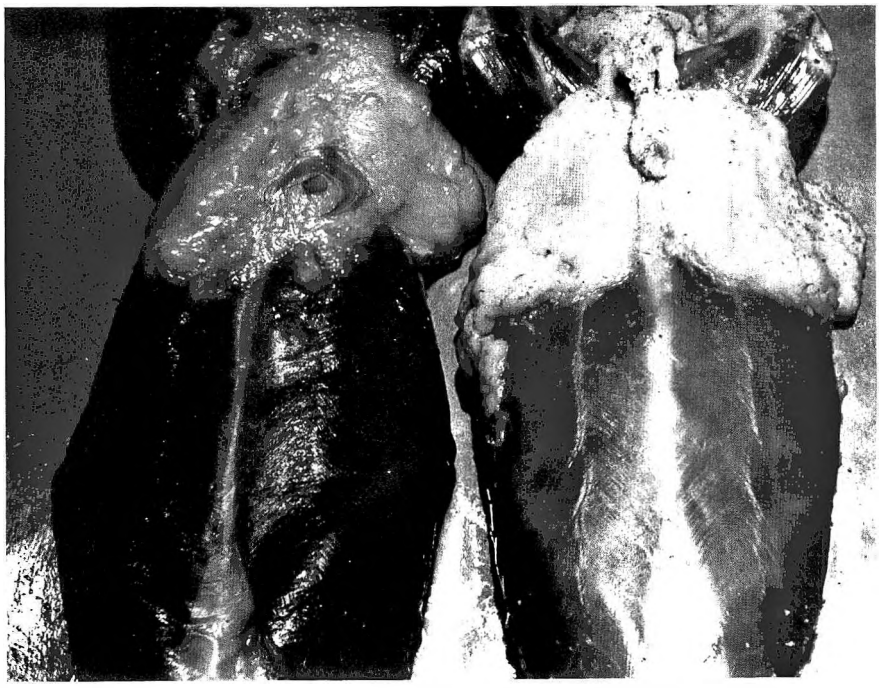

Fig. 1 Skinned carcasses of normal (left) and CF mink illustrating the anemic condition of the latter.

In a sequential trial, 27 CF mink were selected from those groups of the previous experiment in which no protection against CF was evident. Animals were weighed, divided by sex and stratified according to blood hemoglobin levels. Allocation of mink thus arranged was at random within consecutive blocks of three animals. All mink continued to be fed the CF-genic ration and received in addition either (1) no added dietary iron, (2) 17.6 mg of ferrous iron[8] per kg of ration, as fed, or (3) 88.1 mg of ferrous iron[9] per kg of ration as fed. At the end of a 30-day feeding period animals were reweighed and hemoglobin levels measured.

Evidence of anemia present in the CF syndrome is provided by the appearance of skinned carcasses of CF mink in relation to normal mink (fig. 1). Blood data are listed in table 1. Values shown as normal were taken from a sample of 32 standard dark mink (16 males and 16 females) chosen at random from the group fed the adequate control ration. Blood values for "cottons" represent groups of 22 to 27 experimentally-produced CF mink fed the CF-genic ration. The data indicate that in general CF mink have markedly reduced hemoglobin and hematocrit values and slightly reduced total numbers of erythroctyes. Index values (Best and Taylor, '45) show that the amount of hemoglobin within and the volume of individual cells are low for CF mink in relation to normal mink. These conditions are characteristic of a microcytic, hypochromic anemia. Stained smears showed an abundance of poikilocytosis and anisocytosis in blood from CF mink, especially from those severely affected.

Results of supplementing mink fed the CF-genic ration are presented in table 2. Performance of mink fed an adequate control ration as well as those fed the CF-genic ration with no supplementation is given for comparison. No preventive effect due to supplementation with 11 B-complex vitamins was noted. "Cotton" incidence

[8] Supplied as Ferronord, Nordmark Pharmaceutical Lab., Inc., Irvington, N. J.
[9] See footnote 8.

TABLE 1

Blood values of normal and CF mink[1]

Mink	Hemoglobin	Erythrocytes	Hematocrit	Indexes		
				Color	Volume	Saturation
	gm/100 ml	*million/mm³*	%			
Normal	18.7 ± 0.6[2](32)	9.00 ± 0.68(32)	45.0 ± 3.1(32)	1	1	1
"Cotton"	10.8 ± 3.0 (27)	8.50 ± 2.59(22)	28.1 ± 8.8(27)	0.61	0.66	0.93
% of normal	57.8	94.4	62.4			

[1] Figures in parentheses show numbers of mink used in assembling data.
[2] The ± values represent standard deviation.

TABLE 2

Effects of supplementing a CF-genic ration with various nutrients

Treatment	Number of animals	Mortality	CF incidence	Terminal weight[1]		Hemoglobin
				M	F	
		%	%	*gm*	*gm*	*gm/100 ml*
Adequate control ration	61	0	0	1818 ± 240[2]	1063 ± 128	18.7 ± 0.6[3]
Non-supplemented control	10	0	80	1292 ± 284	850 ± 114	11.9 ± 3.4
B-complex vitamins	20	25	83	1079 ± 565	829 ± 259	11.6 ± 3.6
Lysine + tyrosine	12	25	83	1194 ± 252	656 ± 418	10.1 ± 4.1
Copper	10	50	90	1008 ± 505	729 ± 364	11.0 ± 3.7
Iron[4]	20	0	0	1621 ± 273	975 ± 126	16.8 ± 0.8

[1] Measured as weight off test for surviving animals and as death weight for dead animals.
[2] The ± values represent standard deviation.
[3] Hemoglobin values for controls determined on 32 randomly chosen animals.
[4] Supplied as Armidexan, Armour Veterinary Laboratories.

was 83%, growth was markedly subnormal and hemoglobin values were 38% below normal. Supplementation with lysine and tyrosine, important intermediaries in melanin formation, similarly produced no remission of symptoms. Likewise, injection of copper, which is essential in both hemoglobin and melanin formation, offered no protection against the CF condition as 9 of 10 treated mink were classified as "cottons" at the end of the experiment. Iron supplementation gave striking results since none of 20 animals receiving injected organic iron developed the CF condition (fig. 2). Size was significantly greater than in mink not receiving the supplement, as illustrated in figure 3. Blood hemoglobin levels were 41% higher than observed in non-supplemented controls, but about 10% below hemoglobin values of mink fed the adequate control ration. Variation in size and hemoglobin levels was markedly reduced within the iron-supplemented group and was similar to that of the adequately-fed control group.

In a subsequent trial when CF mink fed a CF-genic ration received oral supplements of two levels of iron, hemoglobin regeneration paralleled that of mink receiving no added iron (table 3). Furthermore, all three groups of mink lost weight during the experimental period.

DISCUSSION

Discovery that CF mink were anemic provided a useful criterion for investigating the nutritional basis of this anomalous condition; hence, blood formation, which is relatively rapid as compared with the slower, cyclical process of fur growth, could be used to measure response to supplementation with purified nutrients. Using this measure, it was found that although several individual B-vitamins had no effect, parenteral iron was capable of restoring blood of CF mink to almost normal values.[10]

Supplementing the CF-genic ration with 11 B-vitamins during the growth and furring period proved ineffective in preventing or reducing incidence of CF or its allied symptoms. This observation apparently is in contrast with Norwegian work which has repeatedly stressed the impor-

[10] See footnote 3.

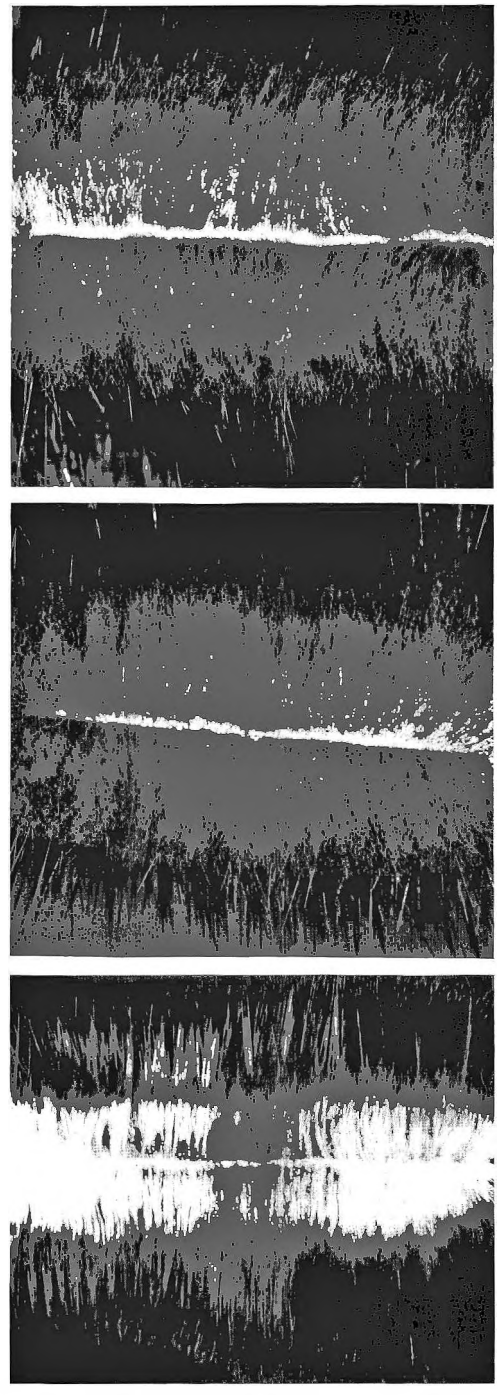

Fig. 2 Pelts (parted to show underfur) of representative mink fed the adequate control ration (top), CF-genic ration plus parenteral iron supplement (center) and CF-genic, non-supplemented ration (bottom). Parenteral iron administered to young mink prior to and during the furring cycle prevented the CF condition evident in the unsupplemented animal.

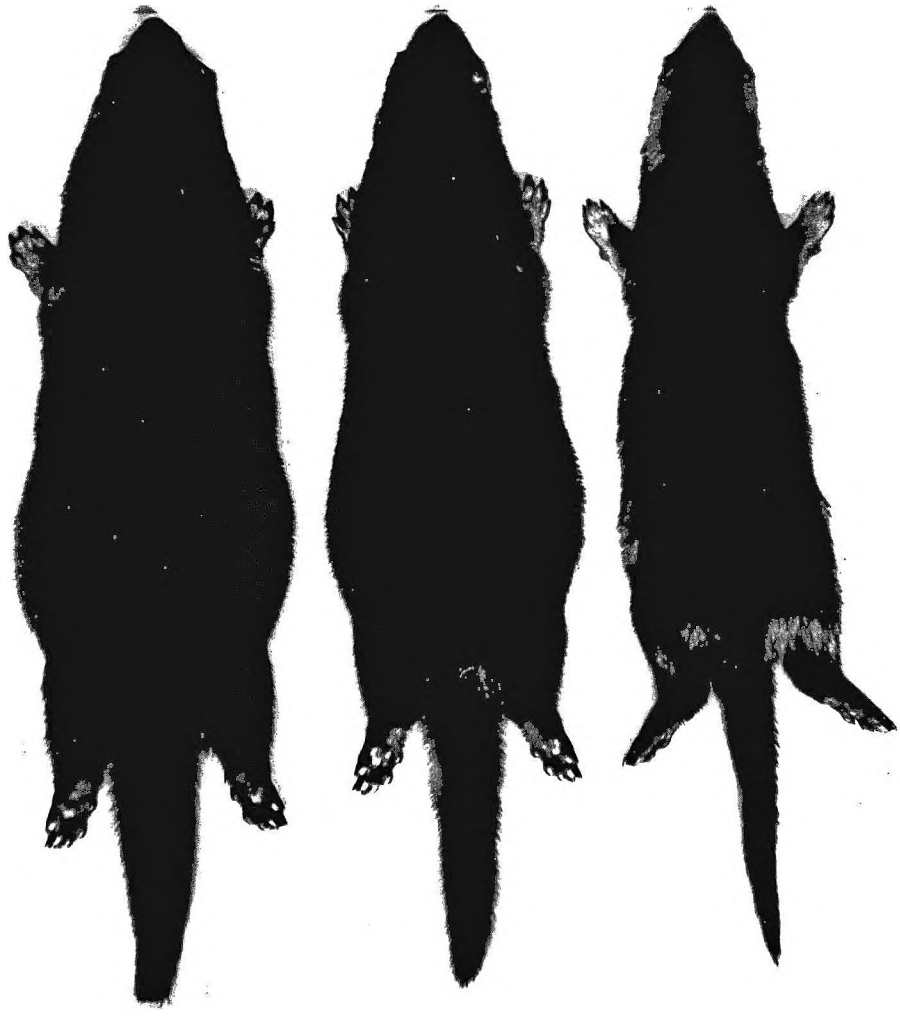

Fig. 3 Effect of parenteral iron on growth. Representative males from the adequately-fed control group (left), the group fed the CF-genic ration + parenteral iron supplement (center) and non-supplemented CF-genic diet groups (right) are shown. Note that the iron-supplemented animal is considerably larger than the non-supplemented animal and approaches the size of the adequately-fed control mink.

TABLE 3

Effects of orally-supplemented iron on weight gain and hemoglobin regeneration of anemic, CF mink

Ferrous iron[1]/ kg ration (as-fed basis)	No. of animals	Hemoglobin levels		Weight change	
		Initial	Regeneration[2]	M	F
mg		*gm/100 ml*		*gm*	*gm*
None	9	10.7±5.2[3]	0.3±3.6	−18±107	−75±40
17.6	9	10.9±5.0	0.3±1.9	−89±213	−47±66
88.1	9	11.7±4.7	0.4±1.5	−25±51	−95±68

[1] Supplied as Ferronord, Nordmark Pharmaceutical Lab., Inc.

[2] Measured as the average increase in hemoglobin level during the 30-day iron-supplementation period.

[3] The ± values represent standard deviation.

tance of adding supplementary B-vitamins to prevent greying of foxes and mink in connection with intensive fish feeding (Helgebostad and Ender, '58). However, it is believed that experimental conditions were sufficiently different so that strict comparison cannot be made. Fish provided the sole source of protein in Norwegian rations, whereas rations here contained protein from horsemeat, meatmeal, skim-milk powder and soybean oil meal in addition to fish protein.

The ineffectiveness of parenteral copper and oral lysine and tyrosine showed that these important components of melanogenesis were not limiting. On the other hand, organic iron when supplied parenterally to mink fed the CF-genic ration induced normal pigmentation of fur, increased weight gains immensely and resulted in essentially normal hemoglobin values. This would indicate that symptoms of CF, induced by feeding raw hake, are essentially those of an iron deficiency. Certainly anemia is the classical symptom of iron deficiency, and anemia of CF mink is of the microcytic, hypochromic type invariably associated with such deficiency. Depressed growth could also be easily related to a deficiency of iron, either indirectly as an effect of the severe anemia or directly since iron is contained in several important enzyme systems. The relation between iron deficiency and depigmentation, however, is not so readily obvious.

The total iron content of the adequate control ration is 114 mg per kg (as-fed basis) and that of the CF-genic ration is 108 mg per kg (as-fed basis), determined by the method of Kennedy ('27). The CF-genic ration contains nearly 95% as much iron as the adequate ration, yet does not supply enough iron to mink for normal growth or blood formation, and further, this lack of iron interferes in some unknown way with pigment formation. When the hake portion of the CF-genic ration is cooked, however, symptoms of CF disappear (Stout et al., '60), demonstrating that the inherent iron content of this ration is quantitatively ample to prevent CF symptoms. From these considerations it appears that raw hake and probably raw whiting contain a factor (possibly a chelating agent) which acts to render unavailable to the animal not only iron contained in the fish but also that of other ration components. Additional proof that dietary iron is unavailable is shown by failure of anemic, CF mink fed the CF-genic ration to respond to daily oral supplementation with iron glycinate (table 3).

As iron has not been linked directly with achromotrichia in the past, the immediate cause of observed depigmentation is speculative. Since achromotrichia has been observed in mink as a symptom of several unrelated nutrient deficiencies (Helgebostad et al., '59; Leoschke and Elvehjem, '59), it seems more plausible to suggest that failure of fur to pigment normally is a symptom of a non-specific dietary deficiency rather than to assume that iron is directly concerned with pigmentation processes. Shortage of an element as vital as iron to normal body physiology undoubtedly would affect overall metabolic reactions and it is conceivable that those processes which are of least consequence to the organism's survival, such as hair pigmentation would likely be first impaired.

SUMMARY

1. Comparison of blood values for "cotton fur" (CF) and normal mink revealed that CF mink exhibit a microcytic, hypochromic anemia.

2. Supplementing groups of mink fed a CF-genic ration with 11 parenterally-administered B-vitamins, parenteral copper, or oral lysine plus tyrosine, did not prevent mink from developing the CF syndrome.

3. Mink fed a CF-genic ration and supplied with parenteral iron did not develop the CF syndrome.

4. Iron glycinate added to a CF-genic ration was incapable of restoring normal hemoglobin values to anemic, CF mink.

5. The effect of iron on pigmentation is thought to be indirect, reflecting low priority of fur pigment formation in the face of nutritional stress.

ACKNOWLEDGMENT

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LITERATURE CITED

Adair, J., and K. G. Davis 1957 1956 Mink nutrition research. Misc. paper 41, Oregon Agricultural Experiment Station, Corvallis.

Best, C. H., and N. B. Taylor 1945 The Physiological Basis of Medical Practice, ed. 4. The Williams and Wilkens Company, Baltimore.

Ender, F., and A. Helgebostad 1947 Fôringens innflytelse på pelskvaliteten hos sølvrev. Norsk Veterinoer-Tidsskr., *12:* 381.

Frost, D. V. 1948 The relation of nutritional deficiencies to graying. Physiol. Rev., *28:* 368.

Helgebostad, A., and F. Ender 1951 Fortsatte undersøkelser over fôringens innflytelse på pelskvaliteten hos sølvrev. IV B-vitaminenes betyding for normal pelsfarge. Norsk Pelsdyrblad, *7:* 1.

——— 1958 Fôrets innflytelse på pelskvaliteten. Norsk Pelsdyrblad, *1:* 1.

Helgebostad, A., and E. Martinsons 1958 Nutritional anemia in mink. Nature, *181:* 1660.

Helgebostad, A., R. R. Svenkerud and F. Ender 1959 Experimentell biotinmangel hos mink og rev. Nordisk Veterinaermedicin, *11:* 141.

Kennedy, R. P. 1927 The quantitative determination of iron in tissues. J. Biol. Chem., *74:* 385.

Klain, G. J., D. C. Hill and H. D. Branion 1957 Effect of lysine-deficiency on hematopoiesis in the chick, including observations on folic acid deficiency. Poultry Sci., *36:* 405.

Leoschke, W. L., and C. A. Elvehjem 1959 The importance of arginine and methionine for the growth and fur development of mink fed purified diets. J. Nutrition, *69:* 147.

McCall, K. B., H. A. Waisman, C. A. Elvehjem and E. S. Jones 1946 A study of pyridoxine and pantothenic acid deficiencies in the monkey (*Macaca mulatta*). J. Nutrition, *31:* 685.

Sjollema, B. 1938 Kupfermangel als ursache von tierkrankheiten, Biochem. Ztschr., *295:* 372.

Stout, F. M., J. E. Oldfield and J. Adair 1960 Nature and cause of the "cotton-fur" abnormality in mink. J. Nutrition, *70:* 421.

Wintrobe, M. M. 1946 Clinical Hematology, ed. 2. Lea and Febiger, Philadelphia.

Cardiac Lesions and Related Findings in Young Vitamin B_6-Deficient Rats[1]

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This investigation was undertaken after we had been shown[2] two previously unreported lesions in young rats that had died while receiving a pyridoxine-deficient diet. There were pale opaque streaks on the hearts of these animals, and their chests were filled with fluid. Cardiac hypertrophy in pyridoxine-deficient rats, first reported by Agnew ('55) has been confirmed by others (Olsen and Martindale, '54) along with enlargement of the kidneys; and a variety of other anatomical findings unrelated to the heart have been described (Follis, '58). Since the Zuckers[3] had found a predominance of the lesions in question in their 14C strain, these rats were used in the present study in greatest numbers, two other strains being included for comparison as "genetic controls." Similar lesions had never been encountered under other circumstances by these investigators.

MATERIALS AND METHODS

Three strains of rats used in these experiments were the same as those reported previously (Seronde et al., '56; Zucker, '53). They have now been maintained for some years in this laboratory. Originally, they were selectively bred for large (14C), medium (13C) and small (9B) body size and for resistance to respiratory disease. Since their establishment here, emphasis in selection has been placed upon fertility and low incidence of ear and lung infections.

Animals were caged individually and were housed in isolation units in which cages, litter (pine shavings), litter-pans, water bottles and food cups were periodically autoclaved. Cages were of the hanging type, with top and three sides of galvanized sheet iron and with ½-inch galvanized wire-mesh bottoms and fronts. Except as noted, they were fed and watered ad libitum. Ambient temperature was kept at approximately 22°C, but relative humidity was often low in winter.

The purified diets, pyridoxine-deficient "2515" and control "2520" were of identical composition to those used in previously reported experiments (Seronde et al., '56).

Experiments were initiated with the animals as litters became available over a two-year period. A primary experimental series from each strain with littermate controls was augmented by large numbers of suitable animals from related projects. All animals reported upon here were weaned at 21 days and placed immediately on their definitive diets, experimental and control. Some control animals were fed ad libitum while for others the caloric intake was restricted to approximate that of the experimental animals. They were observed frequently throughout life, and weighed at least once a week. One group was weighed daily. Nearly all were allowed to die naturally, there being only a few which were killed when found moribund. A series killed at predetermined times contributed to our understanding of certain lesions, but are not enumerated with the present groups. All animals were autopsied. The minimum observations were external appearance, and weight and appearance of the heart and kidneys. Significant positive findings in other organs were noted only grossly. In many instances, however, autopsies were more thorough. Hearts were studied in detail, first being opened in a manner customary with human mate-

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[2] T. F. and L. M. Zucker, personal communication.
[3] See footnote 2.

rial,[4] then rinsed with saline, and examined with a 10-power dissecting microscope. Since reported techniques vary, it should be noted that heart weight here includes that of atria as well as of the ventricular mass. Great veins were removed completely, and approximately one millimeter of aorta and pulmonary artery were left attached to the specimen. This was done to preserve anatomical continuity for subsequent histological study. The entire heart was then divided into 6 blocks in preparation for microscopy, and at least one section was cut from each of these. From this sampling, an estimate of the incidence of microscopic lesions was made in a number of cases. This practice has complicated the presentation in tables 4 and 5 where percentage incidence of myocarditis could be computed with accuracy only from "complete cardiac histology" figures, rather than from the larger series of gross observations. Table 1 shows the distribution of animals by strain and diet, as well as the number of rats, by groups, on which the complete cardiac histology was carried out.

Worcester's fixative (Gray, '54) was most frequently used. Formalin (10%) neutralized with marble chips was used extensively in the earlier series. Rabl's solution (Gray, '54) gave exceptionally good results on hearts from freshly killed animals. Hematoxylin and eosin stains were used routinely. Mallory-Heidenhain's connective tissue stain (Jones, '50) was used as indicated. Foot's modification of Hortega's silver carbonate method was used for staining reticulum fibers (Jones, '50).

TABLE 1

Distribution of animals by type of diet and by strain

Diet	No. of animals	Strain	Animals studied for complete cardiac histology
2515[1]	120	14C	28
2520[2]	47	14C	46
2515[1]	29	13C	28
2520[2]	28	13C	24
2515[1]	25	9B	25
2520[2]	14	9B	14

[1] Experimental diet.
[2] Control diet: combined figure for ad libitum and restricted intake.

Sudan III and oil blue N were used in searching for stainable fat. Histological findings will be given only in general terms in this report.

OBSERVATIONS

The growth of animals fed the experimental diet was slow and eventually ceased. Many lost weight before death (fig. 5). They developed a characteristic hunched posture and accumulation of chest fluid was often indicated by a prominent rounding of the rib cage. Acrodynia was pronounced, particularly in the 9B strain. Neurological disturbances ("fits") were noted (Chick et al., '40) clinically in many animals, but were not investigated morphologically. There was wasting of musculature. Livers at autopsy were pale, as from exsanguination. Lymphoid tissue was much reduced (Stoerk and Eisen, '46) but could be located easily owing to marked atrophy of depot fat. The thymus showed severe involution. The character and overall incidence of findings with which we have been principally concerned is given in table 2.

The incidence and degree of cardiac hypertrophy is shown in figure 1. The control curve represents the best line which could be drawn through plots of the heart weights of animals fed the complete diet

TABLE 2

Principal anatomical findings in 174 animals receiving vitamin B_6-deficient diet

	no.	*%*
Cardiac hypertrophy	166	95
Renal hypertrophy	170	98
Myocarditis	87	50
Cardiac dilatation	76	44
Atrial thrombi	76	44
Hydrothorax	65	37
Pulmonary edema	34	20
Pneumonia	77	44

[4] Essentially this consists first of opening both atria by connecting their natural orifices and extending these incisions to the extremities of the auricular appendages. A cut is then made through the right atrial wall, down through the tricuspid orifice toward the apex of the right ventricle. Here it is turned sharply upward and continued out through the pulmonary orifice. A similar cut is made on the left side, down through the mitral orifice and out through the aortic valve ring. This method exposes the interiors of the chambers for close inspection, while preserving many of the anatomical relationships.

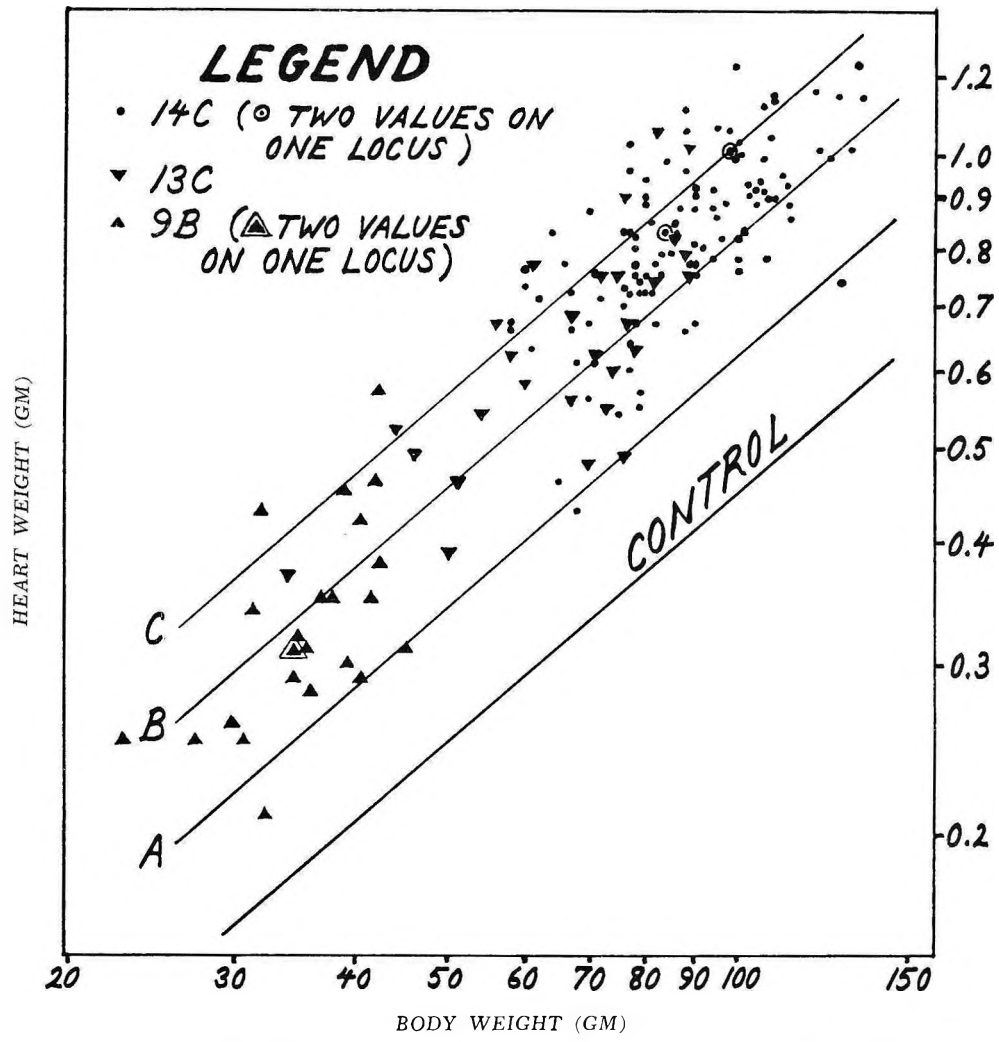

Fig. 1 The heart weight of vitamin B_6-deficient rats is plotted against the body weight, both on logarithmic scales. The line labeled "Control" represents an average of observations on 42 well-fed animals. Values for two of these lie above line A which is chosen as the upper limit of normal. It represents a 35% deviation from the control mean. Lines B and C are described in the text.

ad libitum. It lies above but within 5% of an empirical norm determined by Zucker[5] and has the identical slope. Line A represents the upper limit of variation for the controls and, arbitrarily, no values below it are considered to represent hypertrophy. For purposes of analysis and correlation the area above line A is divided into three zones by lines B and C, designated "slight," "moderate" and "marked" hypertrophy (table 5).

The heart of a strain 14C rat in this experimental series typically appeared enlarged *in situ*, the ventricular mass being widened notably. Part of this increased width could be attributed to dilatation of the right ventricle. Where such increase in size of the right ventricular cavity occurred without sacrifice in wall thickness, hypertrophy of this chamber could be in-

[5] T. F. and L. M. Zucker, personal communication.

ferred as well. The left ventricular wall, however, contributed most of the excess weight, being considerably increased in thickness. Dilatation, when it occurred here, was quite striking with deep cupping of the cavity and rounding of the apex. Hypertrophy of myocardial fibers was unmistakable in histologic sections and when the hearts of vitamin B_6-deficient rats were weighed, then dried to constant weight, no difference in relative water content was found between them and those of control animals. The right atrium was generally well filled with post mortem clot but on the whole was not distended beyond its normal diastolic contours. In a number of instances the left atrium contained a rounded firm white thrombus, usually visible from the outside through the translucent wall of the chamber. These thrombi tended to fill and distend the entire auricular appendage, on occasion even extending part way across the mitral orifice and thereby appearing to threaten the free passage of blood. Microscopically these lesions sometimes showed early and partial organization. Only rarely were thrombi located in other chambers.[6]

Yellow-white opaque streaking of the myocardium was a striking finding, included in table 2 under the designation of myocarditis. Grossly this ranged in extent and severity from sharply defined lesions on the ventricles a dozen square millimeters in area and extending deeply into the myocardium, to vague yellowish opacity which varied from one area to another giving a mildly mottled effect. These lesions were by no means confined to the ventricles, but were difficult to recognize grossly on the atria owing to thinness and pallor of the walls. The gross findings of severe involvement correlated well with a microscopic lesion which might be characterized most simply here as a deficit in the myocardium from which scarring is characteristically absent. Where loss of myocardium was presumed to have been recent the surviving framework of supporting reticulum cells and fibers retained their original pattern (fig. 2). In what were perhaps older cases, affected areas appeared densely populated with mononuclear cells (fig. 3). Polymorphonuclear leucocytes, laden macrophages and necrotic myocardium were unusual in this series. Instead, the myocardial fibers generally appeared to have undergone a relatively swift lysis.

Degenerative changes were common, but could not be clearly related to the myocarditis. They varied widely in severity and distribution. Figure 4 illustrates a case with severe widespread vacuolation of myocardial fibers.

Hydrothorax generally included mediastinal edema and pericardial effusion (table 2). Occasionally there was excessive moisture in the peritoneal cavity and in the layers of the ventral body wall. The volume of recoverable chest fluid averaged 4.2 cm^3 and sometimes reached 9 cm^3, enough to cause collapse of almost all lung parenchyma in animals as small as ours (fig. 5), and furnished a very tangible cause of death. Characterization of the fluid as an uncomplicated transudate is brought into question by its almost invariable clotting on standing and by its being anything from a clear "straw color" to a dark opaque bloody red. The animals having died spontaneously, cultures proved inconclusive. Microscopic study of all adjoining tissues in cases of hydrothroax revealed no evidence of relevant inflammation. Aside from atelectasis, the lungs showed little acute congestion or edema, and chronic congestive changes in lungs or elsewhere were not found. This latter might be due to the relatively brief average survival time.

In contrast with the above, clearly recognizable infections were far more common and severe in the experimental series than in the control series, and at times pneumonia could be considered a cause of death. Generally, such pneumonia resembled one caused by *Brucella bronchiseptica* with which we have become familiar in our colony. A few cases of peritonitis could be attributed to massive invasion of the bowel by its native flora.

Observations of renal hypertrophy are presented in figure 6. As in the case of the heart this increase in weight was demonstrated to be not due to increased water

[6] Wilens and Sproul ('38) in a survey of 487 rats found intracardiac thrombi in 31 cases. Most of these were in the left atrium.

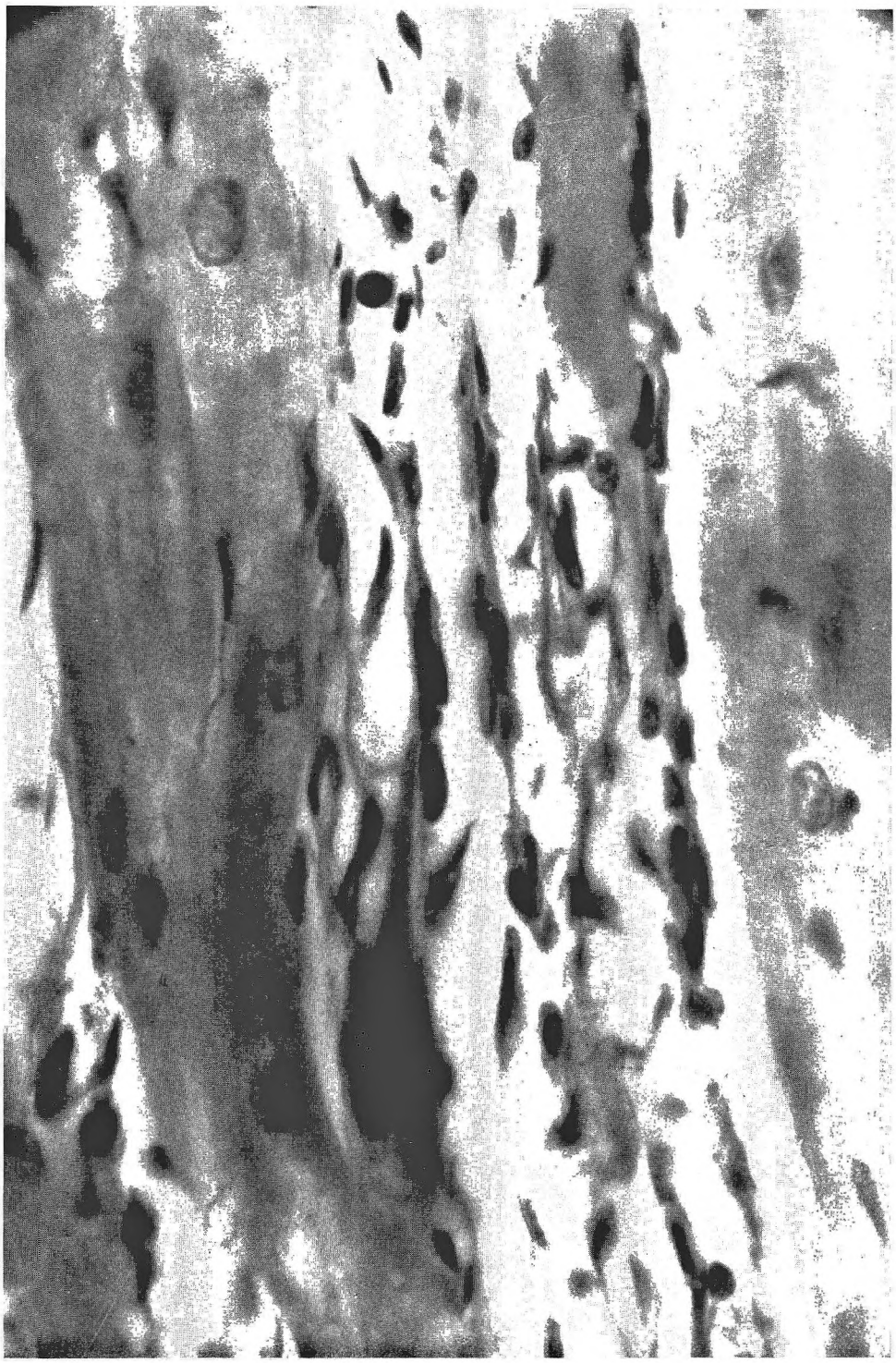

Fig. 2 Intact myocardium lies above and below a central horizontal deficit. Here the dark flattened nuclei of supporting reticulum cells and of capillary endothelium, normally obscured by muscle cells, have become conspicuous together with elongated cytoplasmic projections and reticulum fibers. At the upper left edge of the deficit a myocardial cell has two nuclei, indicative of a regenerative response. High-dry magnification.

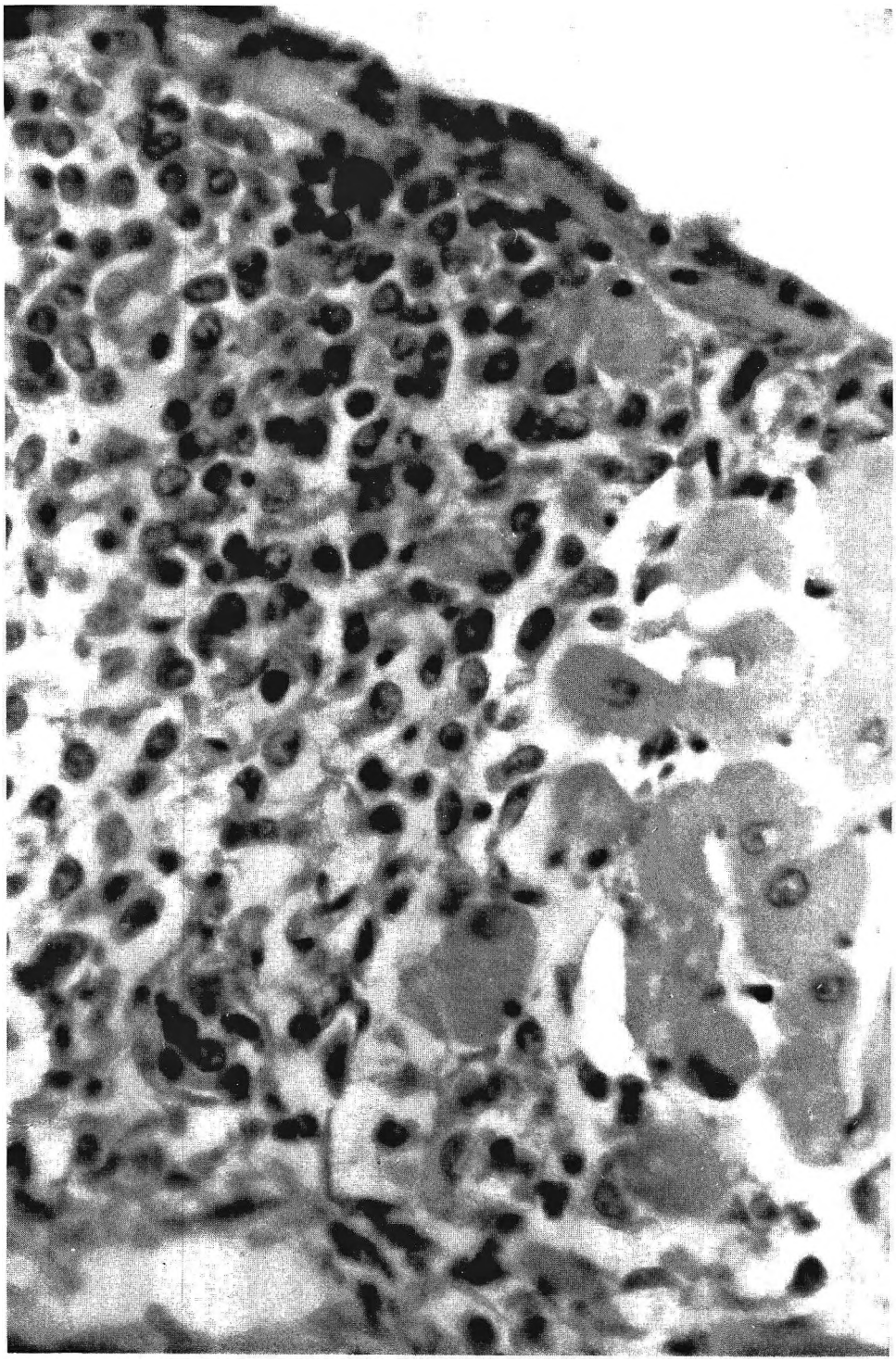

Fig. 3 The plane of section is at right angles to the axes of the myocardial cells visible in the lower central portion of the photograph. Above these, a deficit in the myocardium has become populated with large numbers of histiocytes, or "mononuclear cells." High-dry magnification.

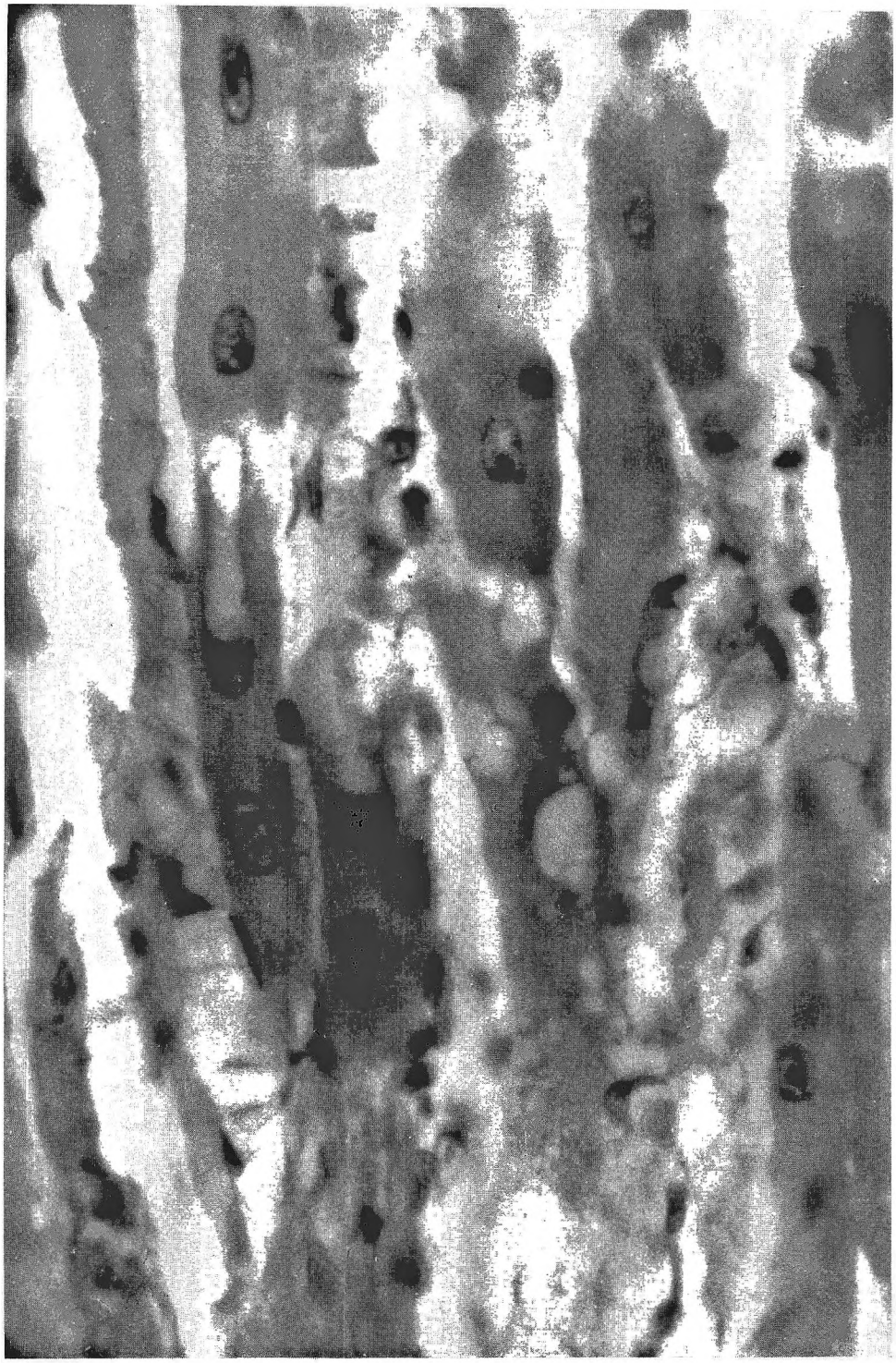

Fig. 4 An area of severely damaged myocardium is illustrated. Cytoplasm and nuclei of myocardial cells are distorted by large vacuoles. The animal, a strain 14C-female survived nearly 12 weeks when receiving the vitamin B_6-deficient diet. Grossly, the myocardium showed semiopaque vague streaking that followed the grain of the muscle. High-dry magnification.

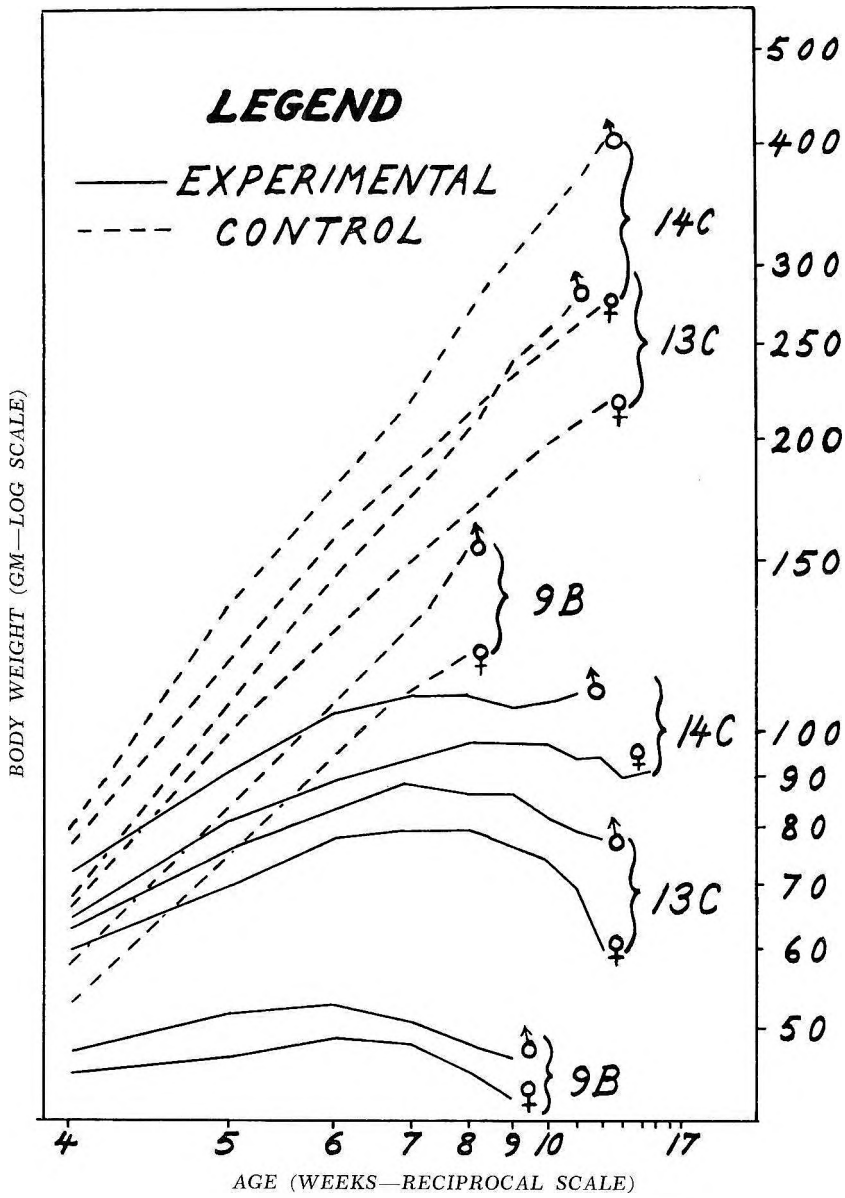

Fig. 5 Growth records of experimental and control groups have been averaged and plotted: body weight on logarithmic scale, age on reciprocal scale. (Zucker, '53).

content. Lines A, B and C have the same significance as in figure 1. Aside from this the only obvious lesion affecting these organs was an occasional very small and presumably inconsequential infarct that could be attributed to emboli from left atrial thrombi. The possibility of more subtle anatomical changes in the kidneys can be pursued only when better-preserved material from freshly killed animals becomes available.

Correlations. Table 3 and figure 5 show observations on growth and survival of both sexes and the three strains. The curves of figure 5 are plotted on log reciprocal coordinates (log body weight; recip-

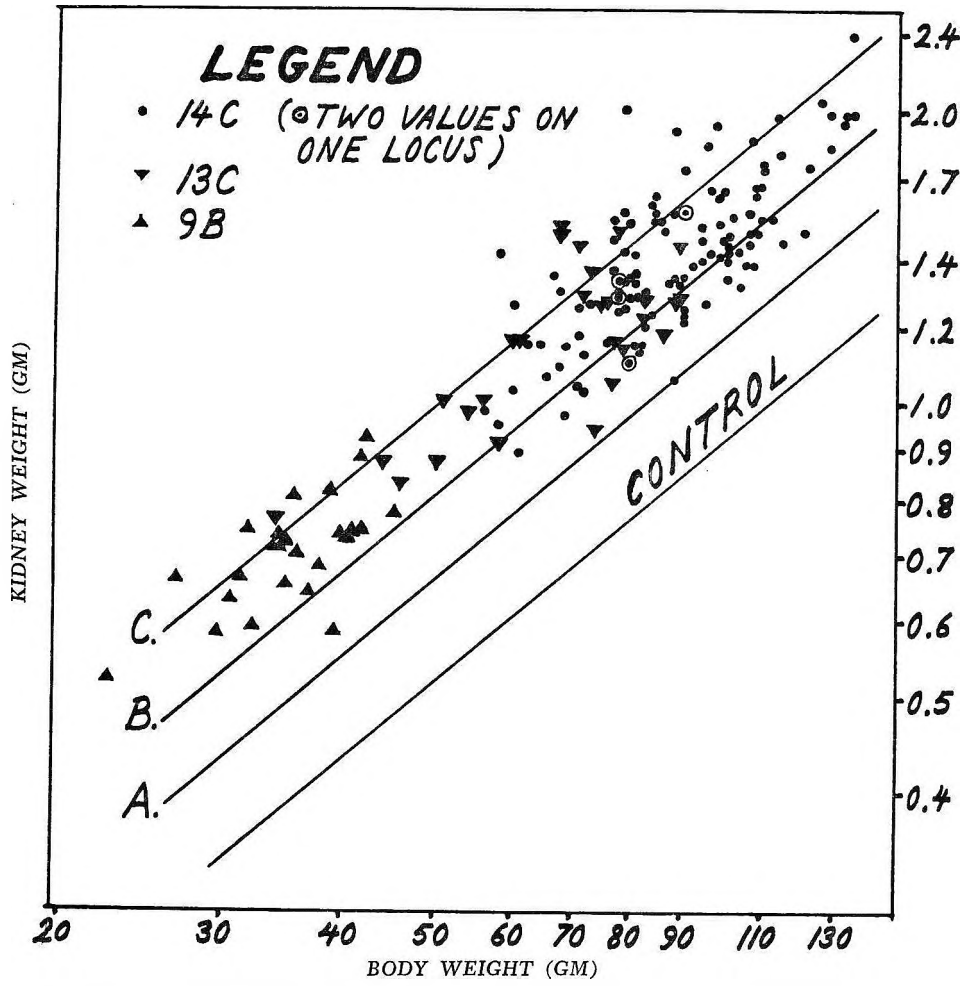

Fig. 6 The kidney weight of vitamin B_6-deficient rats is plotted against the body weight, both on logarithmic scales. The line labeled "Control" represents an average of observations on 43 well-fed animals. Values for two of these lie on or above line A which is chosen as the upper limit of normal. It represents a 25% deviation from the control mean. Lines B and C are described in the text.

rocal time) upon which optimal growth produces a straight line with a greater slope for males than for females (Zucker, '53). When deprived of pyridoxine, the large strain, 14C, lives the longest and gains the most weight; the 13C strain follows; the 9B strain gains least weight and dies earliest. That this is not entirely a matter of relative body size is shown by the substantially greater longevity of females which are in all cases smaller than males of their strain. Moreover, females fed the deficient diet lose more weight than males, which (particularly 14C) tend to hold their weight constant when they can no longer gain, then die suddenly. The terminal upward turn in the 14C-male growth curve, and the irregularity of the 14C-female growth curve may perhaps be related to hydrothorax and attendant edema seen in many of these animals (table 4, part C).

These findings provoked an inquiry into the significance of longevity and sex in relation to the incidence of other lesions we have already described. Table 4, part A, shows that invariably this incidence in-

TABLE 3

Comparative survival by sex and strain of rats fed vitamin B_6-deficient diet

Rats fed diet 2515	Total	Survival (days)	
		28–56	57–114
	no.	%	%
All spontaneous casualties	172	50	50
Males	75	68	32
Females	97	36	64
Strain 14C	100[1]	41	59
Strain 13C	26[1]	65	35
Strain 9B	24[1]	88	12

[1] Adjusted to contain an equal number of each sex.

creases with longevity. Since this is the case, it would be expected that females should in general have a higher incidence of lesions than males. Table 4, part B, shows the extent to which this is true. Females show a 15% greater incidence of severe myocarditis. Males, however, have a substantial preponderance of mild myocarditis, and a moderate one of cardiac dilatation and hydrothorax. It might be inferred from these figures that lesions which happen to be more common in the shorter-lived males take less time to develop and are perhaps more lethal. This is in fact consistent with the natures of these lesions, for myocarditis which is present to some extent even in the controls, might be expected to materialize early in a mild form and progress with time. And cardiac dilatation can appear suddenly, either in a heart that is failing, or in one subjected to sudden stress. We have indirect evidence that chest fluid may accumulate in the course of a day since the volume of chest fluid found at autopsy may correspond exactly to a weight gain observed in the last 24 hours of life.

Table 5 shows a direct correspondence between the degree of cardiac hypertrophy and other lesions including renal hypertrophy.

The graphs in figure 7 are based upon observations of 81 experimental animals in which the heart was surveyed for microscopic lesions. The following conclusions may be drawn from them and are diagrammed in figure 8: (1) hydrothorax is always accompanied by myocarditis; (2) atrial thrombi almost always accompany hydrothorax; (3) most animals with atrial thrombi have myocarditis; and (4) myocarditis almost always accompanies pulmonary edema. These observations are consistent with the possibility that myocarditis, or its presumed immediate cause, could give rise to hydrothorax and to pulmonary edema, while there is little to indicate much direct relationship between atrial thrombi or hydrothorax and pulmonary edema. That myocarditis or its precursor might give rise to hydrothorax in part through the mediation of atrial

TABLE 4

Percentage incidence of lesions in animals fed experimental diet

	Part A		Part B		Part C		
	Days of survival		Males	Females	Strain		
	28–56	57–114			14C	13C	9B
	%	%	%	%	%	%	%
(No. of rats)	(86)	(86)	(75)	(99)	(120)	(29)	(25)
Cardiac dilatation	33	55	49	39	53	28	16
Atrial thrombi	36	51	43	44	50	48	8
Hydrothorax	31	43	43	33	46	35	0
Pulmonary edema	16	23	20	19	28	3	0
Pneumonia	36	51	47	42	52	31	24
Myocarditis (no. of rats)[1]	(53)	(28)	(37)	(44)	(28)	(29)	(25)
Severe	32	50	30	45	61	48	0
Mild	15	29	32	9	18	24	16

[1] This figure applies to myocarditis only. In contrast with other items in this table, the incidence of myocarditis could be arrived at only in animals where "complete cardiac histology" had been carried out. See table 1.

TABLE 5

Relation of cardiac hypertrophy to other lesions in animals fed a vitamin B_6-deficient diet

Associated lesions	Cardiac hypertrophy		
	Marked	Moderate	Slight
	%	%	%
(No. of rats)	(40)	(87)	(39)
Cardiac dilatation	63	45	18
Atrial thrombi	58	41	33
Hydrothorax	55	33	28
Pulmonary edema	33	18	10
Pneumonia	65	47	18
Renal hypertrophy:			
Marked	28	10	5
Moderate	58	66	51
Slight	15	22	44
Myocarditis			
(no. of rats)[1]	(12)	(37)	(29)
(Severe and mild)	83	68	35

[1] This figure applies to myocarditis only. In contrast with other items in this table, the incidence of myocarditis could be arrived at only in animals where "complete cardiac histology" had been carried out. See table 1.

thrombi, or at least that atrial thrombi might facilitate the accumulation of chest fluid, is borne out by the observed anatomical position of the thrombi astride the return flow of blood from lungs to heart. In contrast, there seems little relationship between pneumonia and hydrothorax or pulmonary edema.

Controls. The incidence of lesions in the controls is given in table 6. Myocarditis was an unexpected microscopic finding in this series, for no corresponding lesions had been recognized grossly. Of the two cases tabulated as "severe," one was perfectly typical of those seen in the pyridoxine-deficient group. This animal had appeared outwardly vigorous and healthy at the time it was killed 7 weeks after weaning, but it had had diarrhea some 6 weeks before, suggestive perhaps of systemic infection, and its subsequent growth curve contained irregularities. The other was a spontaneous casualty 8 weeks after weaning, one of 4 occurring in the control group. The cause of death was unusual and in marked contrast with all other cases: a fulminating interstitial hemorrhagic myocarditis with hemopericardium. Mild myocarditis occurred in one other spontaneous casualty, but the cause of death was more probably related to a

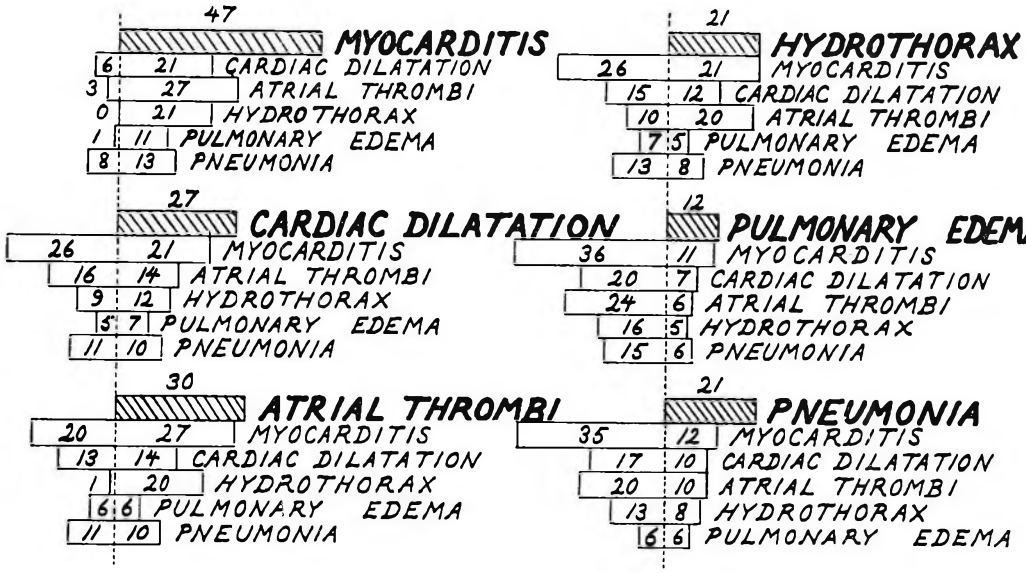

Fig. 7 These graphs show the frequency of coexistence of lesions. Figures represent numbers of individuals. Open bars are each to be compared to the cross-hatched bar at the top of each group, but not to each other. Overlap of any open bar with the cross-hatched bar heading its group (all to the right of the dotted line) indicates coexistence of the two lesions in question, whereas open bars to the left of the dotted line indicate non-coexistence. Thus for example, the upper left graph shows that of 47 animals with myocarditis, 21 had cardiac dilatation, while 6 with cardiac dilatation did not have myocarditis.

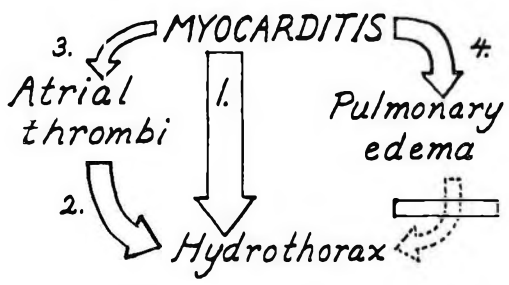

Fig. 8 This diagram illustrates conclusions drawn from figure 7. The relative thickness of the arrows indicates the strength of the relationship. Numerals correspond to those in the text.

peritoneal abcess found after a long clinical course of diarrhea. The remaining 12 animals in which myocarditis was found, had been visibly healthy up to the time of sacrifice, and lesions were small.

DISCUSSION

Myocarditis was found among the control animals and was clearly aggravated in pyridoxine deficiency. Most of the lesions in the well-fed animals appeared as trivial and incidental microscopic findings which probably would have been ignored had they not been recognized to differ only in size from the large "serious" lesions of the experimental series. In a survey for cardiovascular disease by Wilens and Sproul ('38) similar small foci of mononuclear cells were common in a large number of adult rats fed a stock diet. The frequency of latent virus infections, including those of the myocardium has been recognized increasingly since that time (Saphir, '49); and while it must be emphasized that we have not yet undertaken virus studies on our material, the morphological character of the lesions we observed is consistent with the diagnosis of virus myocarditis. The well-documented loss of capacity of animals deficient in pyridoxine to produce antibiodies, vital in antiviral defense, further supports this hypothesis. Schmidt ('48) has described massive and sometimes blood-tinged pericardial and pleural effusions in proven cases of fatal virus myocarditis.

We have described a cardiac syndrome, resulting from pyridoxine deficiency, which became evident when a particularly susceptible strain of rats was studied. To the commonly observed hypertrophy which seems basic in this deficiency have been added cardiac dilatation, mural thrombosis and hydrothorax. In comparing the resultant entity with some descriptions of others, we see first a close correspondence between our findings and those of Street et al. ('41) in pyridoxine-deficient dogs which had cardiac hypertrophy and dilatation with hydrothorax and congestive failure. Correspondence is more conjectural in the two entities which follow. The beriberi syndrome (Follis, '58) is recognized

TABLE 6

Numerical incidence of lesions in 89 control animals[1]

	Diet treatment		Total lesions
	Restricted[2] (38 rats)	Ad libitum-fed (51 rats)	
	no.	*no.*	*no.*
Cardiac hypertrophy	0	0	0
Renal hypertrophy	0	0	0
Myocarditis			
Severe	0	2[3]	14
Mild	4[4]	8[5]	
Atrial thrombi	0	0	0
Hydrothorax	0	1[6]	1
Pulmonary edema	0	0	0
Pneumonia	0	0	0

[1] Males, 49; females, 40.
[2] Caloric intake restricted to approximate that of deficient animals.
[3] Strain 14C, male.
[4] Strain 14C, 3 males, one female.
[5] Strain 14C, 4 males; strain 13C, 3 males, one female.
[6] Strain 14C, one male; very bloody. One of two with severe myocarditis. See text.

to be one of multiple deficiencies which varies with the genetic background of the patient. Sometimes it shows incomplete or poor response to thiamine therapy alone. It is characterized not only by cardiac hypertrophy and dilatation, but often by voluminous hydrothorax, and in some series at least by mural thrombi (Dock, '40). Another multiple-deficiency state, reported by Higgenson et al. ('52), is characterized by cardiac hypertrophy and dilatation, with mural thrombi in a large percentage of cases. Pleural and pericardial fluid occur much less conspicuously, ascites being more prominent. In relation to these examples, Levy's chemical evidence of pyridoxine deficiency in a group of human patients with cardiac failure due to various causes is of particular interest (Levy et al., '59). All of these findings combine to suggest that pyridoxine may eventually be found to play a role in certain problems of human heart disease.

SUMMARY

Rats receiving a pyridoxine-deficient diet at weaning survived about two months, then died, showing cardiac and renal hypertrophy, myocardial lesions, cardiac dilatation, atrial thrombi and hydrothorax in a significant number of cases. The interrelationships of these lesions and their possible meaning is discussed.

LITERATURE CITED

Agnew, L. R. 1955 Cardiac, renal and hepatic hypertrophy in pyridoxine deficient rats. Proc. Soc. Exp. Biol. Med., *90:* 452.

Chick, H., M. M. El Sadr and A. N. Worden 1940 Occurrence of fits of an epileptiform nature in rats maintained for long periods on a diet deprived of vitamin B_6. Biochem. J., *34:* 595

Dock, W. 1940 Marked cardiac hypertrophy and mural thromboses in ventricles in beriberi heart. Tr. A. Am. Physicians, *15:* 61.

Follis, R. H., Jr. 1958 Deficiency Disease. Charles C Thomas, Springfield, Illinois.

Gray, P. 1954 The Microtomist's Formulary and Guide. Blakiston Company, New York.

Higginson, J., A. D. Gillanders and J. F. Murray 1952 The heart in chronic malnutrition. Brit. Heart J., *14:* 213.

Jones, R. McC. 1950 McClung's Handbook of Microscopical Technique, ed. 3. Paul B. Hoeber, Inc., New York.

Levy, H. A., M. G. Wohl and A. Szutka 1959 Pyridoxine deficiency in congestive heart failure. Proc. Soc. Exp. Biol. Med., *101:* 617.

Olsen, N. S., and W. E. Martindale 1954 Studies on chronic vitamin B_6 deficiency in the rat. II. Changes in tissue metabolism. J. Nutrition, *53:* 329.

Saphir, O. 1949 Virus myocarditis. Mod. Concepts Cardiovas. Dis., *18:* 43.

Schmidt, E. C. H. 1948 Virus myocarditis. Pathologic and experimental studies. Am. J. Path., *24:* 97.

Seronde, J., Jr., T. F. Zucker and L. M. Zucker 1956 Thiamine, pyridoxine and pantothenic acid in the natural resistance of the rat to a corynebacterium infection. J. Nutrition, *59:* 287.

Stoerk, H. C., and H. N. Eisen 1946 Suppression of circulating antibodies in pyridoxine deficiency. Proc. Soc. Exp. Biol. Med., *62:* 88.

Street, H. R., G. R. Cowgill and H. M. Zimmerman 1941 Some observations of vitamin B_6 deficiency in the dog. J. Nutrition, *21:* 275.

Wilens, S. L., and E. E. Sproul 1938 Spontaneous cardiovascular disease in the rat. I. Lesions in the heart. Am. J. Path., *14:* 177.

Zucker, T. F. 1953 Problems in Breeding for Quality, in Rat Quality, a Consideration of Heredity, Diet and Disease (symposium). National Vitamin Foundation, Inc., New York, p. 48.

The Biological Unavailability to the Chick of Zinc in a Sesame Meal Ration[1]

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In feeding trials to evaluate the nutritive quality of sesame meal protein, chicks on a semipurified ration developed symptoms resembling those reported in zinc deficiency even though they were raised in galvanized cages and received added zinc in the salt mixture. The chicks showed a reluctance to move about, marked leg deformities, shortened shanks, poor growth and in some experiments frizzled feathers. These symptoms resembled those described for a zinc deficiency in poultry by several investigators[2] (Kratzer et al., '59; Morrison and Sarett, '58; O'Dell et al., '58; Roberson and Schaible, '58; Supplee et al., '58; Pensack et al., '58; Young et al., '58; Day and Hill, '59; Moeller and Scott, '58). Control chicks under similar conditions fed a semipurified ration containing soybean oil meal as the source of protein showed no signs of this deficiency.

The addition of zinc to the rations and determination of the zinc content of the sesame meal were undertaken to ascertain whether the symptoms observed were due to a lack of zinc. A chelating agent was used and the sesame meals were autoclaved (Kratzer et al., '59) to determine whether the zinc was bound and was not available to the chick.

EXPERIMENTAL

Day-old New Hampshire × Connecticut Randombred chicks were reared in electrically-heated galvanized batteries with galvanized wire floors and given feed and tap water ad libitum. There was an equal number of males and females in each group. The chicks were weighed weekly over the 4-week experimental period and the leg-deformity scores determined at the last two weekly weighings. These scores were determined by rating the chicks on a scale from zero to 4: zero being normal, 1—one leg slightly deformed; 2—both legs slightly deformed; 3—one leg slightly deformed, the other severely deformed; and 4—both legs severely deformed. Shortened shanks are characteristic of zinc deficiency and were thus considered a deformity.

In experiment 1, 16 chicks were used, in duplicate groups for each ration. In experiment 2, there were 10 chicks in triplicate groups for each ration in a random block design. An analysis of variance was run on the weight of the chicks after 4 weeks on the diets. In addition to the sesame meal rations a 50% protein soybean oil meal ration was used for comparison of growth and leg scores. A ration containing isolated soybean protein[3] was also fed in experiment 2. The basal ration for the chicks fed the sesame meal is given in table 1. For the soybean oil meal ration, the sesame meal of the basal ration was replaced isonitrogenously by 50% protein soybean oil meal, and the 9 gm of L-lysine hydrochloride by 3 gm of DL-methionine plus 6 gm of sucrose. Similarly for the isolated soybean protein ration, the sesame meal of the basal ration was replaced by the isolated soybean protein, and the 9 gm of L-lysine hydrochloride by 6 gm of DL-methionine and 3 gm of glycine. It will be noted that there are

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[1] South Carolina Experiment Station Technical Contribution no. 334.

[2] Zeigler, T. R., R. M. Leach, Jr. and L. C. Norris 1958 Zinc requirement of the chick. Federation Proc., *17*: 498 (abstract).

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

TABLE 1
Composition of basal ration

	per kg
Sesame meal,[1] gm	311 to 346
Barnett salts,[2] gm	60
Vitamin mixture[3], gm	5
Corn oil,[4] gm	55
L-Lysine·HCl, gm	9
Choline chloride, gm	2
Inositol, gm	1
Sucrose, gm	to 1000
D-α-tocopheryl acetate, mg	10
Vitamin D_3, I.C.U.	200
Vitamin A, U.S.P. units	10,000
Procaine penicillin, mg	10

[1] The protein content of the sesame meals varied from 58 to 64%; the amount used provided 200 gm of protein.

[2] The salt mixture was composed of the following in grams: $CaCO_3$, 3803; K_2HPO_4, 2580; $CaHPO_4 \cdot H_2O$, 1190; NaCl, 1340; $MgSO_4 \cdot 7H_2O$, 816; Fe citrate·$6H_2O$, 220; $MnSO_4 \cdot H_2O$, 40; KI, 6.4; $CuSO_4 \cdot 5H_2O$, 2.4; $ZnCl_2$, 2.

[3] Barnett et al. ('56).

[4] The lipid content of the sesame meals varied from one to 9%; thus the amount of corn oil added was adjusted so that the fat content of the ration was 5.5%.

5 gm of zinc chloride per 10,000 gm of salt mixture which gave 5.5 ppm of added zinc to the basal rations. A water solution of zinc chloride was mixed with the ration when supplementary zinc was added.

The sesame meals were made from decorticated K-10 sesame seed[4] which was pressed without additional heat, so that the temperature in general did not exceed 80°C to 90°C. The meals were made from the 1957 and 1958 crop of sesame seed (1957 and 1958 meal), and were very light colored in comparison with commercial hydraulic pressed sesame meal. The 1957 sesame meal contained 24% of lipids (ether-extract) as received, the 1958 sesame meal, 19% of lipids. These meals were extracted in the laboratory with cold hexane so that the lipid content was reduced to 1 to 2% for the 1958 sesame meal used in experiment 1 and for the 1957 meal used in experiment 2; the 1957 sesame meal for experiment 1 contained 9% of lipids. The zinc content of the 1958 sesame meal (19% lipids) was 108 ppm; that of the 1957 sesame meal (9% lipids) 117 ppm, as determined by the Vallee-Gibson procedure (Vallee and Gibson, '48). The rations for experiment 2 had the following zinc content: sesame meal, 52 ppm; soybean oil meal, 29 ppm; and isolated soybean protein, 12 ppm. The zinc content of the sesame meal and soybean oil meal rations in experiment 1 were similar to those in experiment 2. The L-lysine content of the 1957 sesame meal was 23 γ per gm; that of the 1958 sesame meal 19 γ per gm on the fat-free basis. The L-lysine of the sesame meal in the ration plus that of the added L-lysine hydrochloride totaled 1.4% of L-lysine in the sesame meal rations.

The autoclaved sesame meal for experiment 1 was prepared by mixing the sesame meal with an equal weight of water and autoclaving in glass dishes in a layer 1¾″ deep for 30 minutes at 15 pounds pressure. This was a rather dry preparation so that in experiment 2, the meal was similarly autoclaved with twice its weight of water; this gave a spongy preparation with no visible excess water. Both were dried and reground before incorporating in the ration.

Sesame meal was mixed with twice its weight of water containing enough ethylenediaminetetracetic acid, disodium salt (EDTA) to chelate 50 ppm of zinc with 50% additional to assure an excess (430 ppm of EDTA in the ration). This was allowed to stand in a plastic container at room temperature for 24 hours, dried at 49°C and reground. A control product was treated similarly with water but no chelating agent. Both products were observed to heat considerably during this time. In the dry-chelate ration, the same amount of EDTA was mixed with the sucrose of the ration.

No special effort was taken with the environment of the chicks to avoid zinc; besides raising the chicks in galvanized batteries, the rations were mixed in a galvanized container. Since the original rations with which the zinc deficiency was noted contained zinc chloride in the salt mixture, the same salt mixture was used in subsequent rations. Only in autoclaving the meal or the wet treatment with the chelating agent was care taken to use plastic or glass containers to prevent addi-

[4] The authors wish to thank Mr. Roy Anderson, Sesa-Kraft, Inc., Paris, Texas, for the sesame meals used in the experiments.

tional zinc getting into the ration from the surface of containers.

RESULTS AND DISCUSSION

The chicks fed the sesame meal rations grew poorly and exhibited marked leg deformities (table 2). Addition of 30 ppm of zinc to the ration led to an increase in weight and reduction of the leg deformities; addition of 60 or 120 ppm of zinc gave a further increase in weight. While the leg score is a subjective test, the lowered score for the chicks given 120 ppm of zinc and the slight increase in weight suggest that 120 ppm was a more adequate supplement than 60 ppm.

Feeding the 1957 sesame meal autoclaved for 30 minutes did not result in a significant increase in weight or reduction of leg deformities in the chicks. The addition of 30 ppm of zinc to this autoclaved meal ration, however, resulted in a significant gain in weight and reduction of leg deformities when compared with non-treated sesame meal. This ration gave a gain in weight and reduction in leg deformities comparable with those obtained with rations having 60 ppm of added zinc. This suggests that neither the 30 ppm of added zinc, nor autoclaving alone, supplied enough zinc but that the combination provided sufficient zinc for good growth and prevention of leg deformities. Doubling the water added to the sesame meal before autoclaving did not increase the availability of the zinc to the chick. Allowing sesame meal to stand with double its weight of water for 24 hours did not increase the availability of its zinc. With the 1958 meal, autoclaving led to a significant increase in weight but no reduction in leg deformities.

The use of EDTA, either as a solution with the meal itself or added dry to the ration, led to an increase in growth and reduction of leg deformities comparable with the addition of 60 or 120 ppm of zinc to the ration. Since the sesame meal of the ration contained 45 ppm of zinc and enough EDTA was added to free at least 50 ppm of zinc, evidently the EDTA was capable of releasing the zinc from

TABLE 2

Effect of treatment of sesame meal and the addition of zinc on the growth and leg deformity of chicks fed sesame meal rations

Protein source	Meal treatment	Added zinc	Average weight and leg deformity score at 4 weeks			
			Experiment 1		Experiment 2	
		ppm	*gm*	*score*	*gm*	*score*
1957 Sesame meal	none[1]	0	301	2.5	327	2.3
	none	30	349	0.6		
	none	60	374	0.9	409	0.7
	none	120			421	0.3
	autoclaved	0	341	1.9[2]	340	2.5[3]
	autoclaved	30	368	1.0[2]		
	+EDTA in ration[4]	0			404	0.3
	+EDTA, wet	0			430	0.4
	+water	0			323	2.4
1958 Sesame meal	none	0	225	2.4		
	none	60	387	0.7		
	autoclaved	0	329	2.3[2]		
Isolated soybean[5] protein	none	0			366	1.2
	none	60			386	0.4
Soybean oil meal	none	0	394	0.2	403	0.3
		60			428	0.4
L.S.D. (0.05)			41		39	

[1] In two previous experiments chicks fed the sesame meal rations had leg deformities with a score of 2.3 and 2.4.

[2] Sesame meal autoclaved with an equal weight of water.

[3] Sesame meal autoclaved with double its weight of water.

[4] The sesame meal received no treatment but dry EDTA was added to the ration.

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

its sesame meal-bond or was capable of destroying the zinc-binding power of the sesame meal so that extraneous zinc was available to the chick.

The addition of zinc to the isolated soybean protein ration did not lead to as striking improvement in growth as the addition of zinc to the sesame meal rations. Although leg deformities were present and were reduced by the addition of zinc to the ration, the condition was not as striking as that found with sesame meals. This is probably due to the greater zinc-binding capacity of the sesame meal.

Chicks fed the 50% protein soybean oil meal ration showed a slight increase in growth on addition of 60 ppm of zinc to the ration, but under the conditions of the experiment this was not significant; the incidence of leg deformities using either of these rations was negligible. Since the soybean oil meal ration contained 29 ppm of zinc in addition to that available from the chicks' environment, this was to be expected.

While the 29 ppm of zinc contained in the soybean oil meal ration resulted in good growth and lack of leg deformities, the addition of 30 ppm of zinc to the sesame meal rations already containing about 52 ppm of zinc did not result in good growth. Apparently not only is the zinc present in the sesame meal rendered unavailable, but also the sesame meal can combine with added zinc.

The sesame meals used in these experiments differ from most commercial meals in that the seed was decorticated before pressing, and the temperature of pressing was relatively low. The variety used, K-10, and its provenance could also possibly be factors which would differentiate these meals from other commercial ones. Experiments are under way with sesame meals made from non-decorticated K-10 seed grown in Texas, and with a different, new variety grown in South Carolina. These have been "cold-pressed," and pressed at elevated temperatures to determine which, if any, of the above factors are contributing to the zinc binding capacity of the meals used in the above experiments.

SUMMARY

Chicks fed purified rations containing sesame meal, made from decorticated K-10 seed as the sole source of protein, showed gross signs of zinc deficiency although the rations contained about 52 ppm of zinc. The chicks were raised in galvanized batteries and given tap water. Growth was significantly improved and leg deformities were greatly reduced when 60 or 120 ppm of zinc were added to the ration. Autoclaving the sesame meal led to significantly improved growth in some cases but did not prevent leg deformities in any case. Addition of a solution of ethylenediaminetetracetic acid, disodium salt (EDTA) to the sesame meal, or of dry EDTA to the ration greatly reduced leg deformities and resulted in as good growth as 120 ppm of zinc.

The addition of zinc to the sesame meal rations resulted in a much greater increase in growth and decrease in leg deformities than the addition of zinc to isolated soybean protein rations.

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LITERATURE CITED

Barnett, B. D., M. Lapidus, H. R. Bird and F. M. Strong 1956 Adenosine as growth factor for chicks fed purified diet. Proc. Exp. Biol. Med., *92:* 372.

Day, E. J., and J. E. Hill 1959 Zinc, potassium and certain other minerals as additives to practical turkey starter and grower rations. Poultry Sci., *38:* 646.

Kratzer, F. H., J. B. Allred, P. N. Davis, B. J. Marshall and P. Vohra 1959 The effect of autoclaving soybean protein and the addition of ethylene-diamine-tetracetic acid on the biological availability of dietary zinc for turkey poults. J. Nutrition, *68:* 313.

Moeller, M. W., and H. M. Scott 1958 Studies with purified diets. 3. Zinc requirements. Poultry Sci., *37:* 1227.

Morrison, A. B., and H. P. Sarett 1958 Studies of zinc deficiency in the chick. J. Nutrition, *65:* 267.

O'Dell, B. L., P. M. Newberne and J. E. Savage 1958 Significance of dietary zinc for the growing chick. Ibid., *65:* 503.

Pensack, J. M., J. N. Hensen and P. D. Bogdanoff 1958 The effects of calcium and phosphorus on the zinc requirements of growing chickens. Poultry Sci., *37:* 1232.

Roberson, R. H., and P. J. Schaible 1958 Zinc requirement of the chick. Science, *127:* 875.

Supplee, W. C., G. F. Combs and D. L. Blamberg 1958 Zinc and potassium effects on bone formation, feathering and growth of poults. Poultry Sci., 37: 63.

Vallee, B. L., and J. G. Gibson 1948 An improved dithizone method for the determination of small quantities of zinc in blood and tissue samples. J. Biol. Chem., 176: 435.

Young, R. J., H. M. Edwards and M. B. Gillis 1958 Studies on zinc in poultry nutrition. 2. Zinc requirement and deficiency symptoms of chicks. Poultry Sci., 37: 1100.

Selenium and Exudative Diathesis in Chicks and Poults[1,2]

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Schwarz et al. ('57) and Patterson et al. ('57) have shown that Factor 3 concentrates would prevent the gross symptoms of exudative diathesis in chicks fed a vitamin E-free Torula yeast basal diet of Scott et al. ('55) and selenium was found to be the active constituent of Factor 3. Dried brewers' yeast which also prevented exudative diathesis (Scott et al., '55), was found to be free of vitamin E but an effective source of Factor 3 (Schwarz, '55). Dietary selenium at 0.1 ppm of the diet replaced vitamin E or dried brewers' yeast in reversing the lowered protein content and albumin to globulin ratio (A/G) in the serum, reduced red cell count, hemoglobin values and the elevated cell volume in blood associated with exudative diathesis in chicks (Reid et al., '58).

In the present studies, supplements of varying levels of selenium as sodium selenate, dried brewers' yeast and ashed brewers' yeast were compared with vitamin E in their ability to prevent the symtoms of exudative diathesis in chicks and poults fed a Torula yeast diet. Analyses of blood were made to provide additional information on the relationship of such supplements to the condition of exudative diathesis.

EXPERIMENTAL

The Torula yeast basal diet used by Creech et al. ('57) was assigned to 10 groups of White Plymouth Rock chicks, one-day old.[3] Magnesium, potassium, copper and zinc were omitted from the mineral mixture since these elements were present in the Torula yeast in rather high concentrations (N.R.C., '56). Supplements added to the basal diet were vitamin E, selenium, dried brewers' yeast, ashed brewers' yeast, corn distillers' dried solubles and condensed fish solubles (table 1). Selenium was supplied as an aqueous solution of sodium selenate[4] (Na_2SeO_4). The ration was calculated to contain 22% of protein and was considered to be adequate in all micronutrients except vitamin E. Dried brewers' yeast was substituted on an isonitrogenous basis for Torula yeast. Dried brewers' yeast in the presence of 10% concentrated HCl or 1% CaO, was charred at a low temperature and was subsequently charred at 400°C to 500°C for 24 hours in an electric muffle furnace.

All diets were mixed fresh each week and rancidity indices determined at the mixing time and after one week's storage to test the antioxidant nature of selenium. The index used was the milliequivalents of peroxide present in 100 kg of feed. A sample of feed, calculated to contain one gram of fat was extracted with peroxide-free ethyl ether. After evaporation of the ether, peroxide number was determined in the extracted fat by titration with 0.002 N sodium thiosulphate.

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[2] The following products were supplied through the courtesy of the indicated companies: choline chloride by Hoffman-Taff, penicillin and other B-vitamins by Merck Sharp and Dohme, *d-α*-tocopheryl acetate by Distillation Products Industries, condensed fish solubles by Philip R. Park, Aureomycin by American Cyanamid Company, stabilized vitamin A by Stabilized Vitamins, vitamin D_3 by Charles Bowman and Company, menadione sodium bisulfite by Heterochemical Corporation, butylated hydroxytoluene by Koppers Company, and Torula yeast by Lake States Yeast Corporation.

[3] Dried Torula yeast contains the dried cells of *Candida utilis* and is produced by the Lake States Yeast Corporation, Rhinelander, Wisconsin.

[4] E. H. Sargent and Company, Detroit.

TABLE 1

Effect of selenium on growth, incidence of exudative diathesis and associated blood changes in chicks fed a vitamin E-free Torula yeast diet

Supplement to basal diet	Av. weight 28 days	Incidence of exudative diathesis 28 days	First appearance of symptoms	Red blood cells	Hemoglobin	Packed cell volume	Mean cell volume	Hemoglobin per cell
	gm	*%*	*days*	*ppm*	*gm/weight*	*%*	*10^{-7} mm³*	*10^{-8} mg*
None	264	95	17	2.10	6.3	29.8	141	3.00
d-α-Tocopheryl acetate (20 mg/pound)	290	—[1]	—	2.50	8.6	33.9	135	3.44
10% Dried brewers' yeast	295	—	—	2.51	8.4	33.7	134	3.34
10% Dried brewers' yeast (acid ashed)	254	90	17	—	6.0	27.6	—	—
10% Dried brewers' yeast (alkali ashed)	275	45	21	—	7.0	30.4	—	—
0.1 ppm Selenium (sodium selenate)	299	0	—	—	8.6	33.0	—	—
0.05 ppm Selenium (sodium selenate)	315	0	—	2.48	8.5	32.5	131	3.42
0.025 ppm Selenium (sodium selenate)	285	50	20	—	6.9	29.7	—	—
10% Corn distillers' dried solubles	245	0	—	—	—	—	—	—
10% Condensed fish solubles	300	0	—	—	—	—	—	—

[1] The — indicates no appearance or was not determined.

In the second study, the supplements, selenium, vitamin E, and dried brewers' yeast were added in all possible combinations.

The vitamin E-deficient diet for turkey poults was that used by Creech et al. ('57) with zinc, magnesium, potassium and copper omitted from the mineral mixture. Beltsville Small White poults, obtained from dams fed a complete practical diet, were divided into 4 groups of 15 each, and fed supplements of vitamin E and sodium selenate as indicated in table 2. Glucose monohydrate was replaced by corn starch in the diet of one group.

Birds in all studies were housed in electrically heated batteries with raised wire floors, and feed and water were supplied ad libitum. Body weights were determined weekly throughout the study and daily observations were made for the appearance of symptoms of exudative diathesis.

TABLE 2

Effect of selenium on the production of exudative diathesis in turkey poults fed a vitamin E-free Torula yeast diet

Supplement to basal diet	Weight at 28 days	Incidence of exudative diathesis at 35 days
	gm	*%*
None	220.0	70.0
d-α-Tocopheryl acetate, 20 mg per pound	225.0	—
Selenium (sodium selenate), 0.5 ppm	230.0	—
None[1]	233.0	—

[1] Starch used as source of carbohydrate in place of glucose monohydrate.

Five birds were removed for biochemical studies when sufficient birds in the basal group showed symptoms of exudative diathesis. Only birds having gross symptoms of exudative diathesis in the affected groups were selected for biochemical investigation.

Blood, taken by cardiac puncture, was allowed to coagulate at room temperature for one hour. The serum was then separated by centrifugation at 5000 rpm for 5 minutes and analyzed by paper electrophoresis.[5] The A/G ratio in the serum was calculated by the procedure recommended by the manufacturer of the apparatus. Total protein determinations were made by using the biuret reaction following the method of Kingsley ('42).

Blood for hematological measurements was taken by puncture of the wing vein. Measurements included erythrocyte counts, packed cell volume and hemoglobin determinations. Mean erythrocyte volume was determined according to the method of Wintrobe ('42).

RESULTS AND DISCUSSION

The determination of the rancidity in feed offered to the chicks indicated that very little oxidation had taken place in the lard used in this study prior to mixing the diet. After one week, the rancidity of the fat in the feed had increased to some extent. Vitamin E retarded rancidity effectively in the diet but selenium and dried brewers' yeast were totally ineffective as antioxidants (table 3).

The first symptom of exudative diathesis in the birds fed the basal diet appeared on the 17th day and increased to an incidence of 95% by the 28th day. The symptoms were characterized by an exudate and hemorrhage in the subcutaneous tissues of the abdomen, lower portion of the breast and on the inner sides of the thigh. The addition of selenium, as sodium selenate, at 0.1 or 0.05 ppm of the diet prevented the development of the gross symptoms of exudative diathesis in chicks up to 4 weeks old (table 1). Symptoms of exudative diathesis were likewise prevented by the addition of 20 mg of *d*-α-tocopheryl acetate per pound of diet or by dried brewers' yeast at 10% of the diet. Previous reports, however, showed that exudative diathesis in chicks was prevented by 0.1 ppm of selenium supplied in the diet as sodium selenite or selenocystathione (Schwarz et al., '57) or by 0.3 ppm of selenate selenium (Patterson et al., '57).

Vitamin E, dried brewers' yeast or selenium (0.1 and 0.05 ppm) promoted growth of the chicks, the maximum stimulation (about 20%) being exerted by 0.05 ppm of selenium. Evaluation of the activity of dried brewers' yeast reveals that it might be comparable with that resulting from 0.1 ppm of selenium.

Corn distillers' dried solubles and condensed fish solubles prevented exudative diathesis when used at 10% of the diet, indicating that these are effective sources of vitamin E-active factors, probably selenium. Birds fed 10% of corn distillers' dried solubles had diarrhea which might be due to the high mineral content of the supplement. Fish solubles promoted growth comparable with dried brewers' yeast (table 1).

Dried brewers' yeast ashed after acidification with concentrated HCl, did not prevent exudative diathesis nor did it stimulate growth (table 1) and the deficiency symptoms of the birds fed this supple-

[5] Spinco-R-Paper Electrophoresis and Model RB Analytrol, Spinco Division, Beckman Instruments, Inc., Belmount, California.

TABLE 3

Effect of selenium on the rancidity index of feed stored for a week

Supplement to basal diet	Rancidity index[1] of stored feed	
	0-day	7th day
None	0.20	42.0
d-α-Tocopheryl acetate, 20 mg/pound	0.21	30.1
Dried brewers' yeast, 10%	0.25	42.0
Selenium (sodium selenate), 1 ppm	0.22	44.0

[1] Milliequivalents of peroxide in 100 kg of feed, stored at 80°F.

ment were as severe as those of the birds fed the basal diet. The active factor preventing exudative diathesis was lost entirely under the conditions of ashing. When ashing of the yeast was carried out in the presence of 1% calcium oxide, a lower incidence of exudative diathesis was observed and the appearance of the symptoms was delayed by 4 days. Probably the active factor in brewers' yeast is volatile and capable of forming a less volatile salt with calcium as the cation. Selenium at 0.025 ppm was approximately 50% effective in preventing exudative diathesis in a 4-week period and the appearance of the symptoms was delayed by three days. The response due to 0.025 ppm of selenium was approximately the same as that with alkali-treated brewers' yeast ash with respect to protection against exudative diathesis and promotion of growth.

Red blood cell counts, packed cell volume and hemoglobin levels were reduced in the chicks having exudative diathesis. The cell volume, however, was increased slightly by the deficiency. The anemia observed in the deficient chicks thus was a macrocytic hypochromic anemia, which is in accord with the observation of Creech et al. ('58). The reduction of the hemoglobin per cell in the affected birds attracts attention. It is not known whether this change is due to the deficiency of vitamin E or to emaciation. Selenium at 0.05 ppm, or dried brewers' yeast at 10% of the diet, could replace vitamin E in maintaining the normal level of the volume and count of red cells and the hemoglobin content. Acid-ashed dried brewers' yeast was ineffective in maintaining these factors whereas alkali-ashed dried brewers' yeast and 0.025 ppm of selenium were partially effective in this regard (table 1).

The sodium, potassium, and calcium content of the serum was not affected by any of the supplements (table 4). Sodium and potassium are known to be involved in the osmotic mechanism of the blood. Dried brewers' yeast, vitamin E and 0.1 ppm of selenium exerted similar effects on the total serum protein and A/G ratio, but the addition of 0.05 ppm of selenium elevated the serum protein level to a greater extent than the other supplements did (table 4). The data for alkaline ash of dried brewers' yeast and 0.025 ppm of selenium were taken from birds which had exudative diathesis. These supplements exerted a partial protection of the normal A/G ratio of serum. In fact this partial protection means that by the time the birds of the basal group showed gross symptoms of a deficiency the birds fed the alkaline ash of dried brewers' yeast, or 0.025 ppm selenium, were only mildly affected by the symptoms since the supplements had delayed the onset of exudative diathesis.

In the second study, symptoms of exudative diathesis were observed in 85% of the chicks fed the vitamin E-free Torula yeast diet by the end of the 4th week (table 5). The chicks were fed the experimental diet for 5 weeks and by the end of the experimental period all of the birds fed the basal diet had died. Addi-

TABLE 4

Effect of selenium on sodium, potassium, calcium, total protein and A/G ratio of the serum of chicks fed a vitamin E-free Torula yeast diet

Supplement to basal diet	Serum composition			Total protein	A/G ratio
	Sodium	Potassium	Calcium		
	meq/l	*meq/l*	*mg/100 ml*	*gm/100 ml*	
None	159.0	5.5	8.0	2.01	0.13
d-α-Tocopheryl acetate, 20 mg/pound	154.0	5.3	9.1	3.80	0.84
Dried brewers' yeast, 10%	150.0	5.5	8.5	3.79	0.72
Dried brewers' yeast (acid ashed), 10%	159.0	5.7	—	2.50	0.20
Dried brewers' yeast (alkali ashed), 10%	157.0	5.0	—	2.60	0.29
Selenium[1], 0.1 ppm	150.0	5.2	9.0	3.80	0.81
Selenium[1], 0.05 ppm	156.0	5.8	8.4	4.04	0.79
Selenium[1], 0.025 ppm	—	—	—	2.80	0.40

[1] As sodium selenate.

TABLE 5

Effect of selenium on growth and occurrence of exudative diathesis in chicks fed a vitamin E-free Torula yeast diet

Supplement to basal diet	Weight		Blood hemoglobin
	21 days	35 days	25 days
	gm	*gm*	*gm/100 ml blood*
None	235[1]	—	7.00
Vitamin E[2]	286[1]	568	8.13
Dried brewers' yeast[2]	285	555	8.33
Selenium[4]	294	563	8.07
Vitamin E[2] and selenium[4]	291	560	8.00
Selenium[4] and dried brewers' yeast[3]	287	550	8.70
Dried brewers' yeast[3] and vitamin E[2]	290	549	8.66
Vitamin E,[2] dried brewers' yeast[3] and selenium[4]	289	560	8.33

[1] Symptoms of exudative diathesis appeared on 17th day and occurrence was 85% by end of 4th week; all chicks died by 5th week.
[2] As 20 mg *d*-α-tocopheryl acetate/pound of diet.
[3] Dried brewers' yeast, 10% of diet.
[4] As sodium selenate, 0.05 ppm.

tion of either 20 mg/pound of *d*-α-tocopheryl acetate or 10% of dried brewers' yeast to the diet produced equivalent growth responses of about 21% at the end of the third week. The supplementation of 0.05 ppm of selenium resulted in a growth response of 25% over that of the birds fed the basal diet. The growth responses obtained by the different combinations of the supplements were no greater than those due to any single supplement. The average three-week weights of chicks fed these three supplements, singly or in combination, were within the limit of experimental variation. The weights in the 5th week also supported the same conclusion, but since all the birds fed the basal diet had died, no comparison could be made between the supplemented and unsupplemented groups. The hemoglobin content of the blood of the deficient birds was lower by about 16% than that of the birds receiving any of the supplements; however, no significant differences existed among the hemoglobin values of the birds receiving the different treatments (table 5).

Exudative diathesis appeared in turkey poults on the 25th day of feeding the Torula yeast basal diet (table 2), the symptoms being a mild abdominal edema and exudation as described by Creech et al. ('57). All the birds fed the basal diet died by the end of the 5th week. The symptoms were completely prevented by *d*-α-tocopheryl acetate (20 mg/pound) or by 0.5 ppm of selenium as sodium selenate. Most of the birds, fed the basal diet had edema and exudates which might have increased the weight of the birds. The affected birds had only mild edema and died before the edema became severe. The use of corn starch in place of glucose in the Torula yeast diet for the turkey poults prevented the appearance of symptoms of exudative diathesis.

The possibility exists that vitamin E, or more probably selenium, occurs as a contaminant in corn starch in sufficient quantities to prevent the condition. It is also possible that this observation is related to the report by Manfre et al. ('58) that the microbial population in the crop of the turkey is greatly increased by substitution of glucose[6] for starch.

SUMMARY

Symptoms of exudative diathesis were produced in chicks fed a vitamin E-free Torula yeast diet. The condition was prevented by the addition of vitamin E or of dried brewers' yeast to the basal diet. Corn distillers' dried solubles and condensed fish solubles were also active in this respect.

Selenium fed at 0.1 ppm or 0.05 ppm as sodium selenate in the diet successfully replaced vitamin E or dried brewers' yeast

[6] Cerelose.

in the prevention of the gross symptoms of exudative diathesis in chicks and in preventing the increase in cell volume, the reduction in blood hemoglobin content, in red cell count, total serum proteins and in the A/G ratio. The incidence of exudative diathesis in turkey poults was prevented by the addition of 0.05 ppm of selenium (selenate). Unlike vitamin E, the dried brewers' yeast and selenium did not prevent the rancidity of feed fat.

In the absence of vitamin E, selenium (selenate) promoted growth when fed at 0.1 or 0.05 ppm in the diet, the latter level being slightly more effective. In the presence of vitamin E, no growth response was observed by the supplementation of selenium or dried brewers' yeast in the diet. The growth response due to dried brewers' yeast was comparable with that resulting from 0.1 ppm selenium.

Selenium, vitamin E, or dried brewers' yeast did not affect the sodium, potassium or calcium level of the serum of the chicks. The alkaline (calcium oxide) ash of dried brewers' yeast had partial effectiveness against the condition of exudative diathesis, equivalent to that due to about 0.025 ppm selenium (selenate) in the diet. No protection was observed when dried brewers' yeast, ashed in the presence of 10% concentrated HCl, was added as a supplement to the diet. Selenium, vitamin E, or the use of starch as a carbohydrate source completely prevented the development of exudative diathesis in turkey poults fed a Torula yeast diet.

LITERATURE CITED

Creech, B. G., G. L. Feldman, T. M. Ferguson, B. L. Reid and J. R. Couch 1957 Exudative diathesis and vitamin deficiency in turkey poults. J. Nutrition, *62:* 83.

Creech, B. G., M. M. Rahman, B. L. Reid and J. R. Couch 1958 Exudative diathesis in chicks. Ibid., *64:* 55.

Kingsley, G. R. 1942 The direct biuret method for determination of proteins as applied to photoelectric and visual colorimetry. J. Lab. Clin. Med., *27:* 840.

Manfre, A. S., H. O. Wheeler, G. L. Feldman, R. H. Rigdon, T. M. Ferguson and J. R. Couch 1958 Fungi in the crop of the turkey. Am. J. Vet. Res., *19:* 689.

National Research Council Committee on Feed Composition 1956 Composition of Concentrate By-Product Feeding Stuffs. Publication 449, Washington, D.C.

Patterson, E. L., R. Milstrey and E. L. R. Stokstad 1957 Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exp. Biol. Med., *95:* 617.

Reid, B. L., M. M. Rahman, B. G. Creech and J. R. Couch 1958 Selenium and development of exudative diathesis in chicks. Ibid., *97:* 590.

Schwarz, K. 1955 Modern Brewery Age, 53: 72. Cited by Schwarz et al. ('57).

Schwarz, K., J. G. Beiri, G. M. Briggs and M. L. Scott 1957 Prevention of exudative diathesis in chicks by Factor 3 and selenium. Proc. Soc. Exp. Biol. Med., *95:* 621.

Scott, M. L., F. W. Hill, L. C. Norris, D. C. Dobson and T. S. Nelson 1955 Studies on vitamin E in poultry nutrition. J. Nutrition, 56: 387.

Wintrobe, M. M. 1942 Clinical Hematology. Lea and Febiger. Philadelphia.

Dietary Hormones and Fat and Serum Cholesterol, Transaminases and Copper in Swine[1]

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The study of Gottieb and Lalich ('54) showed that about one third of the swine reaching the age of three years had sclerotic aortas. Since then several studies on the effect of various dietary regimes on the atherosclerotic process in swine have been reported.

Bragdon et al. ('57) observed that addition of butter to the diet of swine caused a significant elevation in serum cholesterol, but corn oil was without effect. Peifer and Lundberg[2] found that both corn oil and beef tallow fed at two levels of energy intake raised serum cholesterol. With maintenance intake of total calories the beef tallow gave slightly higher values than corn oil. Roswell et al. ('58) reported that blood cholesterol did not increase when swine were fed isocaloric diets rich in butter or margarine. Recently, Barnes et al. ('59a, '59b) reported studies on the effect of dietary fat and protein on serum cholesterol in young and adult swine. They found that fat in the form of beef tallow caused an increase in serum cholesterol in young and adult swine. Low protein also caused a hypercholesteremia, but only when evidence of protein deficiency was manifested. They suggested that information obtained from swine on the atherosclerotic process as affected by the diet may be of help in unraveling the mystery of atheroschlerosis and heart disease in man. Consequently we initiated a study in which swine were maintained on different dietary regimes and the blood analyzed for cholesterol, transaminases and copper. The results of these analyses are reported herein.

EXPERIMENTAL

Purebred Duroc pigs, 9 to 10 weeks old, were used. In the testosterone study, 30 barrows were assigned randomly to the following 5 treatments: controls and testosterone additions of 20 mg per day; zero mg to 125 pounds of body weight and 20 mg per day thereafter; 20 mg per day to 125 pounds of body weight and zero mg thereafter; and zero, 5, 10, 15, 20, 30, 40 and 50 mg per day at 10-day intervals. In addition, 6 boars were included for comparison with the barrow controls. The methyltestosterone, U.S.P. grade, was mixed with the complete feed mixture. In the fat-stilbestrol experiment, 36 barrows were assigned randomly to 6 treatments. The design of this experiment is described in table 1. The diethylstilbestrol[3] was mixed with the complete feed mixture so that each pig received a minimum of 2 mg per day. The pigs were housed in individual concrete-floor pens and received feed and water ad libitum. The composition of the various diets is shown in table 2. The pigs in the testosterone study received a diet containing 16% of protein until they reached the weight of 100 to 110 pounds and then changed to a 14% protein diet. In the fat-stilbestrol experiment, the initial diet contained 14% of protein, which was lowered to 10% when the pigs attained 100 to 110 pounds. The pigs were killed upon reaching approximately 200 pounds. Blood was obtained about one hour after the pigs were removed from the feed.

Serum total cholesterol was determined by the method of Zak ('57); serum copper by the procedure of Peterson and Bol-

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[1] Published with the approval of the Director as Journal Series Paper no. 76.

[2] Peifer, J. J., and W. O. Lundberg 1958 Influence of total calories, fat calories and fat unsaturation on blood lipides. Federation Proc., *17:* 288 (abstract).

[3] Stilbosol, supplied by Eli Lilly and Co., Indianapolis.

TABLE 1

Effect of dietary hormones and fat on serum cholesterol, transaminases and copper

Treatment	Cholesterol[1]	SGOT[2]	SGPT[2]	Copper[3]
Testosterone				
Controls (6)[4]	187 ± 11	48.4 ± 6.6	36.4 ± 5.8	2.16 ± 0.28
20 mg/day (6)	143 ± 26[6]	60.8 ± 10.5	36.8 ± 6.0	2.08 ± 0.33
20 mg/day from 125 pounds (6)	152 ± 12[7]	63.9 ± 14.0	37.6 ± 5.3	2.15 ± 0.17
20 mg/day to 125 pounds (6)	170 ± 31	60.8 ± 12.5	41.1 ± 6.1	1.86 ± 0.41
Graded levels (6)	136 ± 30[6]	66.0 ± 18.8	34.9 ± 5.3	2.10 ± 0.25
Boars (6)	138 ± 13[6]	52.6 ± 11.0	32.2 ± 3.6	1.76 ± 0.23
Fat—stilbestrol				
Controls (6)	180 ± 13	54.7 ± 15.1	42.8 ± 3.4	2.11 ± 0.11
Stilbestrol (6)	177 ± 24	54.5 ± 10.6	40.5 ± 7.3	2.40 ± 0.18[6]
5% Tallow (5)	209 ± 31	48.0 ± 16.9	42.6 ± 4.9	2.15 ± 0.25
5% Tallow + stilbestrol (6)	214 ± 21	54.4 ± 6.7	39.0 ± 3.2	2.32 ± 0.28[6]
10% Tallow (6)	288 ± 39[7,8]	52.1 ± 9.3	37.1 ± 4.3	2.02 ± 0.40
10% Tallow + stilbestrol (6)	201 ± 27	48.1 ± 6.5	38.8 ± 5.1	2.32 ± 0.19[6]

[1] Milligrams per 100 ml of serum.
[2] Sigma-Frankel units per milliliter of serum [glutamic oxalacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT)].
[3] Micrograms per milliliter of serum.
[4] Figures in parentheses show number of animals assayed.
[5] Standard error of the mean.
[6] Significant difference from controls at $P < 0.01$.
[7] Significant difference from controls at $P < 0.05$.
[8] Significant difference from stilbestrol group at $P < 0.05$.

TABLE 2

Composition of diets

	Level of protein in diet	Testosterone[1] experiment	Fat-stilbesterol[2] experiment		
			Control	5% Tallow	10% Tallow
	%	%	%	%	%
Corn	16	79.3	—	—	—
	14	83.8	83.8	77.8	71.8
	10	—	92.8	86.8	80.8
Soybean meal	16	16.5	—	—	—
	14	12.0	12.0	13.0	14.0
	10	—	3.0	4.0	5.0
Alfalfa meal[3]		2.0	2.0	2.0	2.0
Mineral mix[4]		2.0	2.0	2.0	2.0
Vitamins[5]		0.1	0.1	0.1	0.1
Aureomycin[6]		0.1	0.1	0.1	0.1
Stabilized beef tallow		—	—	5.0	10.0

[1] Added as methyltestosterone.
[2] Added as diethylstilbestrol to supply each pig a minimum of 2 mg per day.
[3] Dehydrated alfalfa meal (17% protein).
[4] Mineral mixture supplied 273.9 gm $CaCO_3$; 273.9 gm steamed bone meal; 273.9 gm NaCl; 19.55 gm Fe_2O_3; 195 mg $CoCO_3$; 3.9 gm $MnSO_4 \cdot H_2O$; 50 mg KI; 3.12 gm $CuSO_4 \cdot 5H_2O$; and 58.69 gm cane molasses per 100 pounds of feed. In addition, zinc carbonate was added to supply 50 ppm of zinc.
[5] Vitamin premix supplied 2 mg riboflavin, 4 mg Ca pantothenate, 9 mg niacin and 10 mg choline chloride per pound of feed.
[6] Aurofac 1A, American Cyanamid Co., New York; supplied 1.8 mg of Aureomycin per pound of feed.

lier ('55); and the serum enzymes, glutamic oxalacetic transaminase and glutamic pyruvic transaminase, were measured by the method of Reitman and Frankel ('57).[4] The data were treated statistically and the multiple range test as proposed by Duncan ('55) was used to test significance of the means.

RESULTS

The data illustrating the effect of testosterone, stilbestrol and fat on serum total cholesterol are shown in table 1. Feeding testosterone at a level of 20 mg per day or graded levels of 10 mg per day at 10-day intervals to growing swine significantly lowered the cholesterol level ($P < 0.01$). It was also decreased in animals receiving testosterone from the weight of 125 pounds to the end of the trial ($P < 0.01$). Evidence that testosterone caused a reduction in serum cholesterol is demonstrated by the observation that when it was not fed after the pigs reached 125 pounds, the cholesterol level was the same as that of the control barrows. Additional evidence is indicated by the results that boar pigs had a lower serum cholesterol than barrows ($P < 0.01$) and essentially the same as those animals fed testosterone. In contrast with the lowering effect of testosterone, stilbestrol did not cause a change in serum cholesterol. The stabilized beef tallow caused a slight elevation of serum cholesterol when it was fed at the level of 5% and a significant increase at the 10% level ($P < 0.01$).

The results showing the effect of hormones and fat on serum glutamic oxalacetic and glutamic pyruvic transaminases and copper are shown in table 1. The data clearly show no effect on the enzymes. No definite trend was found for serum copper as influenced by testosterone. On the other hand, stilbestrol, added to the daily ration, caused a significant increase ($P < 0.01$).

DISCUSSION

Many factors have been shown to influence serum cholesterol concentration. Among them are the level and type of dietary fat, the amount of cholesterol and cholic acid in the diet, the intake of plant sterols, dietary amounts of a number of the vitamins, the protein intake and endocrine factors.

Of these, the effect of hormones is of particular interest in the present study. However, although considerable work has been done on this subject, the picture is quite variable. Normally, serum cholesterol is higher in the female animal than in the male. This has been reported by Fillios et al. ('58) to be related to estrogenic activity. These investigators found that endogenous cholesterol biosynthesis was higher in female rats than in males. It was further noted that estradiol treatment stimulated endogenous cholesterol biosynthesis in castrated rats.

Various trials have been reported on the effect of endocrine administration on serum cholesterol in experimental animals. Testosterone treatment of male and female rabbits lowered the serum cholesterol level, whereas estradiol increased it (Fillios and Mann, '56). For the male rat, estradiol raised the serum cholesterol (Fillios, '57; Priest et al., '57) but stilbestrol, progesterone and other substances related to active estrogens had little effect (Priest et al., '57). Testosterone has been found to prevent hypercholesteremia in cholesterol-fed cockerels (Pick et al., '59).

Castration also plays a role. Castration of both female and male cockerels (Pick et al., '59) and rats (Fillios, '57) resulted in hypercholesteremia. Estradiol was found (Fillios, '57) to increase cholesteremia in castrates of both male and female rats, while testosterone was found to lower the response of the female castrates. It is of interest to note that estradiol and testosterone treatment of castrated male and female rabbits (Fillios and Mann, '56) and testosterone administered to castrated male and female chickens (Pick et al., '59) did not affect serum cholesterol.

In the present study, we found that testosterone fed to castrated pigs lowered serum cholesterol, in contrast with its effect on the rabbit and chicken, but similar to that on the castrated female rat. It should also be pointed out that as in the case of the castrated rat and chicken, castration of male pigs resulted in higher serum cholesterol levels. Stilbestrol, the synthetic estrogenic compound, did not change serum cholesterol in barrows. This

[4] Reagents were purchased from Sigma Chemical Company, St. Louis.

finding is similar to that observed for the male rat (Priest et al., 57).

Johnson et al. ('59) reported that parenteral administration of testosterone or estradiol to humans increased significantly the content of serum copper. The work presented herein for swine shows that, although stilbestrol increased serum copper, administration of testosterone had no effect. The lack of agreement for testosterone could be due to the difference in method of administration.

SUMMARY

Young, growing barrow pigs were fed either varying levels of testosterone or a single level of stilbestrol supplemented with 5 or 10% of beef tallow and the serum was analyzed for cholesterol, glutamic oxalacetic and glutamic pyruvic transaminases and copper.

Whereas it was found that testosterone caused a marked reduction in serum cholesterol, stilbestrol had no apparent effect. Castration resulted in hypercholesteremia. Beef tallow fed at 5 and 10% of the diet produced higher levels of serum cholesterol.

Stilbestrol caused an increase in serum copper, but testosterone had no effect. None of the factors studied influenced the serum transaminases tested.

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LITERATURE CITED

Barnes, R. H., E. Kwong, G. Fiala, M. Rechcigl, R. N. Lutz and J. K. Loosli 1959a Dietary fat and protein and serum cholesterol. I. Adult swine. J. Nutrition, *69:* 261.

Barnes, R. H., E. Kwong, W. Pond, R. Lowry and J. K. Loosli 1959b Dietary fat and protein and serum cholesterol. II. Young swine. Ibid., *69:* 269.

Bragdon, J. H., J. H. Zeller and J. W. Stevenson 1957 Swine and experimental atherosclerosis. Proc. Soc. Exp. Biol. Med., *95:* 282.

Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.

Fillios, L. C. 1957 The gonadal regulation of cholesteremia in the rat. Endocrinology, *60:* 22.

Fillios, L. C., and G. V. Mann 1956 The importance of sex in the variability of the cholesteremic response of rabbits fed cholesterol. Circulation Res., *4:* 406.

Fillios, L. C., R. Kaplan, R. S. Martin and F. S. Stave 1958 Gonadal regulation of cholesterol metabolism. Am. J. Physiol., *193:* 47.

Gottlieb, H., and J. J. Lalich 1954 The occurrence of arteriosclerosis in the aorta of swine. Am. J. Path., *30:* 851.

Johnson, N. C., T. Kheim and W. B. Kountz 1959 Influence of sex hormones on total serum copper. Proc. Soc. Exp. Biol. Med., *102:* 98.

Peterson, R. E., and M. E. Bollier 1955 Spectrophotometric determination of serum copper with biscyclohexanoneoxalyldihydrazone. Anal. Chem., *27:* 1195.

Pick, R., J. Stamler, S. Rodbard and L. N. Katz 1959 Effect of testosterone and castration on cholesteremia and atherogenesis in chicks on high-fat, high-cholesterol diets. Circulation Res., *7:* 202.

Priest, R. E., M. T. Schroeder, R. Rasmussen and R. W. Wissler 1957 Quantitative study of influence of estrogenic substances on serum lipids of rats fed atherogenic diet. Proc. Soc. Exp. Biol. Med., *96:* 298.

Reitman, S., and S. Frankel 1957 A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Path., *28:* 56.

Roswell, H. C., H. G. Downie and J. F. Mustard 1958 The experimental production of atherosclerosis in swine following the feeding of butterfat and margarine. Canad. Med. A. J. *79:* 647.

Zak, B. 1957 Simple rapid microtechnic for serum total cholesterol. Am. J. Clin. Path., *27:* 583.

The Effect of Pyridoxine on Cholesterol Metabolism[1]

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Rhesus monkeys which had received a pyridoxine-deficient diet have been reported to develop degenerative changes in the arterial system (Rinehart and Greenberg, '49). These observations in monkeys were confirmed and extended to the dog.[3] Fibrous aortic plaques were noted in pyridoxine-deficient dogs and in dogs depleted of pyridoxine by the administration of desoxypyridoxine.

Significantly higher serum cholesterol levels were noted in rhesus monkeys and chicks fed cholesterol and a pyridoxine-deficient diet than in those fed cholesterol and pyridoxine (Greenberg and Rinehart, '51; Dam et al., '58). Previous studies have therefore indicated that pyridoxine may be involved in the metabolism of cholesterol. Whether pyridoxine is involved in the anabolism or in the catabolism of cholesterol was determined in the present study with the aid of acetate-2-C^{14}, mevalonic acid-2-C^{14} and cholesterol-4-C^{14}.

METHODS

Rats, three weeks old, were caged individually in two groups; one group was fed a pyridoxine-deficient diet and the other received this diet with added pyridoxine (table 1). In two months the rats fed the pyridoxine-deficient diet showed gross signs of pyridoxine deficiency as indicated by retarded growth, acrodynia and capillary fragility.

The in vivo metabolism of sodium acetate-2-C^{14}. Five rats from each group were injected intraperitoneally with 5 μc of sodium acetate-2-C^{14} per 100 gm of body weight (specific activity 5.17 mc per mmole). Three hours after the injection blood was drawn by heart puncture for serum cholesterol determinations. The rats were then decapitated and the livers removed for analysis. The radioactivity in the fatty acids, proteins and cholesterol in the livers was determined.

TABLE 1
Composition of diet

	gm/100 gm ration
Glucose[1]	75
Corn oil	3
Vitamin-free casein	18
Wesson salts[2]	4
Water-soluble vitamins[3]	0.3
Fat-soluble vitamins[4]	

[1] Cerelose.
[2] Wesson ('32).
[3] The mixture contained in milligrams/100 mg of mix: choline chloride, 93.5; thiamine, 1.24; riboflavin, 1.24; pyridoxine·HCl, 1.24; Ca pantothenate, 2.68; and folic acid, 0.30. Pyridoxine·HCl was excluded in the mixture prepared for the pyridoxine-deficient diet.
[4] Fat-soluble vitamins were dissolved in corn oil and two drops administered by medicine dropper each week. The mixture contained 5.00 gm vitamin A, 0.0065 gm vitamin D, 2.536 gm vitamin E per 100 gm of corn oil.

Serum and liver cholesterol determinations. Whole blood was collected in a stoppered centrifuge tube, allowed to clot at room temperature, and centrifuged for 20 minutes at 2500 rpm. The serum was decanted and the total serum cholesterol determined (Schoenheimer and Sperry, '34).

The determination of liver cholesterol differed from that of serum cholesterol only in the method of extraction of cholesterol and the solvents used for the purification of the cholesteryl digitonide precipitates. A sample of the wet liver was partially homogenized and 1 gm of the homogenate was weighed into a tared 50-

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[2] Portion of a thesis presented by S. N. Shah as partial fulfillment of the requirement for the degree of Doctor of Philosophy.
[3] Mushett, C. W., and G. Emerson 1956 Arteriosclerosis in pyridoxine deficient monkeys and dogs. Federation Proc., *15:* 526 (abstract).

ml Erlenmeyer flask. Ten milliliters of 10% potassium hydroxide solution in 95% ethyl alcohol were added to the flask. Samples were allowed to stand over night at 50°C, transferred to a 50-ml volumetric flask and diluted to the mark with an alcohol acetone mixture 15:25. A 5-ml aliquot was then transferred into a 12-ml centrifuge tube, neutralized with glacial acetic and cholesterol precipitated as cholesteryl digitonide with digitonin solution as in the serum cholesterol determination. The precipitate was purified according to the procedure used in the serum cholesterol analysis, with the exception that acetone water 2:1, acetone, acetone ether 1:2, and ether were the solvents used for washing the precipitates.

Radioactivity determinations. 1. *Cholesterol.* Cholesterol was precipitated as cholesteryl digitonide as described for the determination of liver cholesterol. Purified cholesteryl digitonide was dissolved in hot methanol. The solution was plated on a tared aluminum planchet with the aid of a Spinco spinner and the radioactivity determined with the aid of a Packard gas flow counter and a Baird atomic scaler. The amount of precipitate plated ranged from 0.5 to 2.0 mg. Self-absorption corrections were taken into account. The results were calculated as counts per minute per milligram of cholesterol by multiplying the counts per minute per milligram of cholesteryl digitonide by 4.18. The counter efficiency was 51.4%.

2. *Fatty acids.* A weighed sample of partially homogenized liver was placed in a 50-ml Erlenmeyer flask. Ten milliliters of 10% potassium hydroxide in 95% ethyl alcohol were added and the sample was allowed to stand over night at 50°C. The contents of the flask were concentrated to 3 to 4 ml and then diluted with 10 ml of distilled water. This aqueous solution was extracted with petroleum ether three to 4 times to remove the unsaponifiable lipid fraction. The aqueous portion was then acidified and re-extracted three times with petroleum ether. The combined extracts were dried over anhydrous sodium sulphate and the solvent was evaporated. One to two milligrams of fatty acids were plated on an aluminum planchet and the radioactivity was determined as in the case of cholesterol. The results were expressed as counts per minute per milligram of fatty acids.

3. *Protein.* A portion of the liver was homogenized with 10% trichloroacetic acid to precipitate the protein. The protein fraction was further purified by washing the precipitate with 5% trichloroacetic acid, acetone, acetone ether 1:1 and ether in order to wash the precipitates free from lipids. Five to 10 mg of this protein fraction were weighed into a scintillator bottle and dissolved in 2 ml of hyamine hydroxide by heating it at 50°C for 4 to 5 hours. Ten milliliters of scintillator solution which contained 0.6% diphenyloxazole in toluene were added. The samples were mixed well and the radioactivity determined with the aid of Packard Tri-carb scintillation counter at tap 3 (880–890 volts). The efficiency of the counter was 48.9%. The results were expressed as counts per minute per milligram of protein.

The in vivo incorporation of mevalonic acid-2-C^{14} into liver cholesterol. Five rats from each group were injected intraperitoneally with mevalonic acid-2-C^{14} (1.5×10^6 cpm/250 gm of body weight, specific activity 4 μc/mg). Two hours after the injection, the rats were decapitated and the livers removed. The radioactivity in liver cholesterol was then determined.

The effect of the reversal of pyridoxine deficiency on the in vivo incorporation of sodium acetate-2-C^{14} into liver cholesterol. Six pyridoxine-deficient rats were divided into two groups. One group was injected intraperitoneally with 55 μg of pyridoxine hydrochloride per day for two days. The other group received saline injections. Each of the pyridoxine-deficient groups of rats and 4 rats which had been receiving a synthetic diet plus added pyridoxine were then injected intraperitoneally with 5 μc of sodium acetate-2-C^{14} per 100 gm of body weight. Three hours after the injections, the rats were decapitated and the livers removed. The radioactivity in liver cholesterol was then determined.

The effect of pyridoxine deficiency on the excretion of cholesterol-4-C^{14} via the bile in bile cannulated rats. Three pyridoxine-deficient and 4 normal stock rats were used in this experiment. These rats were anesthetized with ether, their bile

ducts cannulated with polyethylene tubing and circulation of bile maintained for 24 hours by external connection of the bile cannula with polyethylene tubing (size PE 50) inserted into the distal portion of the bile duct. The rats were then injected with an emulsion (Bergstrom and Norman, '53) containing cholesterol-4-C^{14} (1.427×10^5 cpm/rat). The enterohepatic circulation was stopped by disconnecting the polyethylene tube and the bile was collected continuously during three consecutive 24-hour periods. A 20-λ aliquot of rat bile was spread as a thin film on an aluminum planchet, dried under the infrared lamp on the Spinco spinner, and the radioactivity determined with the aid of Packard gas flow counter and Baird atomic scaler. The values were corrected for mass absorption. The cumulative percentage recovery of cholesterol-4-C^{14} excreted in bile was calculated.

RESULTS AND DISCUSSION

The incorporation of sodium acetate-2-C^{14} into liver cholesterol by pyridoxine-deficient animals was markedly increased when compared with pyridoxine-supplemented animals, or from 377 to 2224 cpm/mg, respectively (table 2). When the pyridoxine deficiency was reversed by the injection of pyridoxine hydrochloride, there was a corresponding reversal of the enhanced incorporation of acetate into cholesterol (table 3). The cholesterol extracted from the livers of the pyridoxine-deficient animals which had been supplemented with two 55-μg injections of pyridoxine hydrochloride had an average specific activity of 571 as compared with 1639 and 377 for the pyridoxine-deficient and pyridoxine-supplemented animals, respectively. However, the incorporation of mevalonic acid-2-C^{14} into liver cholesterol and the rate of excretion of cholesterol-4-C^{14} via bile in bile cannulated rats was not affected by pyridoxine deficiency. The cholesterol extracted from the livers of pyridoxine-deficient animals which had been injected with mevalonic acid-2-C^{14} had an average specific activity of 1226 as compared with 1264 for pyridoxine-supplemented rats (table 4). The cumulative excretion of cholesterol-4-C^{14} in bile duct-cannulated rats over a 72-hour period averaged 40.4%

TABLE 2

Serum and liver cholesterol levels and comparison of the incorporation of sodium acetate-2-C^{14} into cholesterol, fatty acids and proteins of livers of pyridoxine-deficient and normal rats

Group (5 rats each)	Weight of rats	Weight of rat livers	Serum cholesterol	Liver cholesterol	Radioactivity in liver		
					Cholesterol	Fatty acids	Proteins
	gm	*gm*	*mg/100 ml*	*mg/gm*	*cpm/mg*	*cpm/mg*	*cpm/mg*
Pyridoxine-deficient	91.2 ± 7.9[1] (82–100)[2]	3.8 ± 0.73 (3.1–4.8)	68.4 ± 12.7 (55.4–81.4)	2.96 ± 0.31 (2.50–3.36)	2224 ± 1249 (1074–4180)	363 ± 314 (110–875)	28 ± 8 (16–35)
Pyridoxine-supplemented	184 ± 4.1 (181–191)	6.9 ± 0.82 (5.6–7.7)	68.6 ± 10.6 (54.3–89.4)	3.21 ± 0.41 (2.74–3.79)	377 ± 381 (108–1040)	277 ± 99 (226–435)	21.0 ± 4 (14–24)

[1] Standard deviation of the mean.

[2] Numbers in parentheses indicate range.

TABLE 3

Effect of injecting 55 μg of pyridoxine hydrochloride on the incorporation of acetate-2-C¹⁴ into liver cholesterol

Group	Rat no.	Specific activity liver cholesterol	
		Single	Average
		cpm/mg	*cpm/mg*
Pyridoxine-deficient	1	1254	1639 ± 543[1]
	2	2261	
	3	1404	
Injected with 55 μg pyridoxine·HCl	1	606	571 ± 41
	2	581	
	3	526	
Pyridoxine-supplemented	1	271	378 ± 83
	2	472	
	3	397	
	4	372	

[1] Standard deviation of the mean.

for those on a deficient diet and 40.6% for those on a pyridoxine-supplemented diet (table 5).

The rat has been reported to resist changes in serum cholesterol level when subjected to dietary stresses (Katz and Stamler, '53). The present results indicate that this is also true in pyridoxine-deficient rats as no change was noted in the serum and liver cholesterol levels. It could reasonably be expected that the increased synthesis of cholesterol which accompanied the pyridoxine deficiency would be reflected in an increase in the serum or liver cholesterol level. Since no such change was observed, it is possible that there was an increased rate of degradation of cholesterol via the bile acids and other steroids or the cholesterol was deposited elsewhere in the carcass. However, no change in the degradation of cholesterol via the bile in pyridoxine-deficient as compared with normal animals was noted.

TABLE 4

Effect of pyridoxine deficiency on the incorporation of mevalonic acid-2-C¹⁴ into liver cholesterol

Group (5 rats each)	Specific activity liver cholesterol
	cpm/mg
Pyridoxine-deficient	Av. 1226 ± 146[1] (1103–1400)
Pyridoxine-supplemented	Av. 1264 ± 196 (1003–1500)

[1] Standard deviation of the mean.

This observation may rule out the possibility of increased catabolism of cholesterol in pyridoxine deficiency.

Since no increase in the incorporation of mevalonic acid-2-C¹⁴ into cholesterol was observed, the change in the rate of incorporation of sodium acetate-2-C¹⁴ into cholesterol was apparently due to an alteration in the utilization of acetate. However, there was no increase in the utilization of acetate for fatty acid synthesis and, in agreement with previous studies (Beaton et al., '53), no increase in the utilization of acetate for protein synthesis. It would thus appear that the change in acetate metabolism might be explained on the basis of the difference in energy requirement between the normal and pyridoxine-deficient rat. In the pyridoxine-deficient rat, growth rate was significantly retarded. Therefore, the energy requirements and the amount of two carbon fragments needed for the building of tissue were reduced; thus extra acetate became available and may have been incorporated into cholesterol. This view is supported by the fact that when the pyridoxine deficiency was reversed, there was a significant increase in growth accompanied by a decrease in cholesterogenesis from acetate.

The production of aortic fibrous plaques in monkeys and dogs as a result of pyridoxine deficiency led Schroeder ('55) to speculate on the role of pyridoxine in the etiology of human atherosclerosis. He suggested that the American diet is marginal in pyridoxine. Furthermore, it contains a

TABLE 5

Effect of pyridoxine deficiency on the excretion of cholesterol-4-C^{14} via bile in bile duct-cannulated rats

Rat no.	Weight of rat	Time	Activity excreted	Cumulative excretion
	gm	*hours*	*cpm*	*%*
		Pyridoxine-deficient rats		
1	132	24	38,148	31.8
		48	45,045	
		72	57,532	
2	148	24	51,224	
		48	79,800	47.5
		72	79,220	
3	135	24	78,186	
		48	78,036	42.1
		72	30,195	
		Pyridoxine-supplemented rats		
1	218	24	27,573	
		48	74,175	46.3
		72	113,390	
2	225	24	50,907	
		48	60,390	37.3
		72	54,048	
3	207	24	16,359	
		48	50,490	26.5
		72	50,464	
4	217	24	49,950	
		48	101,024	52.4
		72	80,975	

high proportion of refined carbohydrates and saturated fats, both readily available sources of two carbon fragments. The present data seem to suggest that if such a diet is low in pyridoxine it may lead to a greater synthesis of cholesterol than one adequate in pyridoxine.

SUMMARY

The effect of pyridoxine deficiency on the *in vivo* synthesis of cholesterol from acetate and mevalonic acid, and on the excretion of cholesterol-4-C^{14} via bile in bile cannulated rats was studied. The results indicated that pyridoxine deficiency enhanced the incorporation of labeled acetate into liver. It was shown that this enhanced incorporation of labeled acetate into cholesterol in deficient rats could be reversed by injecting pyridoxine hydrochloride. The synthesis of cholesterol from mevalonic acid, and excretion of cholesterol-4-C^{14} were not altered by pyridoxine deficiency.

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LITERATURE CITED

Beaton, J. R., J. L. Beare, J. M. White and E. W. McHenry 1953 Biochemical changes in vitamin B_6 deficiency in rats. J. Biol. Chem., *200:* 715.

Bergström, S., and A. Norman 1953 Metabolic products of cholesterol in bile and feces of rat. Steroids and bile acids. Proc. Soc. Exp. Biol. Med., *83:* 71.

Dam, H., G. Kristensen, G. K. Nielsen and E. Søndergaard 1958 Effect of pyridoxine deficiency on cholesterol and polyenoic fatty acids in chicks. Acta Physiol. Scand., *44:* 67.

Greenberg, L. D., and J. F. Rinehart 1951 Plasma cholesterol levels of cholesterol fed control and pyridoxine deficient monkeys. Proc. Soc. Exp. Biol. Med., *76:* 580.

Katz, L. N., and J. Stamler 1953 Experimental Atherosclerosis. Charles C Thomas, Springfield, Illinois.

Rinehart, J. F., and L. D. Greenberg 1949 Arteriosclerotic lesions in pyridoxine-deficient monkeys. Am. J. Path., *25:* 481.

Schroeder, H. A. 1955 Is atherosclerosis a continued pyridoxal deficiency? J. Chronic Dis., *2:* 28.

Schoenheimer, R., and W. M. Sperry 1934 A micromethod for the determination of free and combined cholesterol. J. Biol. Chem., *106:* 745.

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

The Effect of Certain Factors on Nitrogen Retention and Lysine Requirements of Adult Human Subjects

I. TOTAL CALORIC INTAKE[1]

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Minimum lysine requirements of men and women have been investigated in this laboratory, using maintenance of nitrogen equilibrium as the principal criterion (Clark et al., '57, '60). Interpretation of data concerning amino acid requirements is difficult because the ingestion of adequate quantities of individual essential amino acids is only one of several factors that influence nitrogen retention. The existence of any other condition that alters nitrogen utilization might conceivably lead to an erroneous estimate of the requirement for a particular amino acid.

A systematic investigation has been undertaken of the effect on nitrogen retention of modifying specific experimental conditions when the lysine intake is controlled. Such studies should contribute to a better understanding of human lysine requirements and nitrogen metabolism. Lysine is particularly useful for this purpose because it acts primarily in synthesis of tissues.

Investigators have long recognized that an adequate intake of food energy conserves body nitrogen. Despite generous amounts of high-quality protein, men (Young et al., '57) and women (Young, '52) exhibited negative nitrogen balances when calories were decreased as necessary to induce weight loss. Energy requirements of women were increased when the diet contained purified amino acids (Leverton, '54). Men attained nitrogen equilibrium with a lower caloric intake when whole casein furnished dietary nitrogen than when equivalent amounts of essential amino acids were provided in free form (Rose, Coon and Lambert, '54).

Both free and peptide-bound amino acids are present in the experimental diet used in this laboratory. Information was needed concerning caloric requirements of men and women under these conditions and the extent to which the energy value of the diet could be modified without altering nitrogen balance.

Whereas insufficient calories reduce nitrogen retention, provision of excessive calories over a period of several weeks causes undesirable increments in weight and reduces the acceptability of the experimental diet.

PROCEDURE

The experimental method has been presented in detail elsewhere (Clark et al., '57). The subjects, who are described in table 1, were graduate students or advanced undergraduate students between 21 and 28 years old. Their minimum lysine requirements were established in separate tests (Clark et al., '57, '60).

The diet[2] provided between 8.97 and 9.02 gm of nitrogen daily, of which white wheat flour and cornmeal contributed 41%, cornstarch and fruits 4%, purified essential amino acids 8%, and a mixture of glycine, glutamic acid and diammo-

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TABLE 1

Description of subjects

Subject	Sex	Weight[1]	Basal Cal. per 24 hour	Activity	Lysine requirement[2]
		kg			*mg/day*
BB	F	64	1640	mod. active	350
GM	F	68	1130	mod. active	670
PC	F	58	1250	active	700
BC	M	65	1700	very active	500
JM	M	67	1670	mod. active	400
LM	M	85	1750	mod. active	850
EO	M	63	1820	mod. active	500

[1] At beginning of experiment.
[2] Estimated in other experiments (Clark et al., '57, '60).

nium citrate 47%. The total quantities of essential amino acids were similar to those in 20 gm of egg protein (Clark et al., '57), except that lysine was reduced below this amount in some tests. Cereals supplied from 30 to 75% of the daily allotments of essential amino acids except lysine which varied from 25 to 90% for different individuals. In the initial 12-day adjustment interval, all subjects received 1400 or 1500 mg of lysine to facilitate attainment of nitrogen equilibrium (Clark et al., '60). Thereafter some subjects continued to receive this quantity of lysine throughout the experiment, and others were given 500 or 700 mg.

Sources of energy in the basal diet which was eaten by all subjects were, in grams: wheat flour, 159; cornmeal, 21; cornstarch, 50; sucrose, 34; butter oil, 20; hydrogenated vegetable fat, 36; and fruits or juices, 300. Flour and cornmeal were incorporated in biscuits and cornbread, cornstarch in wafers and pudding. Foods in the basal diet provided 1700 Cal. as calculated (Watt and Merrill, '50). Carbohydrate, fat and protein contributed 63, 32 and 5%, respectively, of the calories in the basal diet. It was estimated that purified amino acids and diammonium citrate provided 150 Cal. The total energy values of individual diets were increased to the quantities indicated in figures 1 and 2 and table 2 by including variable amounts of butter oil, cornstarch, sucrose, candy and/or carbonated beverage. The percentage of total calories obtained from fat was held between 31 and 35% for each subject. The basal diet, amino acid mixtures and supplementary foods together supplied between 1770 and 4350 Cal.

Basal caloric expenditures of the subjects were considered in planning the initial allotment of calories, as well as information concerning energy needs of these or other individuals under similar conditions. The subjects kept daily records of their activities during the experiment. The highest caloric intake was always tested first, then stepwise decreases of approximately 5, 10 or 15% were made. Total decrements varied from 20 to 40% of the initial energy value.

RESULTS AND DISCUSSION

Subjects GM and LM were tested over a wider range of caloric intakes than other subjects. Mean daily nitrogen balances of GM when calories were reduced stepwise to 60% of the initial intake are shown in figure 1. Subject GM consumed 1500 mg of lysine, equivalent to 225% of her minimum requirement, throughout the experiment. She gained weight with an

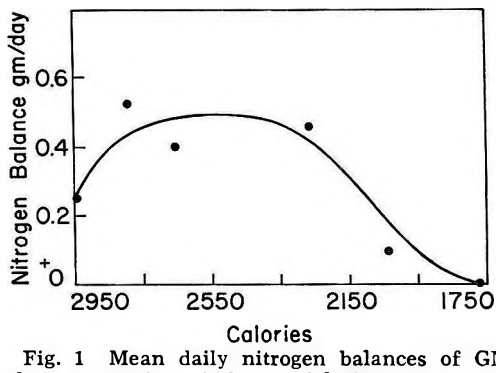

Fig. 1 Mean daily nitrogen balances of GM when consuming 1500 mg of lysine.

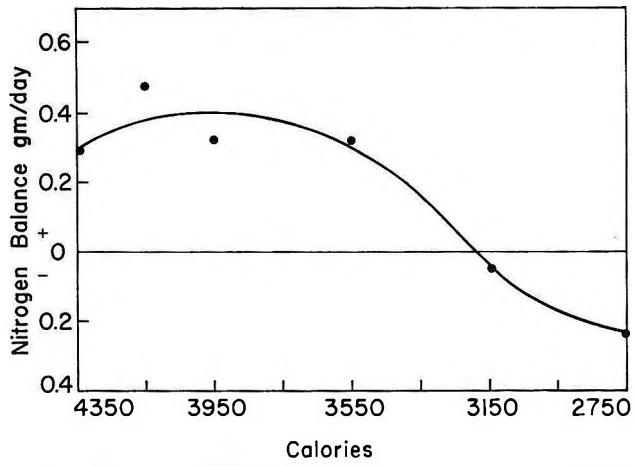

Fig. 2 Mean daily nitrogen balances of LM when consuming 1500 mg of lysine.

intake of 2650 Cal. or more, maintained it with 2300, and lost weight with intakes of 2150 Cal. or less. Nitrogen retention was not altered noticeably by a stepwise reduction from 2950 to 2300 Cal. Mean daily nitrogen balances during 6-day metabolism periods were, respectively, +0.25, +0.52, +0.40 and +0.46 gm when the diet supplied 2950, 2800, 2650 and 2300 Cal. However, GM retained only 0.10 gm of nitrogen with an intake of 2150 Cal., and just maintained equilibrium with 1750 Cal. Whereas she stored almost 0.40 gm of nitrogen daily when calories were adequate, GM retained little or none when the energy value of the diet was insufficient to maintain weight, although she had gained weight earlier in the experiment. Mean daily fecal nitrogen varied from 0.91 to 0.70 gm, being lowest at the 2150- or 1750-Cal. level.

The possibility that nitrogen retention might decline as the experiment progressed, even though calories were adequate, cannot be ignored. However, unpublished data indicate clearly that nitrogen retention of almost all subjects tested in another series increased steadily with time when dietary treatment remained constant, and in no case did it decrease.

Subject LM maintained weight when receiving 3950 Cal., gained with higher intakes and lost when caloric value was reduced below this point. Mean daily nitrogen balances (fig. 2) in successive periods with intakes of 4350, 4150, 3950 and 3550 Cal. were +0.29, +0.48, +0.32 and +0.32 gm, respectively. There was no apparent difference in nitrogen retention as calories were decreased through a range of 20%, but further caloric restriction caused a downward trend. Nitrogen balances of −0.05 and −0.23 gm were associated with intakes of 3150 and 2750 Cal. Fecal nitrogen decreased as calories were lowered. This man evidently was unable to maintain nitrogen equilibrium when the energy value of the diet was insufficient, despite ingestion of almost twice as much lysine as he needed when calories were adequate. The provision of excessive calories did not improve nitrogen retention of either LM or GM, but in both cases a caloric intake below a critical level decreased it.

Mean body weights and nitrogen balances of three men and two women during consecutive 6-day periods are presented in table 2 in the same sequence as caloric intakes were tested. Subject BB retained 0.87 and 0.62 gm of nitrogen in two adjacent periods at a level of 2900 Cal.; 0.91 gm with 2600 Cal.; and 0.59 gm with 2350 Cal. A caloric allowance of 2100 resulted in mean daily nitrogen balances of only +0.28 and +0.08 gm in two periods, during which daily balances were frequently negative. In the final period, she retained little nitrogen although the diet supplied 4 times the amount of lysine that permitted equilibrium when calories

TABLE 2

Mean daily nitrogen balances of men and women when caloric intakes were varied

Subject	Lysine	Total Cal.[1]	Body weight	N in diet	N in urine	N in feces	N balance
	mg		*kg*	*gm*	*gm*	*gm*	*gm*
BB	1400	2900	64.5	9.02	7.48	0.67	+0.87
	1400	2900	64.3	9.02	7.61	0.79	+0.62
	1400	2600	64.1	9.02	7.41	0.70	+0.91
	1400	2350	64.1	9.02	7.69	0.74	+0.59
	1400	2100	63.8	9.01	8.02	0.71	+0.28
	1400	2100	63.3	9.01	8.31	0.62	+0.08
PC	700	2950	57.7	9.00	7.98	0.67	+0.35
	700	2950	57.7	9.00	8.14	0.58	+0.28
	700	2700	57.8	9.00	8.40	0.56	+0.04
	700	2400	57.9	9.00	8.04	0.66	+0.09
	700	2700	57.7	9.00	8.23	0.68	+0.30
	700	2400	57.8	9.00	8.10	0.54	+0.36
BC	1500	3400	65.2	9.00	7.92	0.81	+0.27
	500	3400	65.4	9.00	8.35	0.75	−0.10
	500	3400	65.6	9.00	8.32	0.83	−0.15
	500	2950	65.4	9.00	8.45	0.72	−0.17
	500	2950	65.1	9.02	8.57	0.83	−0.38
	500	3400	65.5	9.02	8.13	0.72	+0.17
	1500	3400	65.9	9.02	6.97	0.84	+1.21
	1500	3400	65.9	9.02	7.50	0.76	+0.76
	1500	3400	66.4	9.02	7.06	0.70	+1.26
	1500	2950	66.5	9.02	8.13	0.68	+0.21
	1500	3200	66.4	9.02	7.42	0.76	+0.84
	1500	3200	66.5	9.02	7.93	0.53	+0.56
JM	600	3500	66.7	9.02	7.85	0.68	+0.49
	600	3500	67.3	9.01	7.83	0.91	+0.27
	600	2950	67.1	9.01	8.33	0.76	−0.08
	600	2950	67.2	9.01	8.25	0.64	+0.12
	600	2650	67.0	9.01	8.52	0.65	−0.16
EO	1500	3500	64.3	9.00	7.18	1.03	+0.79
	1500	3400	64.1	9.00	7.10	0.94	+0.96
	1500	3300	63.7	9.00	7.31	0.89	+0.80
	500	3500	63.4	9.00	7.89	0.79	+0.32
	500	3200	63.6	9.00	7.93	0.85	+0.22
	500	3000	63.0	9.00	8.18	0.86	−0.04
	500	3000	63.1	9.00	8.13	0.91	−0.04

[1] From the basal diet, amino acid mixtures and supplementary foods.

were adequate. The mean nitrogen balance (+0.75 gm) for the 12 days in which 2900 Cal. were available was 0.57 gm greater than the mean balance (+0.18 gm) for the 12 days at 2100 Cal A linear relation existed between caloric intake and nitrogen balance below 2600 Cal.

The lysine intake of PC corresponded with her minimum requirement. Three different caloric intakes were tested in two periods each (table 2). Respective mean nitrogen balances for the two periods in which her intake was 2950 Cal. were +0.35 and +0.28 gm; 2700 Cal., +0.04 and +0.30 gm; and 2400 Cal., +0.09 and +0.36 gm. Although PC complained of unusual fatigue during the first period in which she was given 2400 Cal., no unfavorable effect on nitrogen retention or body weight was observed in 6 days, after which calories were increased to 2700. More clear-cut results might have been obtained if 2400 Cal. had been tested in two consecutive periods.

The treatments imposed on BC were planned on the basis of a previous experiment in which he maintained nitrogen equilibrium and body weight for 5 successive periods when consuming 500 mg of lysine with either 3050 or 3300 Cal. available. The present series extended for 80 consecutive days. In the first experi-

mental period BC was in positive balance, +0.27 gm, with 1500 mg of lysine and 3400 Cal. Thereafter, lysine was reduced to 500 mg for 5 periods. Nitrogen balances were negative, −0.10 and −0.15 gm, with 3400 Cal., and −0.17 and −0.38 gm with 2950 Cal. No marked change in weight was observed during this interval. Nitrogen balance increased to +0.17 gm, an improvement of 0.55 gm per day, when 3400 Cal. were restored. However, the loss of appetite, severe fatigue and difficulty in studying which were characteristic of the interval of low caloric intake did not disappear in 6 days.

Therefore, lysine was increased to 1500 mg and held constant until the experiment terminated. Nitrogen balances were +1.21, +0.76 and +1.26 gm in three successive periods during which 3400 Cal. were available. A slight gain in weight occurred. Since the cumulative negative nitrogen balance of 0.80 gm from 4 earlier periods was compensated fully during the first two periods in which 3400 Cal. were provided, the continued high level of nitrogen storage after that time is difficult to explain. BC responded immediately and unfavorably when only 2950 Cal. were given in the next period, even though lysine was held at three times his minimum requirement. Since the mean balance for the period dropped to +0.21 gm and some daily nitrogen balances were negative, it seemed unwise to prolong this treatment. An intermediate intake of 3200 Cal. permitted retention of +0.84 and +0.56 gm of nitrogen in the last two periods of the series. Probably 3200 Cal. represented the critical intake for this lean active subject who was apparently very sensitive to caloric adjustments.

Nitrogen balances of JM were positive, +0.49 and +0.27 gm, when 3500 Cal. were given, but bordered on equilibrium, −0.08 and +0.12 gm, when he had only 2950 Cal. (table 2). The downward trend was aggravated when 2650 Cal. were given for the final period, the balance being −0.16 gm. When the data were plotted, a linear relation between caloric intake and nitrogen retention was apparent. The ability of this subject, who ingested 150% of his minimum lysine requirement, to retain nitrogen was decreased markedly when the energy value of the diet was reduced. Little effect on body weight was noted.

EO had nitrogen balances of +0.79, +0.96 and +0.80 gm, respectively, when the diet provided 1500 mg of lysine and 3550, 3400 and 3200 Cal. Reduction of caloric intake by 10% apparently did not alter nitrogen retention. In a separate test, the minimum lysine requirement of EO was established at 500 mg, which then was tested in 4 successive periods in which 3500, 3200, 3000 and 3000 Cal. were provided. The corresponding nitrogen balances were +0.32, +0.22, −0.04 and −0.04 gm. When lysine intake was minimal, reduction of calories by 13% apparently had an adverse effect on nitrogen retention.

It is evident that for each individual tested, a critical caloric intake existed at which satisfactory retention of nitrogen occurred, but below which nitrogen retention decreased in direct relation to the reduction in calories. A decrement in calories of sufficient magnitude could change a strongly positive nitrogen balance to a state of equilibrium or even to negativity, even though the lysine intake represented three or 4 times the minimum requirement and other essential amino acids were present in adequate amounts. On the other hand, calories could be increased as much as 20% above the critical intake without improving nitrogen storage. Variation among subjects in respect to caloric needs and the extent to which calories could be reduced without altering nitrogen retention was observed. Caloric restriction may be reflected more promptly by changes in daily nitrogen balance than in body weight.

It is apparent that useful data concerning lysine requirements can be obtained only if the energy value of the diet is sufficient, when attainment of nitrogen balance is used as a criterion. Furthermore, in meeting nutritional needs of population groups, consideration must be given to the adequacy of diets in respect to calories as well as lysine.

SUMMARY

The effect on nitrogen retention of varying the caloric intake when the diet pro-

vided quantities of lysine that equalled or exceeded minimum requirements was measured. Amino acids were present in purified form and as peptide-bound amino acids in cereals. Nitrogen balances of men and women were not altered by increasing the energy value of the diet above a critical level which differed for individuals. Reduction of calories below this point, however, caused a decrease in nitrogen retention, so that a subject who previously retained significant amounts of nitrogen might just maintain equilibrium or even be in negative balance.

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LITERATURE CITED

Clark, H. E., E. T. Mertz, E. H. Kwong, J. M. Howe and D. C. DeLong 1957 Amino acid requirements of men and women. I. Lysine. J. Nutrition, *62:* 71.

Clark, H. E., S. P. Yang, W. Walton and E. T. Mertz 1960 Amino acid requirements of men and women. II. Relation of lysine requirement to sex, body size, basal caloric expenditure and creatinine excretion. Ibid., *71:* 229.

Leverton, R. M. 1954 The amino acid requirements of man. In: Symposium on Protein Metabolism. National Vitamin Foundation, no. 8, p. 55.

Rose, W. C., M. J. Coon and G. F. Lambert 1954 The amino acid requirements of man. VI. The role of the caloric intake. J. Biol. Chem., *210:* 331.

Watt, B. K., and A. L. Merrill 1950 Composition of foods: raw, processed, prepared. U. S. Dept. of Agriculture, Handbook no. 8.

Young, C. M. 1952 Weight reduction using a moderate-fat diet. II. Biochemical responses. J. Am. Dietet. A., *28:* 529.

Young, C. M., E. L. Empey, V. U. Serraon and Z. H. Pierce 1957 Weight reduction in obese young men. Metabolic studies. J. Nutrition, *61:* 437.

Effect of Dietary Carbohydrates and Aureomycin on Serum and Liver Cholesterol in Rats

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Dietary factors may have considerable effect on the metabolism and blood concentration of cholesterol. Whereas the effects of fats and proteins have been studied intensively (Portman and Stare, '59), less is known about carbohydrates. These compounds may exert their influence either directly or indirectly by their action on the intestinal flora (Harper and Elvehjem, '57). They may affect the quantity of bile acids in the bile (Portman and Murphy, '58), the principal pathway of cholesterol excretion, as well as their degradation through bacterial action. Thus, fecal excretion of bile acids was found to be greatly depressed in rats treated with antibiotics, presumably because of the lack of microbiological transformation of taurocholate with corresponding better absorption (Lindstedt and Norman, '56). Carbohydrates may interfere with the bacterial reduction of cholesterol to coprostanol (Snog-Kjaerr et al., '55) which is less well absorbed than cholesterol. The importance of the intestinal flora in cholesterol metabolism has been demonstrated by Coleman and Baumann ('57). These authors added antibiotics to diets of rats and found a decrease in fecal excretion of coprostanol and an increase of cholesterol.

Dietary carbohydrates affect also serum cholesterol level. Starch has been reported to depress serum cholesterol in rats (Portman et al., '56) and glucose in chickens (Grant and Fahrenbach, '59). Similarly, changes in the intestinal flora may have some bearing on the regulation of serum cholesterol. Germ-free rats have higher serum cholesterol levels than conventional control animals (Danielsson and Gustafson, '59).

Hypercholesteremia can be produced in rats by incorporation of cholic acid in a cholesterol-containing diet (Swell et al., '53). The bile acid either improves intestinal absorption (Siperstein et al., '52) or retards the rate of catabolism (Pihl, '55).

The present study was undertaken to determine the effect of various carbohydrates on liver and serum cholesterol, and whether this effect is modified by altering dietary conditions. Three carbohydrates, dextrose, sucrose and starch, were tested under 4 dietary conditions: with a basal diet free from either cholesterol or cholic acid, as well as with diets to which either cholesterol or cholic acid or both were added. In further experiments the effects of sorbitol, which exerts a vitamin-sparing action by influencing the intestinal flora (Morgan and Yudkin, '57), and of Aureomycin were studied.

METHODS

Young male rats weighing 40 to 50 gm were used. The basal diet consisted (in per cent) of casein, 18; carbohydrates, 73; soya oil, 5; and salt mixture (U.S.P. XIII no. 2), 4. This diet was supplemented with the conventional vitamins. Glucose, sucrose or cornstarch were used as sources of carbohydrates. The effect of sorbitol was tested in a cornstarch diet. Ten per cent of this sugar was included, and the amount of starch was correspondingly reduced. In some diets 1.5% cholesterol or 0.5% of cholic acid or both were incorporated at the expense of the respective carbohydrate. Chlortetracycline (Aureomycin) was added to the ration at a level of 50 mg/kg of diet.

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Biochemical methods. After 4 weeks the rats were exsanguinated. Serum and liver cholesterol were determined by the method of Abell and associates ('52). For determination of liver cholesterol the livers were saponified at 60°C for 120 minutes. Liver fat was determined gravimetrically after extraction with ether from dried ground liver in a Soxhlet apparatus, and liver nitrogen by the Kjeldahl method.

Statistical methods. Two-way analysis of variance with interaction was applied. The effect of incorporation into the diet of cholesterol and of cholic acid, however, was not studied *per se*. "F" tests were performed separately for first-order effect and interaction. We felt that this procedure was particularly justified for the present material, because the number of degrees of freedom of error was rather large. Since the variance of liver cholesterol was largely influenced by dietary cholesterol and cholic acid, the analysis of variance of this parameter was preformed on a logarithmic basis. Actually the logarithmic transformation stabilized the intraclass variance. The analyses were performed independently for each parameter. The effect of sorbitol was evaluated with Student's "t" test.

RESULTS

Effects of carbohydrates. In the first experiment 12 dietary combinations were studied. The carbohydrate source of 4 diets was dextrose and that of the remaining 8 diets, sucrose and starch. Each carbohydrate was offered in 4 dietary variations: without cholesterol and cholic acid, with cholesterol or cholic acid and with both. Each dietary group included 10 to 12 rats. After feeding these diets for 4 weeks, serum cholesterol and concentrations of water, fat, cholesterol and nitrogen in the livers were examined. The results and their statistical analysis are presented in table 1.

In general, starch promoted the greatest weight increase, followed by sucrose, whereas the weight of rats fed the dextrose diets lagged behind.

As expected, a considerable rise of serum cholesterol was observed, when the diets were supplemented with both cholesterol and cholic acid, whereas the addition of only one of these substances had no effect. Supplementation with either cholesterol or cholic acid led to an increase in liver fat and cholesterol and a corresponding fall in moisture. Addition to the diet of both steroids resulted in a much larger rise in liver cholesterol. The livers of rats maintained on these diets were considerably enlarged, nitrogen per gram of liver being markedly reduced. The increased weight of the liver cannot, however, be attributed to the deposition of fat only, as demonstrated by the significant increase of liver nitrogen per unit of body weight. It appears, therefore, that the weight increase of the liver of rats subsisting on diets containing cholesterol and/or cholic acid is partly the result of an increase of liver cytoplasm.

Variance analysis of the results showed that the different carbohydrates had significant effects on liver fat and cholesterol but not on serum cholesterol and on liver nitrogen (table 1). Generally, feeding starch resulted in a high level of liver fat and cholesterol, and sucrose in a low concentration of cholesterol. There was also a significant effect of the interaction of the different dietary variables (cholesterol, cholic acid) with the different carbohydrates, for serum cholesterol, liver fat and liver nitrogen, but little for liver cholesterol. Thus, it may be impossible to define an effect of a carbohydrate on the level of these substances without consideration of dietary components such as cholesterol or cholic acid. Furthermore, interaction of carbohydrates with dietary cholesterol and cholic acid was less important for liver cholesterol than the action of the carbohydrates by themselves.

Effect of Aureomycin. For studying the effect of Aureomycin 6 groups of rats were used. The basal diet of each group contained cholesterol but no cholic acid. The diets of the three experimental groups provided dextrose, sucrose or starch as source of carbohydrates, and these were supplemented with Aureomycin. The diets of the control groups contained the same carbohydrates but remained unsupplemented. The values for serum cholesterol and concentrations of water, fat and cholesterol in the liver obtained after 4 weeks are shown in table 2.

TABLE 1

Effect of dietary carbohydrates on serum cholesterol and on composition of liver of rats fed specified diets for 4 weeks[1]

Diet treatment			Weight increase	Serum cholesterol	Liver					
									Nitrogen	
Carbohydrate	Cholesterol	Cholic acid			Weight	Water	Fat	Cholesterol	Per gram liver	Per 100 gm body weight
	%	%	*gm*	*mg/100 ml*	*gm*	%	%	*mg/100 gm*	*mg*	*mg*
Dextrose	—	—	103 ± 6.4[2]	58 ± 3.6	7.0 ± 0.36	69.0 ± 0.37	4.5 ± 0.50	313 ± 35	31.5 ± 0.56	150 ± 8.2
Sucrose	—	—	105 ± 4.2	75 ± 3.2	7.8 ± 0.41	70.6 ± 0.39	4.4 ± 0.33	295 ± 25	30.6 ± 0.46	153 ± 11.7
Starch	—	—	123 ± 6.3	66 ± 6.4	7.8 ± 0.43	69.9 ± 0.40	5.2 ± 0.43	311 ± 31	30.7 ± 0.60	140 ± 8.9
Dextrose	1.5	—	96 ± 2.6	83 ± 3.9	7.4 ± 0.19	66.7 ± 0.23	9.1 ± 0.38	2164 ± 260	29.3 ± 0.49	157 ± 5.4
Sucrose	1.5	—	107 ± 4.3	71 ± 3.1	9.4 ± 0.45	66.9 ± 0.32	9.6 ± 0.64	1585 ± 129	28.4 ± 0.62	179 ± 2.1
Starch	1.5	—	120 ± 5.7	99 ± 8.4	9.5 ± 0.54	65.4 ± 0.80	11.4 ± 0.74	3801 ± 343	28.9 ± 0.46	173 ± 3.6
Dextrose	—	0.5	83 ± 3.7	74 ± 5.2	7.4 ± 0.24	68.7 ± 0.12	6.7 ± 0.50	1015 ± 72	32.0 ± 0.72	180 ± 3.7
Sucrose	—	0.5	106 ± 3.6	81 ± 4.3	9.3 ± 0.36	68.9 ± 0.36	7.2 ± 0.34	1264 ± 97	30.7 ± 0.56	191 ± 3.5
Starch	—	0.5	94 ± 5.1	71 ± 6.0	8.2 ± 0.40	66.9 ± 0.35	9.5 ± 0.46	1922 ± 183	29.7 ± 0.66	173 ± 4.1
Dextrose	1.5	0.5	93 ± 2.4	166 ± 4.9	10.7 ± 0.31	61.8 ± 0.71	17.5 ± 0.64	6559 ± 476	26.5 ± 0.48	210 ± 5.6
Sucrose	1.5	0.5	104 ± 4.2	258 ± 37.3	13.3 ± 0.65	62.1 ± 0.69	16.4 ± 1.52	5573 ± 509	26.7 ± 0.73	212 ± 5.7
Starch	1.5	0.5	112 ± 3.1	232 ± 17.4	11.2 ± 0.35	61.0 ± 0.48	19.3 ± 0.94	7210 ± 357	26.2 ± 0.56	193 ± 9.7
Pretreatment				66 ± 2.1	2.7 ± 0.15	72.0 ± 0.49	3.8 ± 0.49	231 ± 73	30.0 ± 0.52	177 ± 6.7

[1] Ten to 12 rats/group.
[2] Figures indicate mean ± standard error.

Analysis of variance

Parameter analyzed	Source of variance	Sum of squares	Degrees of freedom	F	P
Serum cholesterol	Carbohydrates	1650	2	0.8	> 0.5
	Diet-carbohydrate interaction	55147	6	4.5	< 0.001
	Error	249271	129		
Liver fat	Carbohydrates	221	2	21	< 0.001
	Diet-carbohydrate interaction	1133	6	34	< 0.001
	Error	713	129		
Log liver cholesterol	Carbohydrates	1.7354	2	19.8	< 0.001
	Diet-carbohydrate interaction	0.6107	6	2.2	~ 0.05
	Error	5.6539	129		
Liver nitrogen/100 gm body weight	Carbohydrates	1006	2	0.5	> 0.5
	Diet-carbohydrate interaction	14110	6	2.6	0.05–0.01
	Error	112233	129		

TABLE 2

Effect of dietary Aureomycin on cholesterol levels of serum and liver and on liver fat. (All diets supplemented with 1.5% of cholesterol)

Carbohydrate	Aureomycin	Weight increase	Serum cholesterol	Liver			
				Weight	Water	Fat	Cholesterol
	mg/kg/diet	*gm*	*mg/100 ml*	*gm*	*%*	*%*	*mg/100 gm*
Dextrose	—	94 ± 5.2[2]	65 ± 8.2	9.1 ± 0.60	64.8 ± 0.97	12.0 ± 1.14	2218 ± 149
Sucrose	—	104 ± 1.7	91 ± 6.8	9.4 ± 0.43	67.7 ± 0.73	8.7 ± 0.62	1385 ± 124
Starch	—	110 ± 7.6	89 ± 4.4	10.0 ± 0.84	65.3 ± 0.44	12.7 ± 0.82	2789 ± 220
Dextrose	50	102 ± 3.7	68 ± 2.8	8.4 ± 0.26	65.8 ± 0.35	10.1 ± 0.63	1849 ± 180
Sucrose	50	98 ± 5.1	89 ± 4.9	9.3 ± 0.38	66.5 ± 0.31	10.1 ± 0.71	2037 ± 123
Starch	50	116 ± 4.3	88 ± 6.7	10.3 ± 0.29	62.6 ± 0.40	15.8 ± 0.64	4190 ± 213

[1] Six rats in each control group; 12 rats in each experimental group.
[2] Figures indicate mean ± standard error.

Analysis of variance

Parameter analyzed	Source of variance	Sum of squares	Degrees of freedom	F	P
Serum cholesterol	Aureomycin Aureomycin-carbohydrate interaction	73	3	0.97	> 0.5
	Error	11854	47		
Liver fat	Aureomycin	9.4	1	1.9	~ 0.1
	Aureomycin-carbohydrate interaction	103.8	2	11.0	< 0.001
	Error	221.1	47		
Liver cholesterol	Aureomycin	3086700	1	8.3	< 0.001
	Aureomycin-carbohydrate interaction	7619350	2	10.3	< 0.001
	Error	17395000	47		

TABLE 3

Effect of dietary sorbitol on cholesterol levels of serum and liver and on liver fat (Diets supplemented with 1.5% of cholesterol)

Rats	Sorbitol/ 100 gm diet	Weight increase	Serum cholesterol	Liver			
				Weight	Water	Fat	Cholesterol
No.	*gm*	*gm*	*mg/100 ml*	*gm*	*%*	*%*	*mg/100 ml*
6	—	110 ± 7.6[1]	89 ± 4.4	10.0 ± 0.84	65.3 ± 0.44	12.7 ± 0.82	2789 ± 220
12	10	103 ± 7.2	96 ± 9.9	8.3 ± 0.93	64.3 ± 0.60	12.8 ± 0.70	3546 ± 234
			Statistical analysis				
t		0.7	0.7	1.4	1.3	0.1	2.4
P		0.5	0.5	0.2	0.2	0.9	0.05–0.02

[1] Figures indicate mean ± standard error.

Supplementation with Aureomycin did not promote growth. The antibiotic, when added to a sucrose or starch diet, increased liver cholesterol; it had no effect with dextrose. Variance analysis proved that the action of Aureomycin on liver cholesterol was not due to chance. A significant effect of interaction was found between Aureomycin and carbohydrates for liver fat and liver cholesterol but not for serum cholesterol. It appears, therefore, that the response of liver fat and cholesterol to Aureomycin depends on the dietary carbohydrate.

Effect of sorbitol. The effect of sorbitol was investigated with 18 rats. Their basal diet contained starch and cholesterol. Sorbitol significantly increased the cholesterol concentration of the liver, whereas no effect was observed on weight increase, serum cholesterol, liver weight, moisture or fat content (table 3).

DISCUSSION

It follows from our data that, generally, none of the carbohydrates studied nor dietary Aureomycin significantly affect the level of serum cholesterol in rats. This is not in agreement with the observations of Portman and associates ('56) who observed lower serum cholesterol in rats fed starch diets than in those fed sucrose or glucose. Our results do not exclude the possibility that under certain dietary conditions carbohydrates may have an effect on serum cholesterol. Similarly, glucose had a hypocholesteremic effect in chicks only, when the diet contained 3% of cholesterol, but not when cholesterol was not added to the diet (Grant and Fahrenbach, '59). Another factor to consider, on which the effect of carbohydrates may depend, is the duration of treatment. In rats fed a diet including cholesterol and cholic acid a greater hypercholesteremia was found with sucrose as compared with starch at the end of three weeks; this difference disappeared, however, after 17 weeks (Fillios et al., '58).

In rabbits, addition of Aureomycin to diets including cholesterol significantly augmented the hypercholesteremia (Nelson et al., '53). In chicks, the hypocholesteremic effect of glucose in diets containing cholesterol was largely abolished by inclusion of Aureomycin (Kritchevsky et al., '58). In rats, administration of Sulfasuxidine increased serum cholesterol with a starch diet, but was without effect with sucrose (Portman et al., '56). The effect of the drug in rats fed starch diets appeared, however, only when cholic acid was included. These examples illustrate the complexity of the action of antibiotics on serum cholesterol.

In our experiments levels of liver cholesterol, in contrast with serum cholesterol, were significantly affected by both carbohydrates and Aureomycin. Sucrose-containing diets generally lowered liver cholesterol, whereas starch increased it. These effects may be modified by the steroid content of the diet. Incorporation of sorbitol or Aureomycin into a starch diet further increased the concentration of liver cholesterol. Aureomycin also increased liver cholesterol with sucrose and starch but not with dextrose.

Adams et al.[1] reported that replacement of sucrose by cornstarch resulted in a lowering of liver fat and cholesterol in rats. This could be confirmed by us for cholesterol but only when the diet contained cholic acid. Generally, however, feeding starch diets resulted in higher liver fat values than diets including sucrose or dextrose.

Liver weight was much smaller in rats fed dextrose diets than in those receiving sucrose or starch, as noted by Adams and associates ('59).

It appears that liver cholesterol, in contrast with blood cholesterol, easily responds to variations in dietary carbohydrates and to the addition of Aureomycin. This may result from the action of carbohydrates on the turnover of cholic acid. Portman and Murphy ('58) found that feeding commercial chow[2] to rats caused a relatively high turnover rate of fecal bile as compared with purified diets containing starch or sucrose. The action of the carbohydrates may also be modified by differences in the energy intake. Rats supplied with a starch diet ad libitum consume greater quantities of food than animals offered a sucrose diet (Harper and Elvehjem, '57). In view of the influence of both Aureomycin and carbohydrates on the intestinal flora, it seems likely, though it has not been proved, that their action is mediated by variations in the intestinal flora. Since bacteria are responsible for many changes in the bile acids and cholesterol in the intestine, it seems at present impossible to postulate a mechanism by which carbohydrates or antibiotics act on cholesterol metabolism.

SUMMARY

The effects of certain dietary carbohydrates and of Aureomycin on serum and liver cholesterol and on liver fat were studied in young rats. The diets differed as to their content of cholesterol and cholic acid.

Incorporation into the diet of cholesterol and/or cholic acid induced an en-

[1] Adams, M., M. Fisher and G. J. Koval 1959 The influence of dietary carbohydrate on kidney and liver damage and serum cholesterol in the rat. Federation Proc., *18:* 178 (abstract).

[2] Purina.

largement of the liver which is the result of deposition of fat as well as of an increase of liver cytoplasm.

Generally, starch in the diet led to higher levels of liver fat and cholesterol than sucrose or dextrose; sucrose lowered liver cholesterol. The effect of carbohydrates on serum cholesterol, liver fat and nitrogen depended on the presence of steroids in the diet.

Supplementation with Aureomycin or replacement of 10% of starch by sorbitol resulted in an increase of liver cholesterol in most dietary combinations.

It seems at present not possible to postulate a mechanism by which carbohydrates or Aureomycin act on cholesterol metabolism.

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LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum. J. Biol. Chem., *195:* 357.

Coleman, D. L., and C. A. Baumann 1957 Intestinal sterols. 3. Effects of age, sex and diet. Arch. Biochem. Biophys., *66:* 226.

Danielsson, H., and B. Gustafson 1959 On serum cholesterol levels and neutral fecal sterols in germ-free rats. Bile acids and steroids 59. Ibid., *83:* 482.

Fillios, L. C., C. Naito, S. B. Andrus, O. W. Portman and R. S. Martin 1958 Variations in cardiovascular sudanophilia with changes in the dietary level of protein. Am. J. Physiol., *194:* 275.

Grant, W. C., and M. J. Fahrenbach 1959 Effect of dietary sucrose and glucose on plasma cholesterol in chicks and rabbits. Proc. Soc. Exp. Biol. Med., *100:* 250.

Harper, A. E., and C. A. Elvehjem 1957 A review of the effects of different carbohydrates on vitamin and amino acid requirements. Agr. Food Chem., *5:* 754.

Kritchevsky, D., W. C. Grant, M. J. Fahrenbach, B. A. Riccardi and R. F. J. McCandless 1958 Effect of dietary carbohydrate on the metabolism of cholesterol-4-C^{14} in chickens. Arch. Biochem. Biophys., *75:* 142.

Lindstedt, S., and A. Norman 1956 The excretion of bile acids in rats treated with chemotherapeutics. Bile acids and steroids 40. Acta Physiol. Scand., *38:* 129.

Morgan, T. B., and J. Yudkin 1957 The vitamin-sparing action of sorbitol. Nature, *180:* 543.

Nelson, D., A. C. Ivy, R. Altschul and R. Willheim 1953 Effect of aureomycin on experimental atherosclerosis and serum cholesterol levels. Arch. Path., *56:* 262.

Portman, O. W., E. Y. Lawry and D. Bruno 1956 Effect of dietary carbohydrate on experimentally induced hypercholesteremia and hyperbetalipoproteinemia in rats. Proc. Soc. Exp. Biol. Med., *91:* 321.

Portman, O. W., and P. Murphy 1958 Excretion of bile acids and β-hydroxysterols by rats. Arch. Biochem. Biophys., *76:* 367.

Portman, O., and F. J. Stare 1959 Dietary regulation of serum cholesterol levels. Physiol. Rev., *39:* 407.

Pihl, A. 1955 The effect of bile acids on cholesterol absorption, deposition and synthesis in the rat. Cholesterol studies 5. Acta Physiol. Scand., *34:* 207.

Siperstein, M. D., I. L. Chaikoff and W. C. Reinhardt 1952 C^{14}-cholesterol. 5. Obligatory function of bile in intestinal absorption of cholesterol. J. Biol. Chem., *198:* 111.

Snog-Kjaerr, A., I. Prange and H. Dam 1955 On the formation of coprosterol in the intestine. Experientia, *11:* 316.

Swell, L., D. F. Flick, H. Field and C. R. Treadwell 1953 Influence of dietary bile on blood cholesterol levels. Proc. Soc. Exp. Biol. Med., *84:* 428.

Effect of Feeding D-Sorbitol on the Intestinal Absorption of Vitamin B_6 and Vitamin B_{12} in Rats[1]

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D-Sorbitol can enhance the absorption of orally administered vitamin B_{12} in man (Chow et al., '57) and in animals (Greenberg et al., '57). It can also increase the absorption of iron by rats (Herndon et al., '58). Morgan and Yudkin ('57) reported that adding D-sorbitol to an adequate diet, deficient in the vitamins of the B group only, will bring about the normal growth rate of weanling rats. The latter finding may be explained either on the basis of increase in intestinal synthesis, enhancement of absorption or more efficient utilization of these vitamins. It is, therefore, of interest to determine whether prolonged feeding of dietary D-sorbitol could affect the absorption and excretion of vitamin B_{12} and B_6. The results of such studies are reported in this paper.

EXPERIMENTAL

Experimental animals. Young adult rats of the McCollum strain from our stock colony were used. One half of them was fed ad libitum a basal diet (table 1) low in vitamin B_6 and B_{12}, with or without supplementation of these vitamins, and were housed in individual cages with a large-mesh wire screen bottom. The other half was given the same diet except that D-sorbitol was added to the basal diet in amounts to replace a part of sucrose. The animals were offered these diets for 12 weeks. On the 7th days of the 4th and 12th weeks, they were placed in individual metabolism cages. Urine specimens were collected for 24 hours and assayed for vitamin B_6 activity.

Determination of vitamin B_{12}. Vitamin B_{12} concentrations were determined in plasma and liver tissue with Skeggs' media (Skeggs et al., '50) with *Lactobacillus leichmannii* 4797 as the test organism. The technique has been described elsewhere (Yamamoto et al., '57).

Determination of vitamin B_6. Vitamin B_6 activity in urine and liver was meas-

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TABLE 1
Composition of the basal diets

Ingredients	Diet		
	1	2	3
	%	%	%
Vitamin-free casein	21	20	—
Soybean	—	—	62
Sucrose	70	71	30
Corn oil[1]	5	5	4
Salt mixture no. 4[2]	4	4	4
Vitamins[3]	$-B_6, -B_{12}$	$-B_6$	$-B_{12}$
Thyroid powder	—	—	0.1

[1] Mazola, Corn Products Refining Company, New York.
[2] Hegsted et al. ('41).
[3] Each kilogram of diet contained the following in milligrams: thiamine, 2.0; riboflavin, 3.0; pyridoxine, 10; Ca pantothenate, 20; niacin, 50; inositol, 100; biotin, 0.1; PABA, 250; folic acid, 0.2; choline chloride, 1,000; vitamin B_{12}, 0.05; α-tocopherol, 23; menadione, 2.1; and, vitamin A, 1,200 and vitamin D, 170 units.

ured using *Neurospora sitophi'ia* 299 as the test organism (Stokes et al., '43).

Measurement of vitamin B_{12} absorption. Adult female rats from our stock colony (average weight, 200 gm) were fasted overnight and then given by mouth one of the following treatments of radioactive vitamin B_{12}[2] labeled with cobalt^{60}: (a) a single dose of 50 mμg or (b) two doses of 1,000 mμg with a 24-hour interval. The feed was returned to the rats 4 hours after the administration of the test dose. The animals were killed 4 days after the treatment (a), or two days after the treatment (b), and the radioactivities in the liver and kidneys were measured. Feces from rats receiving treatment (a) were collected for 4 days, and all specimens were processed for radioactivity measurement according to the procedures described elsewhere (Okuda et al., '59).

Growth study. Weanling rats of the McCollum strain were raised in individual cages and fed ad libitum a basal diet deficient either in vitamin B_6 or B_{12}, with or without supplementation of D-sorbitol. They were weighed weekly.

RESULTS

Effects of D-sorbitol on the growth of rats fed a vitamin B_6-deficient diet. In order to determine whether supplementation of D-sorbitol improves growth rate, two groups of 8 weanling rats were fed basal diet 2 with or without supplementation of 10% of D-sorbitol. A comparison of the growth curves of such groups (fig. 1) clearly demonstrates that the addition of D-sorbitol improved the growth rate of weanling rats fed a vitamin B_6-deficient diet. The difference in the body weight between the two groups became statistically significant after three weeks of feeding, and its magnitude increased progressively. Although the animals were not pair-fed, the feed consumption of both groups was measured daily and found to be practically the same. These data con-

[2] The specific activity was 1 μc per μg; kindly supplied by Merck and Co., Inc.

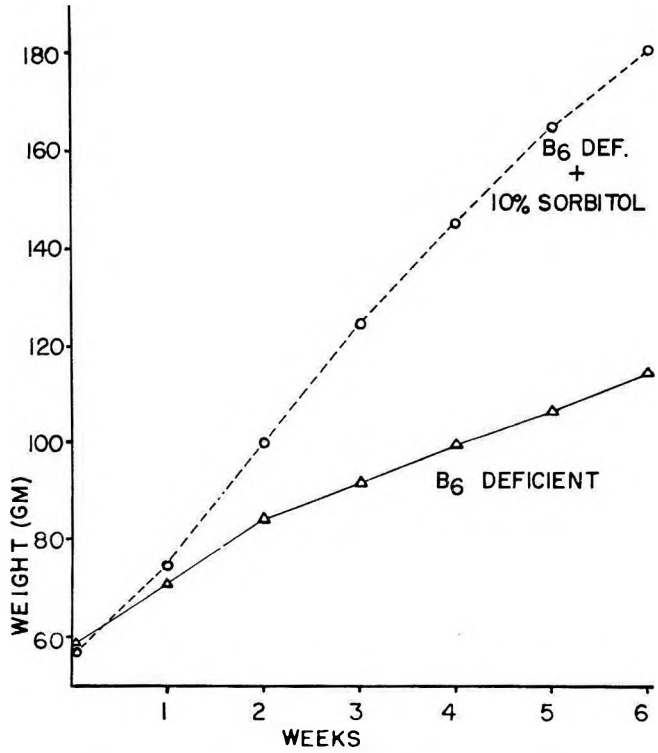

Fig. 1 Effect of D-sorbitol on the growth of weanling rats fed a vitamin B_6-deficient diet.

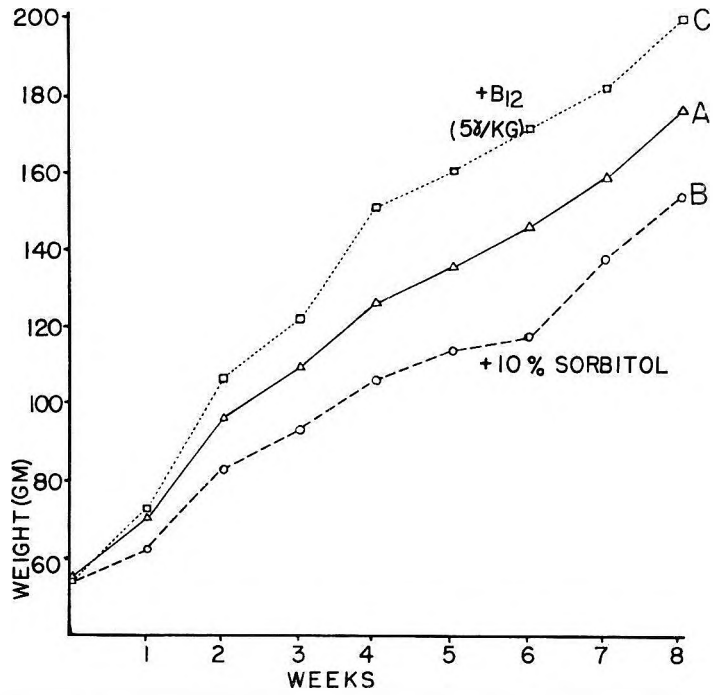

Fig. 2 Effect of D-sorbitol on the growth of weanling rats fed a soybean diet deficient in vitamin B_{12}.

firm essentially the findings of Morgan and Yudkin ('57).

Effect of D-*sorbitol on the growth of rats fed a soybean diet supplemented with desiccated thyroid.* To study the effect of dietary D-sorbitol on the growth of rats fed a vitamin B_{12}-deficient diet, three groups (8 each) of weanling rats were fed basal diet 3 which consisted of a soybean protein containing 0.1% of desiccated thyroid and which was deficient in vitamin B_{12} with (group B) or without (group A) D-sorbitol. Desiccated thyroid was added to the diet to make the animals even more responsive to vitamin B_{12} therapy (Emerson, '49). The third group (C) received the same diet (diet 3) supplemented with 5 μg of vitamin B_{12} per kg of diet. The growth curves of such animals in a typical experiment (fig. 2) demonstrate that sorbitol did not improve the growth of the weanling rats receiving this diet. In fact, there was a possibility of retarded growth, particularly during the first week of the experimental diet (group B diet) although the difference in mean body weights at the end of 8 weeks of feeding between groups A and B was not statistically significant. The group which received vitamin B_{12} in the diet had the highest mean body weight throughout the entire experimental period.

After 8 weeks of feeding, the animals in groups A and B were tested for their ability to absorb radioactive vitamin B_{12} by feeding a single dose of 50 mμg. The results of the study (table 2, study 1) show a reduction of absorption in the sorbitol-treated animals as evidenced by a significant decrease in kidney radioactivity.

Urinary excretion and liver concentrations of vitamin B_6. In order to determine whether D-sorbitol enhances the intestinal absorption of vitamin B_6, and if it does, whether such an effect is limited to vitamin B_6 which has been added to the diet, the following experiment was carried out. Twenty-four young adult female rats were divided into 4 groups: group A, received the basal diet 1 without vitamin B_6 and B_{12}; group B, the same diet supplemented with vitamin B_6 and B_{12}; group C, the basal diet in which

TABLE 2

Absorption of radioactive vitamin B_{12} after prolonged feeding of D-*sorbitol*

Study (Weeks of feeding)	Group[1]	Dosage	Fecal test		Organ uptake	
			Recovery	Absorption	Liver	Kidneys
			mμg	mμg	mμg	mμg
1 (8)	A B_{12}-deficient	50 mμg × 1	26.5 ± 2.71[2]	23.5	4.92 ± 0.54	3.23 ± 0.29
	B B_{12}-deficient + sorbitol	50 mμg × 1	32.3 ± 0.49	17.7	3.76 ± 0.25	2.16 ± 0.15
2 (12)	A B_{12}-deficient	50 mμg × 1	24.1 ± 0.90	25.9	4.63 ± 0.15	3.25 ± 0.14
	C B_{12}-deficient + sorbitol	50 mμg × 1	32.6 ± 1.39	17.4	2.60 ± 0.32	1.72 ± 0.19
	B B_{12}-supplemented	1000 mμg × 2			37.9 ± 7.4	70.4 ± 15.1
	D B_{12}-supplemented + sorbitol	1000 mμg × 2			15.3 ± 2.1	27.4 ± 3.5

[1] Six rats in each group.
[2] Standard error of the mean.

sucrose was partially replaced by D-sorbitol, and group D, the basal diet which was supplemented with vitamin B_6 and B_{12} and containing D-sorbitol. The content of sorbitol in the diets for groups C and D was 10% during the first 6 weeks of feeding and was subsequently increased to 20% during the second 6-week period in order to make the effect of sorbitol more marked. The 24-hour excretion of vitamin B_6 in the urine collected during the last day of the 4th week was measured and found to be 1.04 ± 0.04 μg in group A and 6.52 ± 0.32 μg in group C (table 3), demonstrating a marked increase in excretion ($P < 0.001$).

Effect of prolonged feeding of D-*sorbitol on absorption of vitamin B_{12}.* To study the effect of prolonged feeding of sorbitol on vitamin B_{12} absorption, another set of animals in the various groups similar to those described above (table 3) was given a vitamin B_{12} absorption test at the end of a 12-week feeding period. The animals were fasted overnight to eliminate the effect of any sorbitol remaining in the gastrointestinal tract, and were given radioactive vitamin B_{12} by mouth on the following morning. When the dose of 50 mμg was fed to groups A and C, and the absorption was estimated from the radioactivity in fecal matter and organs, a significantly poorer absorption was demonstrated in group C in comparison with group A (see table 2, study 2). An even greater difference was obtained between groups B and D which received two doses of 1,000 mμg; the uptake of radioactivity was 37.9 ± 7.4 mμg, and 15.3 ± 2.1 mμg in the liver and 70.4 ± 15.1 mμg and 27.4 ± 3.5 mμg in the kidneys, respectively. The decrease in absorption of vitamin B_{12} after 12 weeks of dietary sorbitol also is reflected in a lowering of vitamin B_{12} concentration in the plasma and liver (table 3, last two columns).

DISCUSSION

The data presented here demonstrate clearly that supplementation of a vitamin B_6-deficient diet with D-sorbitol brings about an increase in the urinary excretion and in the liver concentration of vitamin B_6 in young adult rats. These results may be interpreted as evidence that the absorption of vitamin B_6 was increased by the

[3] Herndon, J. F., S. M. Greenberg, E. J. Van-Loon, J. K. Mathues and E. T. Parmelle 1959 Activities of D-sorbitol on the growth of rats deficient in pyridoxine, vitamin B_{12}, biotin or pantothenic acid. Federation Proc., *18*: 529 (abstract).

TABLE 3

Effects of prolonged feeding of D-*sorbitol on urinary excretion and liver content of vitamin B_6 and on liver and plasma vitamin B_{12} concentrations*

Group[1]	Diet	Av. body weight		Urinary excretion of B_6		B_6 in liver	B_{12} in liver	B_{12} in plasma
		Initial	12 weeks	4 weeks	12 weeks			
		gm	*gm*	*µg/24 hours*	*µg/24 hours*	*µg/gm wet tissue*	*mµg/gm wet tissue*	*mµg/ml*
A	$-B_6, -B_{12}$	153	202	1.04 ± 0.04[2]	1.45 ± 0.03	5.6 ± 0.5	160 ± 32	0.107 ± 0.009
B	$+B_6, +B_{12}$	150	209	12.20 ± 1.65	14.15 ± 3.51	8.9 ± 0.4	333 ± 53	0.732 ± 0.032
C	$-B_6, -B_{12}$ +sorbitol	152	200	6.52 ± 0.32	6.88 ± 1.12	9.9 ± 0.6	151 ± 13	0.086 ± 0.010
D	$+B_6, +B_{12}$ +sorbitol	159	218	15.52 ± 1.72	19.35 ± 2.71	10.4 ± 0.3	278 ± 34	0.574 ± 0.030

[1] Six rats in each group.
[2] Standard error of the mean.

sorbitol supplementation. This finding is in harmony with the observation that the growth rate of weanling rats fed a vitamin B_6-deficient diet was improved by the supplementation of 10% of D-sorbitol. This confirms the report by Herndon et al.[3]

The effects of D-sorbitol on vitamin B_{12} absorption under similar conditions were different from those on vitamin B_6. Unlike the latter vitamin, prolonged feeding of D-sorbitol results in apparent reduction of vitamin B_{12} absorption with concomitant decreases in the plasma and liver concentrations of vitamin B_{12} in a manner similar to prolonged dietary deprivation of B_6, (Hsu et al., '57; Ranke et al., '60). In distinct contrast with the growth-promoting effect of sorbitol in B_6 deficiency, sorbitol did not improve and might even have retarded the growth of the weanling rats fed a vitamin B_{12}-deficient soybean diet supplemented with desiccated thyroid. It should be pointed out that such thyroid-treated animals may be different from the ones deprived of vitamin B_{12} in the diet. The influence of sorbitol on the utilization of intestinal vitamin B_6 is apparently different from that on the utilization of vitamin B_{12}. No single mechanism would account for two diversified effects of sorbitol on these vitamins. The effect of sorbitol on vitamin B_6 may be explained on the basis of increased synthesis of vitamin B_6 by intestinal microorganisms, but an effect of sorbitol to increase the concentrations of available vitamin B_6 in the lumen without increasing synthesis cannot be ruled out.

On the surface, our observation of the reduced absorption after prolonged feeding of sorbitol seems to contradict the reported observations on increased absorption when vitamin B_{12} was co-administered with D-sorbitol by mouth to normal rats and human volunteers. Chow et al. ('58) observed a greater increase of vitamin B_{12} levels in plasma in the human subjects receiving 50 µg of vitamin B_{12} and 18 gm of liquid sorbitol as compared with that in control subjects receiving vitamin B_{12} alone after a long period of administration. However, these diametrically opposite results may be explained on the basis of the difference in the physical states of the two mixtures of vitamin B_{12}

and sorbitol. Our unpublished data indicate that sorbitol enhanced absorption of vitamin B_{12} only when a large dose (1,000 mμg) of vitamin B_{12} was used and that without the cecum, this effect cannot be shown in rats. The markedly enlarged ceca of the sorbitol-dosed animals as observed suggest such a possible role of cecum in absorption of vitamin B_{12}. It should be pointed out that, in the present study, the absorption of vitamin B_{12} was measured under a condition in which sorbitol was not present in the lumen when the test dose of vitamin B_{12} was administered. Such a condition would permit one to estimate the effect of prolonged feeding of sorbitol on the absorption capacity of the intestinal wall, rather than a direct effect of sorbitol on absorption of B_{12} or interaction between the two components. The involvement of intrinsic factor in the observed reduction of absorption of vitamin B_{12} seems unlikely, because the absorption of a large dose of vitamin B_{12} has been shown repeatedly not to depend on intrinsic factor (Doscherholmen and Hagen, '57; Okuda, '60). The absorption study using such a dose also demonstrated the reduction of absorption by sorbitol (table 2, group B vs D).

SUMMARY

The effects of D-sorbitol in the diet on the utilization of vitamin B_6 and B_{12} were studied in rats. The following conclusions were drawn:

1. The addition of D-sorbitol to a diet deficient in vitamin B_6 increased the urinary excretion and liver concentrations of vitamin B_6 in adult rats, and markedly improved the growth rate in weanling rats.

2. After feeding D-sorbitol at the level of 10 to 20% in the diet for 8 to 12 weeks, the intestinal absorption of orally-fed radioactive vitamin B_{12} was reduced.

3. The addition of D-sorbitol to a diet deficient in vitamin B_{12} did not improve the growth of weanling rats.

These observations demonstrate a multiple effect of D-sorbitol on vitamins in the intestine. The possible mechanism for the increased utilization of vitamin B_6 by the dietary D-sorbitol has been discussed.

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LITERATURE CITED

Chow, B. F., P. Meier and S. M. Free, Jr. 1957 Absorption of vitamin B_{12} enhanced by D-sorbitol. Am. J. Clin. Nutrition, *6:* 30.

Chow, B. F., H. Prystowsky, A. E. Hellegers and V. Wong 1958 Studies on the absorption of vitamin B_{12} in human pregnancy with especial reference to the effect of D-sorbitol. Am. J. Obst. Gyn., *76:* 91.

Doscherholmen, A., and P. S. Hagen 1957 A dual mechanism of vitamin B_{12} plasma absorption. J. Clin. Invest., *36:* 1551.

Emerson, G. 1949 Growth-promoting activity of vitamin B_{12} in rats receiving thyroid substance. Proc. Soc. Exp. Biol. Med., *70:* 392.

Greenberg, S. M., J. F. Herndon, E. G. Rice, E. T. Parmelee, J. J. Gulesich and E. J. VanLoon 1957 Enhancement of vitamin B_{12} absorption by substances other than intrinsic factor. Nature, *180:* 1401.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. G. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

Herndon, J. F., E. G. Rice, R. G. Tucker, E. J. VanLoon and S. M. Greenberg 1958 Iron absorption and metabolism. III. The enhancement of iron absorption in rats by D-sorbitol. J. Nutrition, *64:* 615.

Hsu, J. M., and B. F. Chow 1957 Effect of pyridoxine deficiency on the absorption of vitamin B_{12}. Arch. Biochem. Biophys., *72:* 322.

Morgan, T. B., and J. Yudkin 1957 The vitamin-sparing action of sorbitol. Nature, *180:* 543.

Okuda, K., J. A. Wider and B. F. Chow 1959 The effect of intrinsic factor on the hepatic uptake of vitamin B_{12} following intravenous injection. J. Lab. Clin. Med., *54:* 535.

Okuda, K. 1960 Vitamin B_{12} absorption in rats, studied by a "loop" technique. Am. J. Physiol., July.

Ranke, B., E. Ranke and B. F. Chow 1960 The interrelationship between vitamin B_6 and B_{12} deficiencies in rats. J. Nutrition, *71:* 411.

Skeggs, H. R., H. M. Nepple, K. A. Valentik, J. W. Huff and L. D. Wright 1950 Observations on the use of *Lactobacillus leichmannii* 4797 in the microbiological assay of vitamin B_{12}. J. Biol. Chem., *184:* 211.

Stokes, J., A. Larsen, C. R. Woodward and J. W. Foster 1943 A neurospora assay for pyridoxine. Ibid., *150:* 17.

Yamamoto, R. S., K. Okuda and B. F. Chow 1957 Effect of carbontetrachloride injury on plasma and liver vitamin B_{12} levels. Proc. Soc. Exp. Biol. Med., *94:* 497.

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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		
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

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