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ANTOINE-LAURENT LAVOISIER

(1743–1794)

Photograph courtesy of the Rockefeller Institute

ANTOINE-LAURENT LAVOISIER

Antoine-Laurent Lavoisier
— *A Biographical Sketch*

(August 26, 1743 — May 8, 1794)

Familiar to historians and scientists as "the father of modern chemistry," Antoine-Laurent Lavoisier was perhaps the greatest and one of the most versatile men of science France has produced. His central role in the Chemical Revolution has tended to dwarf his other achievements which, nonetheless, were considerable. Major contributions to geology and physiology and studies in hydrometry, meteorology, and physics constitute a large part of his activities in the sciences; and, as a civil servant, he produced important and influential writings in the fields of political economy, education, agriculture and public health.

The only son of a wealthy bourgeois family, Lavoisier was born in Paris where he was to spend most of his life. Although expected to enter the law, his father's profession, and equipped with a sound education in the humanities, he soon showed a distinct preference for the sciences. To which branch of science he would devote himself was not immediately clear, but, after completing his formal education in 1763, his uncertainty vanished when he was invited by his father's friend, the naturalist Jean-Étienne Guettard, to collaborate in the preparation of a geologic atlas of all France. He quickly acquired the rudiments of geology and some familiarity with analytical techniques by attending the famous chemical lectures of G.-F. Rouelle, then at the height of his popularity among students of science and the intellectuals of the salons.

The work with Guettard was to be Lavoisier's major scientific preoccupation until 1772, when his attention was drawn to the problem of combustion. But he also found time, in these early years, to prepare two memoirs on the analysis of gypsum, both of which he read before the Royal Academy of Sciences, and to enter a competition, sponsored by the Academy, on the problem of the best means of lighting the streets of a large city. The impressive results of these labors, coupled with the promise he displayed as Guettard's protégé, led to his election to the Academy in 1768. In the same year, he bought a share in the Ferme générale — the company of financiers responsible for the collection of certain indirect taxes under the Old Régime — and embarked upon those activities which were to acquaint him with the political and economic life of France and which, eventually, were to lead to his arrest and execution during the Revolution.

In the summer of 1772 Lavoisier turned his attention to the study of combustion and, in particular, to the calcination (slow roasting) of metals. According to the then prevalent phlogiston theory, combustion was a chemical change involving the loss of an inflammable principle, phlogiston. Satisfactory in some respects, this theory could not account for one striking phenomenon: if metals lose phlogiston during calcination, why then do their calces (oxides) show a *gain* in weight? Within a relatively short time — and by a path too intricate to describe here — Lavoisier recognized the key role played by "air" in combustion. His new theory, upon which was to rest most of his subsequent work in science, was published in 1774 in his first major work, the *Opuscules physiques et chimiques*. Priestley's discovery of oxygen later that year was followed by Lavoisier's modification of his theory which now explained combustion as a chemical combination not with "air," but with a distinctive part of atmospheric air.

The rapid course of the Chemical Revolution has been described many times: the discovery, in quick succession, of several new gases; Lavoisier's realization that water is the product of the burning of

hydrogen; the further elaboration of Lavoisier's theory, and the attack launched on phlogistic chemistry. These developments were followed by the publication, in 1787, of the *Méthode de nomenclature chimique,* the collaborative work of Lavoisier, Guyton de Morveau, Fourcroy, and Berthollet, which embodied in a systematic scheme of classification the discoveries and theories of the new chemistry and which provided the basis of modern chemical terminology. Two years later Lavoisier's *Traité élémentaire de chimie* (1789), often called the first modern textbook of chemistry, presented the new chemistry in terms of the new nomenclature; soon translated into Dutch, French, German, Italian, Spanish, and English, the *Traité élémentaire* was different in style, form, and content from the traditional textbooks which quickly became obsolete.

Lavoisier was fully aware that his explanation of the nature of combustion would be unacceptable unless he could also account for certain closely related phenomena which were easily understood by his fellow chemists in terms of their Protean principle, phlogiston. Three phenomena became the objects of his strenuous research program: the evolution of heat and light in combustions, which his contemporaries described as an effect accompanying the escape of the "matter of fire," or phlogiston; the combustion of "inflammable air" (hydrogen), which its discoverer, Henry Cavendish, could readily interpret in phlogistic terms; and the nature of respiration which, according to the prevailing theory, depended upon the ability of the air to absorb the phlogiston exhaled from the lungs (thus, oxygen, Priestley's "dephlogisticated air," was almost devoid of phlogiston, capable of absorbing it in large quantities, and therefore better able than ordinary air to support respiration and combustion). Of these three problems, the one which called for the most laborious and imaginative in experiment and theory and for a brilliant foray into the field of physiology was that of the nature of respiration.

By 1777 it had long been a familiar technique in chemical experiments to test the "quality" of the air evolved in any reaction by determining if it would support respiration and combustion. The use of small mammals — birds or mice — or a lighted candle for this purpose had shown that air is, in some mysterious fashion, vital to both processes. Robert Hooke and John Mayow, in the seventeenth century, had suggested that there was a particular constituent of common air which served to support respiration and combustion, and Mayow had shown that a small volume of air is actually used up in both instances. But these early proposals bore no fruit, and, by Lavoisier's day, the effect of a particular kind of "air" on birds or on a candle's flame was generally taken to mean only that this "air" was either similar to atmospheric air or tainted by noxious and asphyxiating vapors. It was Lavoisier, with his genius for asking the right question and making the masterful synthesis, who finally perceived that the analogy between combustion and respiration, taken for granted by his contemporaries, was no mere happy accident.

On May 3, 1777, Lavoisier presented to the Academy of Sciences the results of his first, not very extensive, experiments to determine the role in respiration of the gas he now called "air éminemment respirable." After briefly stating Priestley's phlogistic interpretation and offering his own experimental evidence that "air éminemment respirable" is somehow converted during respiration to "acide crayeux aériforme" (carbon dioxide), he went on to state fully the suggestive parallels between combustion and respiration: both processes can be maintained longer in "air éminemment respirable" than in atmospheric air; both take place only until this "air" is used up; both involve only this kind of "air," while the remaining portion of the atmosphere is passive; and both result in the production of carbon dioxide. He even went so far as to compare the red color of blood with that of minium and to suggest that the redness in both instances was to be attributed to the combination with "air éminemment respirable."

Although this early memoir contains almost more conjecture than experiment, it has been summarized here at some length because it marks the start of a series of classic investigations in physiology — many of them carried out in collaboration

with the mathematician Pierre Simon de Laplace and others with a younger assistant, Armand Séguin — which were to occupy Lavoisier's attention until shortly before his death.

Lavoisier's theory, first put forth in 1777, that body temperature is maintained by the heat of combustion evolved in respiration was confirmed quantitatively by the experiments carried out with Laplace during the winter of 1782–83 and presented to the Academy of Sciences on June 18, 1783. In this famous "Mémoire sur la chaleur," Lavoisier and Laplace describe a series of experiments, using the ice calorimeter devised by Laplace, to compare the amount of heat evolved in the combustion of a specified quantity of charcoal with the body heat given off by a guinea pig during a certain time interval. By measuring the amount of carbon dioxide exhaled and the heat lost by the guinea pig in ten hours, and by calculating the amount of heat needed to produce the same volume of carbon dioxide by ordinary combustion, they were able to conclude that, within the limits of experimental error, the amount of heat and carbon dioxide given off by the guinea pig during respiration equalled that produced by the burning of charcoal. Respiration was thus nothing more than a slow combustion which had taken place in the body — in the lungs, as they thought — of the animal and which served to replenish constantly the body heat being lost.

In his work with Séguin, much of which was published posthumously, Lavoisier continued to investigate what he called the three principal regulatory mechanisms of the body: respiration, transpiration, and digestion. He and Séguin conducted experiments in further confirmation of Lavoisier's theory that respiration is a slow combustion, and Lavoisier modified his earlier conclusion to suggest that some of the oxygen consumed during respiration went to form water in the body by combining with hydrogen from some unspecified source. Part of the research program announced in the "Mémoire sur la chaleur" was carried out in 1790 when he and Séguin investigated the function of transpiration in regulating body temperature. Still another series of experiments, with Séguin himself serving as guinea pig, was devised to measure metabolic rates; the coworkers concluded that oxygen consumption increases in conditions of cold, during digestion, and during the performance of work, and that, although the pulse rate also increases under these conditions, body temperature remains virtually constant.

Lavoisier's work in the sciences — his discoveries and theories, highly developed analytical techniques, and clarity of thought and expression — had a marked effect upon his activities in public life. In many cases, he was in fact appointed to particular committees by his fellow Academicians or by Royal Ministers because his scientific pursuits were often closely related to the subjects under scrutiny for purposes of reform. That his interest in reform projects was a lively one and that he was more than a passive member of the many committees on which he served is readily apparent from the fact that he was usually chosen secretary of such groups and was entrusted with the tasks of conducting investigations, preparing reports, and drawing up recommendations. The list of such activities is enormous, as is the range of subjects covered in reports prepared entirely or in part by Lavoisier. In 1780 he served on a commission authorized to investigate the prisons of Paris, and a few years later on a similarly constituted group which studied the city's hospitals. It is difficult to determine precisely what share in these labors is to be attributed to Lavoisier, but many of the recommendations of these groups — concerning, for example, inadequate ventilation and sanitation and the poor diet of prisoners and patients — can plausibly be said to reflect both his research in physiology and organic chemistry and his longstanding interest in problems which combine the theoretical with the practical. Since his very early essay on the lighting of city streets, he had successively attacked such subjects as the Paris water supply, the diet of sailors, the food content of the broth fed to hospital patients, the spoilage of meat and grain, sewage disposal, and the ventilation of theaters and other public places. Some of these studies were undertaken on Lavoisier's own initiative, others assigned by the government to committees of the

Academy of Sciences, and still others tackled by special ad hoc committees selected and convened by one or another of the Royal Ministers.

Two of the most important areas covered by Lavoisier's reform activities were taxation and agriculture. His work in the Ferme générale (1768) and as a member of the Comité d'agriculture (1785) illustrates not only his concern with the applications of science, but also his lifelong interest in economic, social, and technological reform. As in all he undertook, Lavoisier devoted to these fields the same boundless energy, efficiency, and brilliance that characterized his scientific research. His fertile imagination, quick understanding, and considerable practical experience made him one of the least theoretico-philosophical of reformers and one whose proposals were impressively thoroughgoing, practicable, and liberal in spirit.

Ever since his early travels with Guettard, Lavoisier had become increasingly aware of the pressing problems confronting the Old Régime: the backward state of agriculture, the poverty of many peasants and villagers, and the unequal incidence of taxes on the various social classes as well as in the different provinces of France. He soon realized that the only way to effect permanent reform — and not simply to fight a delaying action by propping up the existing system with a few superficial measures — was to act through the agencies of the central government. Research must first be directed toward the definition of any particular problem, a workable solution found, and the government persuaded to put the proposed reforms into effect. Lavoisier probably did not envision these steps occurring in logical sequence, although his work in agriculture suggests just such a long-range plan.

Although there is rich soil in many parts of France, the English traveler and scientific farmer, Arthur Young, noted with disapproval the backwardness of the peasants whose land was being ruined through sheer ignorance of the proper methods of cultivation. Not only were farm implements primitive, but the latest ideas on crop rotation were unknown. The small herds of underfed cattle and sheep could not supply enough fertilizer, and crops were too poor to feed the animals during the winter. The various learned societies for the improvement of agriculture were of no help whatever, according to Young, because their members had little practical experience.

In 1778 Lavoisier bought the estate of Fréchines, near Blois, and spent the next ten or more years turning it into a profitable experimental farm. His object was to solve, first, the problem of the feeding of sheep and cattle during the winter months so that a sizable herd, an adequate supply of manure, and, eventually, flourishing crops would result. Having had no experience in farming, he spent three years experimenting to find the best fodder to raise in a soil which was not especially fertile and which hardened and cracked badly during dry periods. The result was the successful cultivation of meadows, the survival and growth of his flocks, and his introduction of the folding of sheep to provide fertilizer. After ten years, he had more fodder than he could store and the available supply of manure had more than tripled; but it took an additional five years to show a comparable increase in the yield of wheat and in the revenues of the estate.

Lavoisier presented reports of these activities to the Comité d'agriculture, of which he was the secretary, and to the Société d'agriculture de Paris. On the basis of his own experience, he recommended that new methods of crop rotation and the practice of sheep folding be introduced on a wide scale. Just how to do this was a problem for the central government, and Lavoisier fully recognized that the government, far from encouraging agriculture, was in fact depressing the peasant farmers by levying exorbitant taxes on successful proprietors, by forbidding the export of grain — or even its free circulation within French borders — and thus discouraging the raising of large crops, and by subjecting the peasants to a multitude of indirect taxes. If agriculture were to be revived, these practices would not only have to be altered, but the government would have to educate and subsidize enterprising farmers. As Lavoisier had discovered, successful farming requires a considerable outlay of capital and years of patient labor before

any profit appears; only rich landowners were in a position to experiment as he had, but they would not be likely to do so as long as profits were greater and more easily realized from investment in industry or commerce. Lavoisier hoped that, by his own example and by an enlightened government policy, landowners and well-to-do farmers could be induced to develop their property to their own advantage and for the good of the national economy.

The unequal incidence of direct taxes was but one aspect of the complex financial problems which beset pre-Revolutionary France. Indirect taxes, on such items as tobacco, salt, and wine, were even more burdensome, especially when these taxes varied considerably from one province to the next. As a member of the Ferme générale for more than 20 years, Lavoisier was particularly familiar with the problems of assessment and collection of indirect taxes and with the fraudulent practices which arose in connection with government monopolies of vital commodities. The smuggling of salt across provincial borders was common, and it was one of Lavoisier's missions to inspect the deployment of brigades set to catch bands of smugglers. The adulteration of tobacco by the addition of excessive moisture, although not a widespread practice, was greatly resented before and during the Revolution and contributed to the hatred in which the Ferme and its agents were generally held. Since entering the Ferme in 1768, Lavoisier had made the detection of tobacco fraud one of his primary concerns.

Lavoisier's travels, with Guettard and for the Ferme générale, were doubtless instrumental in bringing to his attention the conditions in need of reform. But sweeping reform was not undertaken until the eve of the Revolution, when the provinces were invited by Louis XVI to submit lists of grievances and to send delegates to the Estates General which was to assemble in May, 1789. Lavoisier, who had taken part in the deliberations of the Provincial Assembly of Orléans, was chosen substitute delegate to the Estates General from the Bailliage de Blois and was also active in the affairs of the Commune of Paris. He was able at last to present publicly his schemes for the encouragement of agriculture, the establishment of a national system of education, and the complete overhaul of the complex systems of taxation. In addition to his political activities, he continued to serve on the Régie des Poudres (Gunpowder Commission), was appointed to the Commission of Weights and Measures which was to introduce the metric system, and even became a member of the committee charged with overseeing the demolition of the Bastille. As the Revolution entered its fanatically republican phase, Lavoisier became the eloquent, if unsuccessful, defender of the Academy of Sciences which, along with other learned societies, was finally suppressed in 1793.

Lavoisier's reputation as a scientist and public figure failed to save him from the Revolutionary "justice" meted out during the Reign of Terror. Popular hatred of the Ferme générale, a convenient scapegoat for many evils of the Old Régime, demanded the punishment of the financiers who had reputedly amassed fortunes out of the suffering of the people. Lavoisier and his one-time associates were arrested, given a drumhead trial, and guillotined on May 8, 1794.

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Utilization of Alfalfa Carotene and Vitamin A by Growing Chicks[1,2]

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Recent reports (Camp et al., '55; Ely, '59; Gledhill and Smith, '55; Olsen et al., '59; Williams, '62) have indicated, one way or another, that carotene is not utilized efficiently by poultry. Some doubt that natural carotene carriers are satisfactory sources of vitamin A activity for poultry, mentioning poor ability to convert carotene. Earlier literature (Record et al., '37; Petering et al., '41; Kramke et al., '52)[4] clearly supports the equivalence of units of activity from vitamin A and β-carotene for growth of poultry. The National Research Council ('60) takes that position. But purity of the earlier standards has been questioned, as have possible changes in utilization and requirements by newer, faster growing strains of birds eating high efficiency feeds.

It seemed desirable, therefore, to reappraise the relative utilizations of vitamin A activity of alfalfa meal and vitamin A per se with growing chicks.

GENERAL PROCEDURE

Chicks were depleted of vitamin A reserves for 6 to 14 days, vaccinated against Newcastle disease, distributed at random, 10 or 12 birds/lot, in wire-floor batteries kept in an animal laboratory with controlled temperature and light. The lots were assigned at random in the batteries and positions of batteries were shifted weekly. Fresh feed was prepared weekly, fed ad libitum, and discarded if not eaten within 7 days. The basal diet, a balanced growing feed (table 1), containing no measurable vitamin A or carotene, was fed during the depletion period. During test periods the vitamin A sources were weighed carefully and thoroughly mixed into the basal feed. New supplies of USP vitamin A reference solution[5] and of ANRC vitamin A standard[6] were obtained for each experiment, and kept at 5°. Alfalfa meals were stored at −20°. Quantities of meals added to the feeds were based on monthly carotene determinations (AOAC, '60) using 0.6 μg of carotene as one international unit of vitamin A. Birds were weighed individually; feed consumption was recorded by lots. When even the lowest quality meal was used, less than 2.5% of alfalfa was added to the feed; thus the growth-depression factor probably had no effect (Kodras et al., '51), even if present. Vitamin A was determined on blood serum (Kimble, '39) and liver (Shellenberger et al., '60) of birds selected at random from each lot. Judged by other birds in the same lots, the few death losses were not likely from treatment effects. Negative control birds in each experiment usually died 10 to 24 days after hatching.

Data were treated by analysis of variance and multiple group comparisons. To simplify presentation of results, statistical details are not given; symbols are used wherever possible.

EXPERIMENTAL

Experiment 1. The supplements, alfalfa meal A (high quality, 200,000 IU of vita-

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[1] Contribution no. 25, Department of Biochemistry, and no. 272 Department of Poultry Science, Kansas Agricultural Experiment Station, Manhattan.

[2] Supported in part by a research grant from American Dehydrators Association, Kansas City, Missouri.

[3] Present address: Animal Science Department, University of Illinois, Urbana, Illinois.

[4] Wilson, W. O., C. H. Schroeder and W. A. Higgins 1936 Further studies on vitamin A requirements of chicks. Poultry Sci., *15*: 466 (abstract).

[5] Vitamin A acetate in oil, 100,000 units/gm. Additions to feed based on declared potency. From USP, 46 Park Avenue, New York.

[6] Animal Nutrition Research Council. A gelatin beadlet type stabilized product. Obtained from USP, 46 Park Avenue, New York. Additions to feed based on declared potency (about 10,000 units/gm).

TABLE 1

Composition of basal diet

	kg	*(lb)*
Corn, white, ground	13.62	(30.0)
Sorghum grain, ground	14.30	(31.5)
Soybean oil meal, 44% solvent-extracted	13.17	(29.0)
	gm	
Brewer's dried yeast	908	(2.0)
Non-fat milk solids	908	(2.0)
Steamed bone meal	908	(2.0)
Calcium carbonate	454	(1.0)
Sodium chloride	227	(0.5)
Cottonseed oil	300	
Manganese sulfate premix	138.5	
Vitamin premix	469.5	

Special premix	*gm*
Vitamin B_{12}, 12 μg/lb[1]	30
Vitamin D_3 (15,000 ICU/gm[2])	4
DL-Methionine	46
B-vitamin supplement[3]	23
Choline chloride	6
Wheat middlings	360.5
	mg
Vitamin K (menadione)	23
Total	469.5

Manganese sulfate premix	*gm*
$MnSO_4 \cdot H_2O$	25
Wheat middlings	113.5
Total	138.5

[1] Proferm-12, Commercial Solvents Corporation, New York.

[2] Nopco Chemical Company, Harrison, New Jersey.

[3] Merck 58-A. Composition per lb: riboflavin, 2 gm; D-pantothenic acid, 2.68 gm; niacin, 6 gm; choline chloride, 20 gm.

min A/pound),[7] alfalfa meal B (low quality, 70,000 IU of vitamin A/pound), ANRC vitamin A standard, and alfalfa meal A plus 0.003% of furazolidone,[8] were fed at levels of 400 and 800 units of vitamin A/pound. Further details and results are in table 2. Chicks getting 800 units of vitamin A/pound made, as expected, a highly significant gain over those getting 400 units.[9]

Experiment 2. Vitamin A sources were: Alfalfa meal A (exp. 1), alfalfa meal C (low quality, 45,000 units vitamin A/pound), ANRC vitamin A standard, and USP vitamin A reference solution. Alfalfa meal unrefrigerated from the start of the experiment was fed to two lots of chicks to demonstrate effect of vitamin A loss during storage on growth of chicks and vitamin A accumulations. Two lots were fed alfalfa A and 0.02% of ethoxyquin.[10] Another lot, not included in the experimental design, was given 400 units of vitamin A and 0.10% of ethoxyquin. Supplementation was at 400 and 800 units of vitamin A/pound of diet (table 3). Again birds getting 800 units of vitamin A/pound significantly outperformed those getting 400 units.

Experiment 3. The supplements, alfalfa meal D (high quality, 190,000 units/pound), alfalfa meal E (low quality, 35,000 units/pound), ANRC vitamin A standard, and USP vitamin A reference solution, were added at 400 and 800 units/pound. To study effect of sex on utilization of vitamin A, each source was fed to males and females separately.

As previously, chicks performed significantly better with 800 units vitamin A than with 400 units (table 4). Males gained significantly more than females, and deposited significantly more vitamin A in the livers. Males as a whole gained considerably more with 800 units of vitamin

[7] Indicates approximate potency; additions to feed based on analysis.

[8] Supplied as NF-180, which contains 50 gm/lb of supplement. Hess and Clark.

[9] Only results not readily interpreted from tables are mentioned in experimental sections.

[10] Santoquin. Monsanto Chemical Company.

TABLE 2

Results (experiment 1)

Lot[1]	Source and level vitamin A/lb feed	Wt gain[2]	Serum vitamin A/100 ml[3]	Liver vitamin A/gm[4]	Liver vitamin A/liver[4]	Feed conversion ratio[5]
		gm	*units*	*units*	*units*	
1	Alfalfa A, high quality, 400 units	771	67	0.59	8	3.0
2	Alfalfa A, high quality, 800 units	819	117	1.7	29	2.9
3	Alfalfa B, low quality, 400 units	725	53	0.79	11	2.9
4	Alfalfa B, low quality, 800 units	806	138	1.7	30	2.7
5	ANRC standard,[6] 400 units	761	89	0.69	12	3.0
6	ANRC standard, 800 units	882	155	3.6	70	2.8
7	Alfalfa A, 400 units + furazolidone, 0.003%	758	71	0.71	12	2.9
8	Alfalfa A, 800 units + furazolidone, 0.003%	848	142	2.4	43	2.9
Analysis of variance:[7]						
	Level	**	**		**	**
	Source	ns	*		ns	ns
	Source × level	ns	ns		ns	ns
Multiple range test:[8]		Serum vitamin A — source				
	ANRC standard	alfalfa A + furazolidone	alfalfa B		alfalfa A	

[1] Single Comb White Leghorn × HyLine, straight run chicks, hatched from eggs of a flock maintained with a low-carotenoid diet. Chicks were grouped by weight and assigned at random, 12 birds/lot. Weights of females adjusted to male-weight basis for statistical analysis.
[2] Eight weeks on experiment, following 6-day depletion period.
[3] Three birds/lot.
[4] Two birds/lot. In this and subsequent tables, only content per whole liver analyzed statistically.
[5] No adjustment for sex distribution in lots.
[6] Animal Nutrition Research Council, see footnote 6 in text.
[7] In this and later tables, * is significance at the 0.05 level; ** is significance at the 0.01 level.
[8] Results underscored by same line are not significantly different at the 0.05 level. Sources are listed from left to right in decreasing order of magnitude of values obtained.

A/pound than with 400 units/pound, but for females the difference was small.

Birds of some lots did not do well from about two to four weeks on experiment; there was evidence of disease, but few birds died, and feed intake did not drop markedly. The condition apparently was a mild respiratory ailment. Growth curves for lots 8, 18, and, to a lesser extent, 16, deviated from trends as expected from other work in this laboratory.

Experiment 4. The supplements were alfalfa D (high quality, 190,000 units/pound), alfalfa F (high quality, 130,000 units/pound), alfalfa G (low quality, 70,000 units/pound), and ANRC vitamin A standard (table 5). Again, significantly higher vitamin A values were observed when 800 units of vitamin A/pound were used, and males outgained females. There were a few death losses, some from lymphomotosis.

Experiment 5. Alfalfa D (high quality meal, 190,000 units/pound) and ANRC vitamin A standard were fed at 1200 units/pound of feed to determine whether differences occurred when using required levels of vitamin A (NRC, '60), levels higher than needed for optimal growth under our conditions (table 6).

INTERPRETATION AND DISCUSSION

Chicks getting 800 units of vitamin A activity/pound of feed gained significantly more than those getting 400 units/pound, and their blood serum and liver vitamin A content was significantly larger. This was expected, since 400 units of vitamin A/pound is below the optimum for growth. Males consistently outgained females, but

TABLE 3

Results (experiment 2)

Lot[1]	Source and level vitamin A/lb feed	Wt gain[2]	Serum vitamin A/100 ml[3]	Liver vitamin A/100 ml[3]	Liver vitamin A/liver[3]	Feed conversion ratio
		gm	*units*	*units*	*units*	
1	Alfalfa A, high quality, 400 units	939	70	1.7	32	3.3
2	Alfalfa A, high quality, 800 units	986	140	4.0	63	3.2
3	Alfalfa A, unrefrigerated,[4] 400 units	862	57	0.81	16	3.3
4	Alfalfa A, unrefrigerated,[4] 800 units	951	118	2.8	44	3.2
5	Alfalfa C, low quality, 400 units	867	60	0.68	10	3.3
6	Alfalfa C, low quality, 800 units	975	158	2.4	39	3.2
7	ANRC standard, 400 units	944	109	1.0	16	3.1
8	ANRC standard, 800 units	977	142	3.9	57	3.1
9	USP solution, 400 units	964	78	1.0	20	3.2
10	USP solution, 800 units	985	162	5.9	91	3.0
11	Alfalfa A, 400 units + ethoxyquin, 0.02%	902	86	1.1	23	3.3
12	Alfalfa A, 800 units + ethoxyquin, 0.02%	960	149	6.2	117	3.0
13[5]	Alfalfa A, 400 units + ethoxyquin, 0.10%	956	81	1.0	19	3.0
Analysis of variance:[6]						
	Level	**	**		**	*
	Source	*	*		**	ns
	Source × level	ns	ns		*	ns

Multiple range tests:[6]

	Source					
Gains	USP solution	alfalfa A	ANRC standard	alfalfa A + ethoxyquin	alfalfa C	alfalfa A unrefrigerated
Serum vitamin A	ANRC standard	USP solution	alfalfa A + ethoxyquin	alfalfa C	alfalfa A	alfalfa A unrefrigerated
Liver vitamin A	alfalfa + ethoxyquin	USP solution	alfalfa A	ANRC standard	alfalfa A	alfalfa C

[1] HyLine males from a hatchery, assigned 12 chicks/lot, as in experiment 1. Two died in lot 3, and one in each of lots 8, 9 and 11.
[2] Eight weeks on experiment, following 14-day depletion period.
[3] Three birds/lot.
[4] Not refrigerated during time used on experiment. Additions to diet based on analysis at the start.
[5] Not included in the statistical analysis.
[6] Same significance levels and symbolism as in table 2.

only in one of three experiments was the liver vitamin A content of males significantly higher. The difference in gain probably was not a direct effect of sex on vitamin A metabolism, but on growth in general. Although female rats may accumulate larger liver vitamin A stores than males (Moore, '57), chicks fed low-to-moderate levels of vitamin A apparently react differently.

Considering the objectives separately, one can best determine general trends and draw further conclusions from this study. The principal objectives were to study relative utilizations of vitamin A from (a) the ANRC standard and USP reference solution; (b) vitamin A standards and high quality alfalfa meal; and (c) high quality and low quality alfalfa meals; and to study the effect of certain additives on

TABLE 4

Results (experiment 3)

Lot[1]	Source and level vitamin A/lb feed	Wt gain[2]	Serum vitamin A/ 100 ml[3]	Liver vitamin A/ gm[3]	Liver vitamin A/ liver[3]	Feed conversion ratio
		gm	*units*	*units*	*units*	
Males						
1	Alfalfa D, high quality, 400 units	643	51	0.75	10	3.2
2	Alfalfa D, high quality, 800 units	742	114	2.8	38	3.1
5	Alfalfa F, high quality, 400 units	700	69	0.97	13	3.0
6	Alfalfa F, high quality, 800 units	749	120	3.5	49	2.9
9	Alfalfa E, low quality, 400 units	664	68	1.0	13	2.9
10	Alfalfa E, low quality, 800 units	779	132	4.5	73	3.1
13	ANRC standard, 400 units	738	72	1.1	14	3.4
14	ANRC standard, 800 units	867	146	5.0	86	2.8
17	USP solution, 400 units	791	78	0.90	13	2.9
18	USP solution, 800 units	671	90	3.3	41	3.2
Females						
3	Alfalfa D, high quality, 400 units	592	62	1.3	18	3.2
4	Alfalfa D, high quality, 800 units	625	83	2.0	25	3.0
7	Alfalfa F, high quality, 400 units	614	53	0.79	9	3.0
8	Alfalfa F, high quality, 800 units	538	119	4.5	53	3.2
11	Alfalfa E, low quality, 400 units	601	48	1.0	12	3.2
12	Alfalfa E, low quality, 800 units	623	95	3.0	32	3.1
15	ANRC standard, 400 units	621	76	1.0	14	3.0
16	ANRC standard, 800 units	631	147	3.5	45	3.2
19	USP solution, 400 units	628	55	0.70	10	3.0
20	USP solution, 800 units	667	132	3.0	38	2.9
Analysis of variance:[4]						
	Level	**	**		**	ns
	Sex	**	ns		**	ns
	Source	**	**		*	ns
	Source × level	**	ns		*	ns
	Sex × level	*	ns		**	ns
	Sex × source	*	ns		ns	ns

Multiple range tests:[4]

		Source			
Gains	ANRC standard	USP solution	alfalfa E	alfalfa D	alfalfa F
Serum vitamin A	ANRC standard	alfalfa F	USP solution	alfalfa E	alfalfa D
Liver vitamin A	ANRC standard	alfalfa E	alfalfa F	USP solution	alfalfa D

		Source × level			
Gains					
400 unit level	USP solution	ANRC standard	alfalfa E	alfalfa F	alfalfa D
800 unit level	ANRC standard	alfalfa E	alfalfa D	USP solution	alfalfa F
Liver vitamin A					
400 unit level	alfalfa D	ANRC standard	alfalfa E	USP solution	alfalfa F

TABLE 4 (*Continued*)

800 unit level	ANRC standard	alfalfa E	alfalfa F	USP solution	alfalfa D
Gains		Sex × source			
Males	ANRC standard	USP solution	alfalfa F	alfalfa E	alfalfa D
Females	USP solution	ANRC standard	alfalfa E	alfalfa D	alfalfa F

[1] Same type chicks as in experiment 1, 12 chicks/lot. Lost one in lot 10 (accident), one in lot 11, two in lot 13 (illness not definitely determined), one in lot 14 (sexed incorrectly).
[2] Eight weeks on experiment, following depletion for 7 days.
[3] Four birds/lot, except one liver determination lost in lot 12.
[4] Same significance level and symbolism as in table 2.

TABLE 5

Results (experiment 4)

Lot[1]	Source and level vitamin A/lb feed	Wt gain[2]	Serum vitamin A/100 ml[3]	Liver vitamin A/gm[3]	Liver vitamin A/liver[3]
		gm	*units*	*units*	*units*
Males					
1	Alfalfa D, high quality, 400 units	736	67	0.92	15
2	Alfalfa D, high quality, 800 units	779	151	2.4	42
5	Alfalfa F, high quality, 400 units	742	31[4]	0.65	13
6	Alfalfa F, high quality, 800 units	804	138	2.7	49
9	Alfalfa G, low quality, 400 units	724	61	1.2	20
10	Alfalfa G, low quality, 800 units	762	155	4.4	71
13	ANRC standard, 400 units	748	80	1.5	27
14	ANRC standard, 800 units	774	144	4.6	67
Females					
3	Alfalfa D, high quality, 400 units	586	76	1.0	13
4	Alfalfa D, high quality, 800 units	644	122	3.4	48
7	Alfalfa F, high quality, 400 units	637	74	1.3	21
8	Alfalfa F, high quality, 800 units	648	183	3.0	46
11	Alfalfa G, low quality, 400 units	552	43	0.92	14
12	Alfalfa G, low quality, 800 units	641	121	3.3	45
15	ANRC standard, 400 units	606	72	1.0	16
16	ANRC standard, 800 units	659	153	4.6	68
Analysis of variance:[5]					
	Level	**	**		**
	Sex	**	ns		ns
	Source	*	ns		ns
	Source × level	ns	ns		ns
	Sex × level	ns	ns		ns
	Sex × source	ns	ns		ns

Multiple range test:[5]	Source			
Gains	alfalfa F	ANRC standard	alfalfa D	alfalfa G

[1] Ten chicks/lot, from same source as in experiment 3. One chick lost in each of lots 1, 10 and 14; two lost in each of lots 3, 8 and 16; and three lost in lot 15; causes not positively identified, except one in each of lots 8 and 16 had lymphomotosis.
[2] Eight weeks on experiment, following 6-day depletion period.
[3] Four birds/lot.
[4] One serum sample contained no measurable vitamin A.
[5] Same significance levels and symbolism as in table 2.

TABLE 6

Results (experiment 5)

Lot[1]	Source and level vitamin A/lb feed	Wt gain[2]	Serum vitamin A/ 100 ml[3]	Liver vitamin A/ gm[3]	Liver vitamin A/liver[3]	Feed conversion ratio
		gm	*units*	*units*	*units*	
Males						
1	Alfalfa D, high quality, 1,200 units	854	176	7.9	118	2.7
3	ANRC standard, 1,200 units	841	221	12.8	188	2.8
Females						
2	Alfalfa D, high quality, 1,200 units	662	178	11.2	149	3.2
4	ANRC standard, 1,200 units	643	186	9.2	125	2.9
Analysis of variance:						
	Sex	**	ns		ns	
	Source	ns	ns		ns	
	Sex × source	ns	ns		ns	

[1] Chicks of same source and condition as in experiment 3, 12 birds/lot. No losses; one in lot 4 incorrectly sexed.
[2] Eight weeks on experiment, following 7 days depletion.
[3] Four birds/lot.

utilization of the vitamin A activity of alfalfa meal. Evaluation is based mainly on weight gains of depleted chicks fed vitamin A or provitamin A during an 8-week test period; other data are regarded principally as supporting information. Poor growth is one of the most striking signs of vitamin A deficiency.

Rosenberg ('42, p. 85) pointed out that there are no quantitative data to indicate what should be the minimal vitamin A liver storage of any species. If liver storage is to be the measure of responses, one needs to select arbitrarily the vitamin A level to be attained and to vary levels and conditions of supplementation trying to achieve that storage — a difficult experimental procedure. A similar case could be made for serum vitamin A.

Supplementation at 400 and 800 units/pound of feed was chosen because the former is in the critical range as related to growth, and the latter is near that required for optimal growth and for moderate liver vitamin A storage.

Utilization of vitamin A from the ANRC standard and USP reference solution was compared in experiments 2 and 3. In neither experiment was there a significant difference in weight gain at either supplementation level. This was supported in experiment 2 by nonsignificant differences in serum and liver vitamin A data. In experiment 3, however, serum and liver vitamin A were higher when the ANRC standard was given. Chicks getting the USP reference solution were among those possibly affected by an unidentified low-order disease, which may have affected results somewhat. In experiment 3, males had significantly larger weight gains and liver vitamin A storage than females when 800 units, but not 400 units, of ANRC standard were used. These observations suggest no essential differences in utilization of these vitamin A sources for growth, but for vitamin A storage there was possibly a small, but not consistent, advantage from the ANRC standard.

Few published reports compare the reference standards[11] in bioassays — none under conditions comparable to this study. Ames ('61), using a single-dose oral supplementation technique, reported similar liver storage of vitamin A when the ANRC standard and USP reference solution were given. In Ames' paper, Bird reports that the ANRC standard appeared somewhat superior for vitamin A liver storage but not for growth, and Scott reports that the two vitamin A sources produced comparable liver vitamin A storage. The ANRC standard is easier to use, but since it is a dry powder, it requires careful mixing, especially for low-level supplementation. On the other hand, the lack of stability of vitamin A in an oil carrier (Olsen et al., '59) is a problem with the USP reference

[11] Term used hereafter for both USP and ANRC products.

solution. Either standard appears suitable when properly handled.

In experiments 1–5, high quality alfalfa meals were compared with USP or ANRC standards as sources of vitamin A activity. Supplementation in experiments 1–4 was at low and critical levels. Only in experiment 3 was gain with a standard significantly better than with high quality alfalfa. This difference means less than it otherwise might, because gain was not better with one standard than with low quality alfalfa, and possible disease effect, previously mentioned, is not known. In experiments 1 and 3, serum vitamin A levels supported gain data. Liver vitamin A storage was not significantly higher from a standard than from high quality alfalfa meals, except at the 800-unit supplementation level in experiment 3.

Using 1200 units of vitamin A from alfalfa meal and the ANRC standard caused no significant differences, allaying the doubt that alfalfa meal would be inferior to true vitamin A at the NRC requirement level.

Vitamin A was utilized about as well from low quality as from high quality alfalfa meals. In experiment 4 gain was significantly better with one high quality meal, but there were no significant differences in liver or serum vitamin A content.

Thus, within the range of supplementation levels fed, chicks utilized carotene effectively from these alfalfa meals, which contained up to 28% of fiber and from 35,000 to 200,000 units of vitamin A/pound.

Results on utilization of vitamin A activity from true vitamin A and alfalfa meals, using modern feeds and standards, agree with earlier reports by Wilson et al.[12] Record et al. ('37), the later report by Kramke et al. ('52), the conclusion of Petering et al. ('41), and the position of the NRC ('60) subcommittee on poultry nutrition. Using 2270 units of vitamin A/pound of feed from fish oil and from alfalfa meal, Ascarelli and Bondi ('57) did not find significant differences in liver vitamin A storage.

If the objective of feeding chicks is to obtain high liver vitamin A storage, preformed and stabilized vitamin A appear to be most effective under certain conditions (Ely, '59; Olsen et al., '59; Williams, '62). But as Rosenberg pointed out in 1942, there are no quantitative data to indicate what should be the minimal vitamin A storage in the liver. Except possibly for special conditions such as infection (Squibb, '61; Erasmus et al., '60), there is no convincing evidence that the situation is different today.

Unlike liver vitamin A storage, growth and survival (Olsen et al., '59), and growth and feed conversion (Ely, '59) were similar when vitamin A was supplied by stabilized vitamin A and alfalfa meal. However, Ely's vitamin A supplementation was so much above requirements that differences in growth would not be expected.

In view of results of this study, and evidence from the literature, for growth chicks appear to utilize equal units of vitamin A activity of alfalfa meal and vitamin A sources to a similar degree. But under certain conditions, storage of vitamin A by the chick may be somewhat higher if vitamin A per se is fed. In practice, one needs to decide the reason vitamin A is to be fed, and the evidence concerning type and level of supplementation needed for that purpose.

Utilization, the subject of this study, must not be confused with stability. Some earlier studies of utilization of vitamin A sources are difficult to interpret because of stability factors.

Furazolidone did not significantly affect utilization of provitamin A of alfalfa meal for growth, or for liver and blood vitamin A storage. Use of 0.02% of ethoxyquin did not improve gain or vitamin A storage significantly. However, the higher level of ethoxyquin (0.10%) improved gain, but not blood and liver vitamin A storage.

SUMMARY

A comparison was made of utilization of vitamin A activity of the ANRC vitamin A standard, USP reference solution and low and high quality alfalfa meals. Effect of furazolidone and ethoxyquin on utilization of vitamin A activity of alfalfa meal also was studied. Chicks were depleted of vitamin A reserves, fed 400 and 800 IU (4 ex-

[12] See footnote 4.

periments) or 1200 IU of vitamin A (one experiment)/pound of diet for 8 weeks.

Chicks given 800 units of vitamin A gained more and their blood and liver vitamin A content was larger than that of chicks given 400 units of vitamin A. Weight gain of males was superior to that of females.

Gains were similar when the ANRC vitamin A standard and USP reference solutions were used as sources of vitamin A. In one of the two experiments, liver and blood serum vitamin A content was higher when the ANRC standard was used.

In three of 4 experiments, gain of chicks fed high quality alfalfa meal at 400 and 800 units of vitamin A/pound of diet was not significantly different from that of chicks given a vitamin A standard. There were no differences when 1200 units of vitamin A/pound of diet were used. Serum vitamin A was significantly higher in only two experiments, and liver vitamin A in one, when chicks received a vitamin A standard. Vitamin A was utilized about as well from low quality as from high quality alfalfa meals.

Furazolidone did not affect utilization of provitamin A of alfalfa meal. Ethoxyquin at 0.02% of diet did not significantly affect gain, or serum and liver vitamin A levels; gains were improved when 0.10% of ethoxyquin was used.

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Effect of Zinc on Bone Alkaline Phosphatase in Turkey Poults [1]

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Recent studies in the nutrition of poultry (O'Dell and Savage, '57; Morrison and Sarett, '58; Supplee et al., '58; Kratzer et al., '58) have demonstrated a relatively high requirement for zinc. Deficiency symptoms are characterized by marked reduction in growth, ragged and brittle feathers, and shortening and thickening of the long bones. Although much work has been carried out concerning the zinc requirement and the effects of zinc deficiency, little information is available concerning the relationship of enzymes to the deficiency syndrome.

Robison ('23) observed that ossifying cartilage of young rats and rabbits contains a very active phosphatase. The enzyme, alkaline phosphatase, is carried by the osteoblasts and is present in highest concentration where new bone is being formed (Ham, '53). That alkaline phosphatase is involved in bone formation has been well established (Bourne, '56); however, the exact process is not understood.

Wilgus et al. ('36) showed that perosis, an anatomical deformity of the leg bones of young chickens (evidenced by a slipped tendon), is associated with a low intake of manganese. Wiese et al. ('39) observed that the bone alkaline phosphatase activity of perotic chickens is lower than that of birds receiving adequate manganese in the diet. The interrelationship of manganese, alkaline phosphatase and perosis is not known.

Mathies ('58) studied a highly purified enzyme preparation from swine kidney and found that alkaline phosphatase is a metalloenzyme containing approximately 0.15% of zinc. No other metal could replace zinc as part of the active enzyme.

Since alkaline phosphatase has been shown to have a role in bone formation and is a zinc metalloenzyme, it appears possible that a dietary zinc deficiency resulting in abnormal bone formation, might result in a corresponding decrease in bone phosphatase activity.

This report describes experiments in which a highly significant reduction in bone alkaline phosphatase activity was noted in zinc-deficient turkeys.

EXPERIMENTAL

Broad Breasted Bronze poults were fed a practical stock diet for 5 days before being fed the experimental diets (table 1). Zinc was added to the basal diet at levels of 7, 10, 15, 30, 60 and 100 ppm as zinc oxide (table 2). The birds were weighed, banded, and divided at random into groups of 10 poults (5 males and 5 females). They were housed in electrically heated, galvanized batteries and provided with tap water and feed ad libitum. In the first experiment the cages were uncoated. In experiments 2 to 6 the cages, feeders, and waters were coated with an epoxy resin to lower the amount of zinc available from the environment. The birds were raised for 17 days with the experimental diets; at the end of this period they were killed. The legs were removed and stored in a freezer at $-10°C$ until the enzyme assays were made. The entire leg was stored to reduce drying and loss of enzyme activity.

At the time of assay the legs were removed from the freezer, and the tibias separated at the femur-tibio and tibio-metatarsus joints, and cleaned of all muscle and periostium. The cleaned bones were weighed, split into small fragments,

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and water was added in a ratio of 10:1. Four drops of chloroform were added for each 10 ml of mixture. The flask containing the mixture was stoppered and the mixture allowed to autolyze at room temperature for 8 days with daily inversions. The enzyme assay method was a modification of the procedure described in Colowick and Kaplan ('57). The buffer-substrate was prepared from 1 M buffer, pH 10, from 4.45 gm of 2-amino-2-methyl-1-propanol plus 22.5 ml of 1 N HCl, diluted to 50 ml with water which was added to 210 mg of *p*-nitrophenylphosphate in 50 ml of water, with 0.2 ml of 1 M $MgCl_2$ added to the final solution.

The enzyme preparation was strained through 4 layers of cheese cloth and diluted 1:100 for the assay. One-tenth milliliter of the dilute enzyme solution and 0.15 ml of chloroform-water were added to test tubes and placed in an ice bath. To each tube, 0.75 ml of buffer-substrate was added rapidly with an automatic syringe, and the entire rack transferred to a 38°C water bath at zero time. After 30 minutes the rack was returned to the ice bath, and 2.25

TABLE 1

Experimental diet deficient in zinc

	gm/kg
Cornstarch	425
Isolated soy protein[1]	330
Cellulose, powdered[2]	50
Mineral mixture[3]	63
Vitamin mixture[4]	20
Soybean oil, degummed	35
$CaHPO_4$	30
$CaCO_3$	25
DL-Methionine	4.5
Choline chloride[5]	10
Erythromycin	1
Inositol	1
BHT[6]	1
Vitamin A (10,000 IU/gm)	0.5
Vitamin D (1,500 IU/gm)	3
Vitamin E (44 mg/gm)	2
Biotin	0.4 *mg/kg*
Vitamin B_{12}	10.0 *μg/kg*

[1] ADM C-1, Assay Protein, Archer Daniels Midland Company, Cincinnati, Ohio.
[2] Solka Floc, Brown Company, New Hampshire.
[3] Minerals were supplied in mg/kg of diet as follows: NaCl (uniodized), 10.0; $MnSO_4 \cdot H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.65; $CuSO_4 \cdot 5H_2O$, 0.08; $Cu(Ac)_2 \cdot 4H_2O$, 0.02; KI, 0.009; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.25; $MgSO_4 \cdot 7H_2O$, 4.0; KCl, 3.0; K_2HPO_4, 5.0; $Na_2MoO_4 \cdot 2H_2O$, 0.009.
[4] Vitamins were supplied in mg/kg of diet as follows: riboflavin, 10; thiamine·HCl, 10; pyridoxine·HCl, 10; Ca pantothenate, 30; niacin, 120; folic acid, 5; menadione, 10.
[5] Twenty-five per cent in wheat middling carrier.
[6] Butylated hydroxytoluene.

TABLE 2

Effect of graded amounts of zinc added to the basal diet on growth, perosis, and mortality in turkey poults

Added zinc	Exp. 1[1]			Exp. 2			Exp. 3			Exp. 4			Exp. 5			Exp. 6		
	Gain[2]	Perosis score[3]	Mortality[4]	Gain	Perosis score	Mortality	Gain	Perosis score	Mortality	Gain	Perosis score	Mortality	Gain	Perosis score	Mortality	Gain	Perosis score	Mortality
ppm	*gm*			*gm*			*gm*			*gm*			*gm*			*gm*		
0	106	2.2	10/10	54	0.6	5/10	61	0.6	10/10	33	0.4	9/10	31	0.4	9/18	36	0.6	6/10
7		—		109	1.1	10/10	99	1.3	10/10	79	0.9	10/10	57	0.4	5/9		—	
10	232	3.3	10/10		—			—			—			—			—	
15		—		181	2.0	10/10	150	2.3	10/10	161	1.5	10/10	156	1.8	8/9		—	
30				220	1.0	8/10	195	1.5	10/10	223	1.6	10/10	242	1.8	9/9		—	
60	315	0.1	10/10	270	0.6	10/10	236	0.2	10/10	237	0.1	10/10	280	0.0	9/9		—	
60[5]		—			—			—			—		42	0.0	5/9		—	
100		—			—			—			—			—		290	0.0	8/10

[1] Birds in experiment one were raised in uncoated cages.
[2] Mean gain of survivors.
[3] Perosis scores, 0 = normal, 4 = severe.
[4] Survivors over the initial number.
[5] Poults fed a normal diet but restricted to the amount of feed consumed by a group fed a zinc-deficient ration.

TABLE 3

Effect of graded levels of zinc on the specific activity of bone alkaline phosphatase from the tibia of turkey poults

Added zinc	Alkaline phosphatase activity[1]				
	Exp. 1[2]	Exp. 2	Exp. 3	Exp. 4	Exp. 5
ppm					
0	113 ± 19[3]	55 ± 13	38 ± 5	32 ± 8	40 ± 8
7	—	74 ± 6	43 ± 8	29 ± 5	60 ± 19
10	166 ± 26	—	—	—	—
15	—	118 ± 15	67 ± 4	55 ± 10	106 ± 26
30	—	160 ± 33	105 ± 26	134 ± 31	137 ± 23
60	441 ± 12	329 ± 25	132 ± 17	259 ± 41	272 ± 28
60[4]	—	—	—	—	120 ± 20

[1] Activity expressed as optical density units per milligram of protein per hour.
[2] Birds in experiment one were raised in uncoated cages.
[3] Mean and SE of the mean.
[4] Poults fed a normal diet but restricted to the amount of feed consumed by a group fed a zinc-deficient ration.

ml of 0.25 N sodium hydroxide were added rapidly to each tube to stop the reaction. Blanks containing chloroform-water were carried throughout the entire procedure. Optical density was measured on a Beckman DU spectrophotometer at 410 mμ within 6 hours. Also, protein was measured at 260 and 280 mμ. Enzyme activity was expressed as units of optical density per milligram of protein per hour.

Bone ash was determined on tibias from birds in experiment 4. The bones were cleaned as described previously. Fat was extracted with diethyl ether, and the bones were dried in an oven to remove moisture and residual solvent. They were then heated in a muffle furnace until completely ashed.

To show the effect of zinc deficiency on the distribution of the enzyme, the bones were sectioned into thirds by length and the assay run on each section as described previously.

A paired-feeding trial was conducted to determine whether the loss in body weight in zinc-deficient birds influenced the activity of phosphatase. The amount of feed consumed by the zinc-deficient birds on any one day was weighed, and that same amount of feed containing 60 ppm of added zinc was given to the restricted birds on the following day.

RESULTS AND DISCUSSION

A marked reduction in growth, poor feathering, and perosis was observed in poults receiving less than 60 ppm of added zinc in their diet (table 2). High mortality was noted in the basal groups in 3 of the 6 trials. Lesions were observed at the corners of the mouth in birds fed the basal diet with no added zinc.

The specific activity of alkaline phosphatase at different levels of dietary zinc is shown in table 3. The difference in bone enzymatic activity between the birds fed no added zinc and those fed diets containing the 60 ppm of added zinc was statistically highly significant ($P < 0.001$). Greater enzyme activity was noted in poults fed 100 ppm of zinc than in those receiving 60 ppm of zinc (table 4), although the two levels of zinc were compared with the deficient birds in separate experiments. A correlation of 0.99 existed

TABLE 4

Distribution of alkaline phosphatase in the tibia(s) of poults fed high and low levels of dietary zinc

Added zinc	Alkaline phosphatase activity[1]		
	Bone section		
	Proximal	Middle	Distal
ppm			
0	18	95	35
0	33	80	55
0	16	39	19
0	37	88	39
0	41	72	53
0	22	70	63
60	220	49	193
60	174	87	172
60	133	109	161
100	2,765	391	258
100	1,122	187	—
100	844	499	579

[1] Activity expressed as optical density units per milligram of protein per hour.

between enzymatic activity and dietary zinc.

The effect of zinc deficiency on the distribution of alkaline phosphatase of individual poults is shown in table 4. Since alkaline phosphatase activity is highest where new bone is being formed, one would expect the enzymatic activity to be highest at the epiphyseal ends of the bones. This has been shown true in both manganese-deficient and normal chickens (Wiese et al., '41). In poults, however, there was a marked difference in the pattern of enzyme activity in bones from the zinc-deficient birds and in bones from normal birds. In bones from zinc-deficient birds, highest activity was observed in the diaphyseal section, whereas in normal bones the highest activity was at the epiphyseal ends of the bones. This result may have been due to alkaline phosphatase that was present in osteoblasts when the bones were young and the birds were receiving adequate zinc. It is possible that these osteoblasts became enclosed in bone substance as the bones grew older, yet still retained their phosphatase activity. Then, when the birds were transferred from the stock diet to zinc-deficient diets, the phosphatase embedded in bone was not affected. However, the new phosphatase being formed at the ends of the bones reflected the absence of dietary zinc with a loss in enzymatic activity.

When the food intake of poults fed the diet containing 60 ppm of added zinc was limited to the same amount of food ingested by the poults fed the zinc-deficient diet, weight gains were approximately the same (exp. 5, table 2). No symptoms of zinc deficiency, such as poor feathering or perosis, were observed in the feed-restricted poults. Alkaline phosphatase activity of poults fed the restricted diet (table 3, exp. 5) was approximately equal to that of birds receiving diets containing 30 ppm of added zinc. Enzyme activity of poults fed the zinc-deficient basal diet was significantly lower than the activity of the restricted birds ($P < 0.01$). It may be concluded that the reduction in alkaline phosphatase activity in zinc-deficient poults is not due to a general lowering of food intake and subsequent weight loss, but more specifically to an inadequate amount of available zinc.

TABLE 5

Effect of addition of zinc on percentage of bone ash[1]

Added zinc	Ash
ppm	%
0	46.60 ± 2.82[2]
7	46.42 ± 0.53
15	46.03 ± 1.48
30	45.73 ± 0.54
60	45.95 ± 1.00

[1] Bone ash was calculated from the right tibia of birds from experiment 4.
[2] Mean and SE of the mean.

Bone ash was determined from the right legs of the birds in experiment 4 (table 5). No significant differences were found among groups supplied with different levels of dietary zinc. This confirms the observations of Hunt and Blaylock ('53) and Kratzer et al. ('58).

If the role of alkaline phosphatase in bone formation is the precipitation of bone salt as proposed by Robison ('23), one might assume that a severe reduction in phosphatase activity also would reduce bone calcification. Data presented here are not in agreement with this assumption. Poults with a 50% reduction in phosphatase activity had the same bone ash values as the normal birds fed the control diet, indicating that phosphatase is not involved in the mineral deposition of the bone.

From the experimental evidence it was concluded that a dietary zinc deficiency in turkey poults results in a corresponding reduction in alkaline phosphatase activity. The significance of the reduction as related to bone formation and perosis is difficult to determine, since the precise role of alkaline phosphatase in bone formation, and the physiological changes that occur in perosis, have not been elucidated.

SUMMARY

Turkey poults were maintained for 17 days with a purified diet containing isolated soybean protein, supplemented with varying levels of zinc. Poults receiving diets containing less than 60 ppm of added zinc developed deficiency symptoms, including a severe reduction in growth, shortening and thickening of the long bones, and poor feathering.

Alkaline phosphatase activity was severely reduced in the tibia of zinc-deficient poults. A correlation of 0.99 was found between enzyme activity and the level of dietary zinc. Bones from normal birds had highest alkaline phosphatase activity in the epiphyseal sections, whereas the diaphyseal section of the zinc-deficient bones contained highest activity.

Tibias from poults fed varying levels of dietary zinc showed no significant differences in bone ash.

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Influence of Timing of Short-term Supplementation During Gestation on Congenital Abnormalities of Manganese-deficient Rats[1]

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The offspring of manganese-deficient rats exhibit a number of abnormalities, among which are (1) high neonatal mortality (Hurley et al., '58); (2) congenital irreversible ataxia (Hurley et al., '58); (3) delayed development of body-righting reflexes (Hurley and Everson, '59); (4) disproportionate growth of the skull and long bones (Hurley et al., '61a, b); and (5) a localized dysplasia of the proximal epiphysis of the tibia (Hurley and Asling, '63). In addition, abnormal development of the inner ear has been found (Hurley et al., '60; Asling et al., '60).[2]

We have shown previously that congenital ataxia, which is characterized by incoordination, lack of equilibrium, and a pronounced retraction of the head, could be prevented by giving manganese-deficient females a manganese-supplemented control ration continuously from the fourteenth day of gestation. However, if continuous supplementation was not begun until the eighteenth day of gestation, the young were indistinguishable from those of totally deficient females with respect to both ataxia and survival, even though they had received manganese continuously to 28 days of age. These studies showed that the ataxia resulted from a congenital defect or defects which occurred between the fourteenth and eighteenth days of gestation (Hurley et al., '58).

In the experiments to be reported here, the effects of short-term supplementation during pregnancy were studied in an attempt to differentiate these various abnormalities of manganese-deficient offspring in terms of critical periods during gestation.

METHODS

The general procedures and diets used have been reported previously in detail (Hurley et al., '60). Female rats of the Sprague-Dawley strain were maintained from the time of weaning with a fortified milk diet deficient in manganese. Control animals were treated in the same way, except that manganese was added to the diet.[3] At maturity, the females were mated with normal males receiving a stock ration, and the resulting young were studied. Pregnancies were timed by observation of vaginal smears; the day of finding sperm was considered day 0 of gestation. Three groups of deficient females were given the manganese-supplemented control ration for a period of 24 hours on day 14, day 16, or day 18 of gestation, respectively. Other groups received either the manganese-deficient or the manganese-supplemented ration throughout the experiment.

The development of body-righting reflexes was studied by testing the ability of the young to right themselves in air. The rats were tested at 18 and at 28 days of age by dropping them, dorsal surface down, from a height of 12 inches onto a soft, cotton-filled pad. The response was considered positive if the animal landed on all 4 feet in two out of three trials.

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[2] Hurley, L. S., E. Wooten, G. J. Everson and C. W. Asling 1960 Anomalous development of the inner ear in offspring of manganese-deficient rats. Federation Proc., *19:* 327 (abstract).

[3] We are indebted to Merck Sharp and Dohme, Inc., Rahway, New Jersey, and to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for supplies of vitamin B_{12} and ascorbic acid.

Measurements of crown-rump length and tibia length were made with vernier calipers from specimens cleared and stained with alizarin red S, according to the method of Wright et al. ('58). In measuring the tibia, only diaphyseal length was used. For studies of the proximal tibial epiphysis, alizarin-stained specimens of the knee joint were split sagittally and restained.

RESULTS

The effects of short-term supplementation on survival and incidence of ataxia in offspring of manganese-deficient rats are summarized in table 1. In offspring of females receiving manganese throughout the experimental period, 54% of the young born alive survived to 28 days of age, and none showed ataxia. Only 11% of the live young born to unsupplemented females survived to this age, and 81% were ataxic. Supplementation with manganese on day 14 of gestation resulted in nearly normal survival of the offspring (51%) and completely prevented ataxia. Nearly normal survival was also observed in young of females supplemented on day 16, but in this group, 62% of the survivors were ataxic. When females received manganese on day 18 of gestation, 39% of their offspring were alive at 28 days of age, and 84% of these were ataxic. Thus, supplementation on day 18 improved survival substantially as compared with the unsupplemented group without affecting the incidence of ataxia, whereas supplementation on day 16 produced normal survival with a high incidence of ataxia.

Body weights at birth and at weaning (28 days of age) are also summarized in table 1. One day of supplementation during gestation had only a slight effect on body weights, and did not correlate with incidence of ataxia.

The response of the rats to the air-righting test is shown in table 1. All of the manganese-positive controls showed a positive response at 18 days of age, whereas only one-half of the unsupplemented animals reacted positively at this time. At 28 days of age, 36% of the unsupplemented rats were still negative in response. In offspring of deficient females supplemented on day 14 of gestation, 22% were unable to right themselves in air at 18 days of age, but all showed a positive response by 28 days. The group supplemented on day 18 of gestation was even more severely affected than the unsupplemented animals, with over 80% negative at both ages.

Body lengths, tibia lengths, and the incidence of tibial epiphyseal abnormalities are summarized in table 2. No differences were apparent in the length of the tibia relative to body length between the groups given the 24-hour supplement and the manganese-positive controls, although the offspring of unsupplemented animals showed a shortening of this bone.

All of the unsupplemented animals showed abnormalities of the proximal tibial epiphysis. Perforations of the cartilage plate consisting of bony spicules connecting epiphysis and diaphysis were seen in a high proportion of these rats (12/14); one-half of them (7/14) exhibited an extreme "pocketed" condition, in which

TABLE 1

Effect of short-term supplementation on offspring of manganese-deficient rats

Group	No. of litters	Young at birth		Young at 28 days			Response to air-righting test[1]			
							18 days of age		28 days of age	
		No. born alive	Birth weight	Survival of live young	Body weight	Ataxic	No. tested	Negative	No. tested	Negative
			gm	%	*gm*	%		%		%
Mn+	61	406	6.0	54	62	0	174	0	—	0
Mn−	30	191	5.2	11	40	81	60	53	56	36
Ration supplemented for 24 hours during gestation										
Day:										
14	23	172	5.6	51	48	0	90	22	74	0
16	29	186	5.7	51	52	62	89	71	65	35
18	36	220	5.5	39	45	84	89	84	70	83

[1] Includes litters in addition to those tested for survival.

TABLE 2

Effect of short-term supplementation on bones of manganese-deficient offspring at 28 days of age

Group	Tibia and body lengths					Incidence of tibial epiphyseal abnormalities					
	No. of litters	No. of rats	Body length[1]	Body length[2]	Tibia/body length × 100	No. of litters	No. of rats	Abnormal No.	Abnormal %	No. perforated	No. "pocketed"
			mm	*mm*							
Mn+	17	29	96	18.9	19.8	23	45	2	4	0	0
Mn−	5	8	89	16.1	18.4	10	14	14	100	12	7
Ration supplemented for 24 hours during gestation											
Day:											
14	11	13	87	17.1	19.6	8	14	9	64	0	0
16	13	15	91	17.6	19.4	11	15	12	70	3	0
18	10	22	84	17.0	20.3	15	30	25	83	3	0

[1] Crown-rump length.
[2] Diaphyseal length.

the epiphysis was small, incompletely expanded, deeply sunken into a pocket in the metaphysis, and partially fused with it, so that when viewed from the external surface, the epiphysis appeared to be absent. In addition, reduced size and number of bony trabeculae were noted, as well as lack of development of the bony rim of the epiphysis (Hurley and Asling, '63). Deficient animals that did not show this extreme "pocketed" condition or the bony perforations of the plate nevertheless had abnormal epiphyses, with flat, narrow plates, lack of ventral expansion and poor bony trabeculation.

In none of the groups receiving the 24-hour supplement was the extreme condition of "pocketing" observed, and only a few rats showed perforations. The total incidence of epiphyseal abnormalities was considerably lower in these supplemented groups than in the unsupplemented, ranging from 64 to 83% in the former, as compared with 100% in the latter.

DISCUSSION

Data presented here confirm the previously reported observations on the effect of a maternal dietary deficiency of manganese in rats on the survival and incidence of ataxia in the offspring (Hurley et al., '58). In addition, these data show that one day of supplementation was sufficient to prevent ataxia if manganese was given on day 14, but not if it was given on day 16 or day 18. The previous conclusion is thus substantiated that the critical period for the prevention of ataxia in offspring of manganese-deficient rats is about days 15 and 16 of gestation. Furthermore, these data rule out the possibility that in the previous experiments the preventive effect noted in animals receiving the supplement continuously from the fourteenth day of gestation was due to the greater total amount of manganese received by them in comparison with the females whose supplementation was begun on the eighteenth day; in the present experiments, the total amount of manganese received was the same for all groups — if any differences occurred, in fact, the rats supplemented on day 18 were more likely to have received a larger amount of manganese since they presumably had a higher food consumption.

Post-natal mortality of the offspring, however, was significantly decreased by supplementation on days 16 and 18 as well as on day 14, although ataxia was prevented only in the group supplemented on day 14. The observations on body weights of the young, both at birth and at weaning (28 days), indicate that the influence of the supplementation on survival or on ataxia was not due to a generalized change affecting overall body size or growth. These results suggest that post-natal mortality and ataxia in manganese-deficient young develop through different congenital pathways. The basic biochemical role (or roles) of the element remains unknown, and may be the same throughout development, but specific or even localized tissue needs may change as various structural and enzymatic systems develop

in the embryo. Thus, the events arising from a deficiency of manganese at one stage of development may lead to ataxia, whereas at another stage a different series of events may result in post-natal death.

The results of the air-righting test confirm the previous finding of a delay in the development of body-righting reflexes in offspring of manganese-deficient rats (Hurley and Everson, '59). In addition, they show that supplementation on days 16 or 18 of gestation did not prevent this delay. In the group supplemented on day 18 of gestation, a higher percentage of animals showed a negative response than in the unsupplemented group; this difference may be due to the greatly increased survival of the supplemented animals, which would allow the defect in righting reflexes to manifest itself. Since survival in the unsupplemented group was so low, presumably only the least affected animals were able to survive.

The bone studies reported here confirm the previous report of disproportionate shortening of the tibia in offspring of manganese-deficient rats (Hurley et al., '61a). In addition, they indicate that one day of supplementation during gestation was sufficient to prevent disproportionate growth in this bone, at least as seen at 28 days of age. One day of supplementation also appeared to have a mitigating effect upon the incidence of tibial epiphyseal abnormalities, although it did not prevent them entirely. These results do not permit the assignment of a critical period for the development of the epiphyseal anomaly, but they suggest that if such a period exists, it occurs earlier than day 14 of gestation.

The supplementation procedures described may prove useful in future studies to distinguish between the consequences to an animal of manganese deficiency itself (that is, reduced manganese levels in the body) in contrast to the effects of a congenital abnormality which resulted from a deficiency of manganese during embryonic development.

SUMMARY

The effects of short-term supplementation with manganese during pregnancy were studied in an attempt to differentiate various abnormalities of manganese-deficient offspring in terms of critical periods during gestation. Three groups of manganese-deficient female rats were given a manganese-supplemented ration for a period of 24 hours on day 14, day 16, or day 18. Control groups received either no supplementation or the manganese-supplemented ration throughout the study.

Deficient females supplemented with manganese for 24 hours on day 14 gave birth to young whose survival to 28 days approached that of normal controls; none of these young were ataxic. In contrast, only 11% of the offspring of unsupplemented rats survived to this age, and 81% were ataxic. Supplementation on day 16 also resulted in a survival rate in the young comparable to that of the controls, but 67% of the survivors were severely ataxic. Supplementation on day 18 improved survival to 39%, with a high incidence (84%) of ataxia. Similar differences were observed in results of body-righting tests.

Measurements of body and tibia lengths showed that one day of supplementation during gestation was sufficient to prevent the disproportionate tibial shortening observed in manganese-deficient young. One day of supplementation also appeared to have a mitigating effect upon the incidence of tibial epiphyseal abnormalities, although it did not prevent them entirely.

The results show that one day of supplementation, if it occurred on day 14, was sufficient to prevent ataxia in the young, but not when given on days 16 or 18, and in addition suggest that the post-natal mortality and ataxia of manganese-deficient young develop through different congenital pathways.

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Effect of Acute Amino Acid Deficiencies on Carcass Composition and Pancreatic Function in the Force-fed Rat

I. DEFICIENCIES OF HISTIDINE, METHIONINE, PHENYLALANINE AND THREONINE[1,2]

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Many reports have shown changes produced in the activity of a wide variety of enzymes from many animal tissues and organs as a result of single amino acid deficiencies. Few reports, however, have been concerned with the effects such deficiencies may have on the actual secretion and regeneration of the individual digestive enzymes secreted by the pancreas.

Considerable evidence indicates that when diets low in good quality protein or lacking in tryptophan are fed to rats, atrophy and loss of cytoplasm and degranulation of the pancreatic acinar cells result (Friedman and Friedman, '46; Cole and Scott, '54). Experiments by Sidransky and Farber ('58a) demonstrated that deficiencies of threonine, methionine and histidine, induced by force-feeding diets devoid of the amino acids for as short a time as three days, produced similar morphological changes in the pancreas. Magee and Anderson ('55), using the enzyme content of the resting pancreas as a criterion of secretion, suggested that certain low-quality proteins may lack those amino acid residues necessary to stimulate pancreatic enzyme secretion. A later publication (Hong and Magee, '57) reported that 1% of threonine in a low-protein diet depressed all pancreatic enzyme activities (i.e., suppressed secretion), whereas omission of the amino acid from the diet increased activities of all the pancreatic enzymes, particularly that of the proteolytic enzymes.

Inasmuch as the above mentioned experiments were conducted on the resting pancreas, it is not known whether the changes in enzyme activity were the result of changes in synthesis of new enzyme protein or of excessive or depressed secretion.

The present experiments were performed, therefore, to investigate the effects of acute deficiencies of single essential amino acids on digestion, carcass composition and pancreatic function of rats force-fed diets devoid of either histidine, methionine, phenylalanine, or threonine. Pancreatic function was evaluated by determining the activities of the digestive enzymes in the pancreas and in the small intestine during active digestion of control and experimental diets, or during excessive pancreatic stimulation evoked by a single feeding of soybean trypsin inhibitor.

EXPERIMENTAL

In a preliminary experiment rats were fed ad libitum a diet devoid of methionine, for two weeks. Under these conditions, however, pancreatic and intestinal enzyme activities did not differ significantly from those of pair-fed control animals. Therefore, to facilitate the onset of a deficiency and to avoid superimposing the effects of inanition on those produced by the amino acid deficiency, a force-feeding routine was followed, employing the mouthpiece described by Gillespie and Lucas ('57).

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[2] A preliminary report of this work has been presented previously: Lyman, R. L., and Sue S. Wilcox 1961 Effect of specific amino acid deficiencies on pancreatic enzymes in the rat. Federation Proc., 20: 8F (abstract).

The procedure had the advantages of allowing us to feed a definite and similar amount of diet to both deficient and control animals and, as reported by others (Spector and Adamstone, '50; Sidransky and Farber, '58a), to accelerate the appearance of deficiency symptoms.

Animals and diets. Female, Long-Evans rats weighing 160 to 200 gm were distributed randomly among the groups and maintained in individual screen-bottom cages. They were fed twice a day for 10 days an amount of diet (composition shown in table 1) which averaged about 10 gm daily (actual amount fed shown in table 2). For tube feeding, the diet was homogenized in a blender so that 1 ml contained 0.8 to 0.9 gm of solids. The amount of solids was determined, after drying, every other day. Control groups were fed diets containing the complete amino acid mixture, whereas the experimental diets were achieved by omitting the desired amino acid from the amino acid mixture. Sucrose replaced the amino acid removed. The quantity of diet fed daily was not intended to be equivalent to the amount that the control animals would have eaten voluntarily. However, it provided a dietary intake that allowed the controls to gain, yet did not cause undue distress in those rats fed diets devoid of an essential amino acid.

By the end of the feeding period the animals fed the diets lacking an essential amino acid showed typical, nonspecific deficiency symptoms characterized by heavy porphyrin staining about the nose and mouth, unkempt hair and a noticeable lack of muscle tone.

General procedures. All animals were offered the diet lacking the amino acid ad libitum 24 hours before force-feeding was begun. On the eleventh day after the start of force-feeding, individual rats were tube-fed, at intervals, either 5 gm of diet or 5 gm of diet plus 160 mg of soybean trypsin inhibitor concentrate (SBTI). When fed in this way, SBTI had previously been shown to evoke a pronounced and consis-

TABLE 1

Composition of basal diet and of amino acid mixture

Basal diet		Amino acid mixture[1]	
	%		%
Sucrose	46.5	L-Lysine·HCl	1.78
		L-Arginine·HCl	0.91
Amino acid mixture	28.5	L-Tryptophan	0.27
		DL-Phenylalanine	1.04
Cottonseed oil	9.0	DL-Leucine	3.64
		DL-Isoleucine	2.38
Fortified oil[2]	1.0	DL-Valine	2.80
		L-Histidine	0.68
Salts, USP XIV	4.0	DL-Methionine	0.60
		DL-Threonine	1.62
Cellulose, powdered[3]	10.0	DL-Serine	2.26
		Glycine	0.38
Vitamin mixture[4]	1.0	DL-Tyrosine	1.13
		L-Cystine	0.27
		L-Proline	2.27
		L-Aspartic acid	1.17
		L-Glutamic acid	4.22
		DL-Alanine	1.12
		Total	28.54

[1] Amino acids were purchased from California Corporation for Biochemical Research, Los Angeles. The amino acid mixture was added to the diet to provide L-amino acids in amounts similar to 18% casein. The quantities of leucine, isoleucine, valine and threonine were doubled since the rat does not completely utilize the D-isomers. The value for L-cystine represents an additional 0.2% which was added to all diets except the one devoid of methionine, in which only 0.07% of L-cystine was present.

[2] Fortified cottonseed oil provided/100 gm of diet: vitamin A, 1,700 IU; vitamin D, 100 IU; α-tocopheryl acetate, 6.66 mg; menadione, 0.5 mg.

[3] Cellu Flour, The Chicago Dietetic Supply House, Chicago.

[4] The vitamin mixture was made with sucrose, and provided as mg/100 gm of diet: thiamine, 0.2; riboflavin, 0.3; pyridoxine, 0.25; Ca pantothenate, 2.0; inositol, 10.0; biotin, 0.01; folic acid, 0.02; niacinamide, 1.0; vitamin B_{12}, 0.02; and choline chloride, 150.

TABLE 2

Weight changes and rate of stomach emptying in rats force-fed diets devoid of certain essential amino acids

Diet	No. rats	Diet fed daily	Avg final body wt	Wt change in 10 days	Solids removed from stomach two hours after feeding[1]		Dry weight of intestinal contents two hours after feeding	
					− SBTI[2]	+ SBTI	− SBTI	+ SBTI
		gm	*gm*	*gm*	%	%	*mg*	*mg*
Control	14	10.4	182	+ 5.8	33 ± 2[3](7)[4]	23 ± 3(7)	239 ± 52(6)	258 ± 20(7)
Less histidine	15	10.7	171	−10.5	33 ± 1 (7)	27 ± 2(8)	320 ± 22(7)	374 ± 48(8)
Less methionine	11	10.0	155	−17.5	38 ± 5 (4)	33 ± 3(7)	265 ± 78(4)	250 ± 38(7)
Less phenyl-alanine	11	9.9	152	−11.0	32 ± 2 (5)	27 ± 2(6)	190 ± 42(5)	239 ± 26(6)
Less threonine	13	9.8	160	−14.0	45 ± 3 (6)	40 ± 4(7)	297 ± 43(6)	285 ± 22(7)

[1] Percentage of solids removed following a single feeding of the diet (5 gm).
[2] Soybean trypsin inhibitor.
[3] SE.
[4] Number of animals in group.

tent enzyme response which depleted pancreatic enzyme activities, while at the same time sharply increasing intestinal enzyme activities (Lyman and Wilcox, '60). At two hours and at 23 hours after the eleventh day of feeding, animals were decapitated. Intestinal contents between the pylorus and the ileocecal junction were removed and frozen. Pancreases were removed, cleaned of visible fat under a dissecting microscope, frozen, and lyophilized along with the intestinal contents. Enzyme analyses were performed on the dried material. As an indication of digestion, stomach contents were collected and dried overnight *in vacuo,* then weighed. Adrenals and kidneys were rapidly removed and weighed. Individual carcasses minus the liver, pancreas, and stomach and intestinal contents were analyzed for water, fat and crude protein. The carcasses were dried to constant weight and then fat-extracted with diethyl ether. After extraction, they were ground into a powder, duplicate portions of which were digested with sulfuric acid. The ammonia was distilled from an all-glass, semi-micro steam distillation apparatus. Pancreatic nitrogen was determined on the lyophilized pancreases by the same procedure.

Enzyme analyses for lipase, protease and amylase activities were performed on aliquots of the lyophilized pancreas and intestinal contents after homogenization in water with a Potter-Elvehjem homogenizer. Details of the analyses have been described previously (Lyman and Lepkovsky, '57). Proteolytic zymogen in the pancreas was activated by a 20-minute incubation (37°C) of the sample with an equal volume of a 0.5% suspension of desiccated raw duodenum.[3] The soybean trypsin inhibitor concentrate was prepared as previously described (Lyman and Wilcox, '60).

Statistical evaluation of the results was made by the *t* test (Snedecor, '56). Values with a $P < 0.05$ were considered to have significance.

RESULTS

Table 2 shows some of the general effects of force-feeding the control and the experimental diets.

Digestion of the various diets was compared by measuring the amount of food emptied from the stomach two hours after feeding. Threonine was the only deficiency that appeared to have any effect on this measurement as compared with control values. The amount of food removed from stomachs of the threonine-deficient group was significantly higher than that from the controls ($P < 0.01$). Apparently the

[3] Viodenum, Viobin Corporation, Cleveland, Ohio. The expression "pancreatic protease activity" is used throughout the text to denote the activated zymogen.

animals were able to handle the food however, since it did not accumulate in the small intestine. The presence of SBTI in the diets consistently retarded stomach emptying, yet the inhibitor had no consistent effect on the quantity of material present in the small intestine in any of the groups.

Loss of weight by the deficient animals, despite their being fed essentially the same amount of diet as the controls, which gained weight, has been reported previously (Sidransky and Farber, '58a). A change in body composition was expected to accompany the weight loss in the deficient animals since the caloric intake of those animals relative to body weight was obviously in excess of their needs. However, no significant differences were found, in the proportion of water, fat or protein, between deficient and control groups (table 3).

Adrenal size relative to body weight was slightly larger than might be expected of animals fed ad libitum (Lyman and Krueger, '61), although the additional stress imposed by the amino acid-free diets did not cause any significant enlargement of the gland over that of the control. Kidney weights/100 gm of body weight were significantly above those from the control group ($P < 0.05$). The larger relative size of the kidneys in the deficient animals may have been due to the increased excretion of nitrogenous products that were not being utilized. Grossly, the kidneys appeared to be healthy and functioning normally.

The capacity of the pancreases from the deficient rats to maintain and secrete digestive enzymes in response to dietary stimuli is presented in the upper half of table 4. The omission of any one of the 4 amino acids studied resulted in a reduction in the activities of the digestive enzymes as compared with control values. Histidine deficiency not only produced the most significant reduction in pancreatic nitrogen and enzyme activity, as compared with the control values, but also depressed the levels of nitrogen ($P < 0.02$), protease ($P < 0.01$) and amylase activities ($P < 0.01$) significantly below the levels produced by a lack of each of the other amino acids. A lack of dietary methionine did not significantly reduce the pancreatic nitrogen or lipase activity below that of the control group, but the reductions in protease and amylase activities were significant. In the methionine-deficient group, amylase was not depressed as much as when histidine, phenylalanine or theronine were omitted from the diet. The increased amylase activity compared with that of the other deficient groups was significant ($P < 0.01$). Phenylalanine deficiency had a significant depressing effect on all three of the enzymes, as compared with the controls, but lipase activity was least affected. The omission of threonine from the diet significantly decreased pancreatic lipase and amylase activities, but had no significant effect on protease activity as compared with the control animals. Of the deficient animals, the threonine-deficient groups consistently had the highest pancreatic and intestinal protease activity, but the differences between the values and the other deficient groups were not significant.

TABLE 3

Carcass composition and adrenal and kidney weights of rats fed diets devoid of certain essential amino acids

Diet	No. rats	Carcass composition[1]			Adrenal	Kidney
		Water	Fat	Protein		
		%	%	%	*mg/100 gm body weight (wet wt)*	*gm/100 gm body weight (wet wt)*
Control	14	63.7 ± 1.0[2]	10.3 ± 3.1	19.8 ± 3.1	27.0 ± 1.0	0.86 ± 0.02
Less histidine	13	63.6 ± 0.6	9.5 ± 1.7	19.9 ± 2.5	28.4 ± 0.8	0.93 ± 0.03[3]
Less methionine	11	62.1 ± 0.5	8.5 ± 2.0	21.5 ± 4.5	28.8 ± 1.3	0.92 ± 0.02
Less phenylalanine	11	64.7 ± 1.1	6.4 ± 2.4	20.5 ± 4.2	30.0 ± 2.4	0.97 ± 0.01
Less threonine	13	64.7 ± 0.4	8.2 ± 2.5	20.0 ± 2.2	25.7 ± 0.9	0.96 ± 0.02

[1] Liver, 3 to 4 ml of blood, and stomach and intestinal contents removed prior to analyses.
[2] SE.
[3] Only 9 animals were analyzed.

TABLE 4

Effect on pancreatic nitrogen and on pancreatic and intestinal enzyme activities of rats two hours after being fed diets deficient in certain essential amino acids

Diet	No. rats	Diet						
		Pancreatic nitrogen	Pancreas activity/100 gm body wt			Total intestinal contents activity		
			Lipase	Protease	Amylase	Lipase	Protease	Amylase
		mg/100 gm body weight	*mEq acid/hour*	*mEq tyrosine/10 min*	*units*	*mEq acid/hour*	*mEq tyrosine/10 min*	*units*
Control	7	12.7 ± 0.8[1](6)	76.3 ± 9.6	0.249 ± 0.038	6447 ± 648	3.2 ± 0.5	0.134 ± 0.063	977 ± 64
Less histidine	7	7.3 ± 0.8[2]	23.1 ± 6.8[2]	0.029 ± 0.010[2]	870 ± 256[2]	3.4 ± 1.0	0.134 ± 0.008	350 ± 52[2]
Less methionine	4	10.5 ± 0.6	50.9 ± 15.0	0.065 ± 0.042[2]	3095 ± 724[2]	3.2 ± 0.6	0.103 ± 0.023	301 ± 43[2]
Less phenylalanine	5	10.5 ± 0.4	46.7 ± 8.8[3]	0.096 ± 0.021[4]	1590 ± 303[2]	2.5 ± 1.9	0.086 ± 0.011	350 ± 66[2]
Less threonine	6	10.9 ± 1.0	43.1 ± 5.9[4]	0.183 ± 0.040	1860 ± 354[2]	2.0 ± 0.2	0.167 ± 0.020	390 ± 89[2]
		Diet + pancreatic stimulant (SBTI)[5]						
Complete diet	7	11.6 ± 0.6	15.3 ± 4.1	0.047 ± 0.010	2009 ± 89	42.1 ± 6.5	0.259 ± 0.022	7664 ± 360
Less histidine	8	6.7 ± 0.5[2]	4.4 ± 1.9[3]	0.013 ± 0.004[2]	417 ± 81[2]	18.6 ± 2.5[2]	0.155 ± 0.022[4]	1440 ± 195[2]
Less methionine	7	9.3 ± 0.3[2]	5.7 ± 3.1	0.028 ± 0.007	1072 ± 205[2]	26.7 ± 5.4	0.107 ± 0.018[2]	3700 ± 730[2]
Less phenylalanine	6	9.5 ± 0.5[2]	10.4 ± 3.1	0.022 ± 0.005[3]	816 ± 197[2]	30.1 ± 4.9	0.096 ± 0.022[2]	2120 ± 274[2]
Less threonine	7	9.4 ± 0.6[2]	11.4 ± 3.1	0.050 ± 0.015	740 ± 154[2]	22.8 ± 4.6	0.225 ± 0.046	1660 ± 230[2]

[1] SE.
[2] Difference from control significant ($P < 0.01$).
[3] Difference from control significant ($P < 0.05$).
[4] Difference from control significant ($P < 0.02$).
[5] Soybean trypsin inhibitor.

The reduced pancreatic enzyme activities in the deficient animals were not accompanied by any significant effect on intestinal lipase or protease activity. Intestinal amylase activity in all deficient groups, however, was significantly less than the control values.

In the lower half of table 4 are shown the effects on pancreatic nitrogen and pancreatic and intestinal enzyme activities of a single feeding of SBTI with the diets. Under such conditions, pancreatic nitrogen and enzyme content were depleted severely, relative to the values obtained two hours after the diet only was fed. The loss of pancreatic enzyme activity was accompanied by a significant increase in intestinal enzyme activity, in all groups, above that produced when the diet alone was fed ($P < 0.01$). Therefore, the amino acid-deficient animals not only responded to dietary stimulation, but also had sufficient reserve to be able to secrete, rapidly, relatively large amounts of active enzymes. Although the amount of enzyme activity appearing in the intestinal contents of the deficient group was generally significantly less than that in the controls, the activities were still many times the amount secreted when diets without inhibitor were fed. The results of this experiment, therefore, tended to confirm the previous observations that histidine appeared to be particularly important for maintaining optimal activities of the digestive enzymes. During the stimulated secretion, pancreatic nitrogen and enzyme activities were especially low in the histidine-deficient animals, whereas intestinal lipase and amylase activities were significantly lower in this group than in the control rats. In the other deficient groups, only intestinal amylase activities were consistently less than those in the animals fed the complete diet plus SBTI, although intestinal protease was significantly reduced when either methionine or phenylalanine was omitted from the diet. Also the highest intestinal activity of amylase produced by the deficient groups occurred in the group fed a diet devoid of methionine.

In an effort to see what effect single essential amino acid deficiencies had on the regeneration of the individual digestive enzymes, SBTI was fed in a single feeding, with the diets, to deplete pancreatic enzyme activity. The pancreas was then allowed to regain its enzymes over the next 23 hours. The results of these experiments are presented in table 5. The enzyme activities of the control animals increased above the levels observed two hours after feeding the diet alone. In those groups fed the diets lacking a single essential amino acid, pancreatic protease and amylase activities increased above the levels present two hours after feeding the diet and the increase in all the enzyme activities was many times above that present two hours after feeding the diet plus SBTI (table 4). However, the activities remained significantly below the control enzyme levels for all deficiencies except for the group fed the diet devoid of histidine. The high pancreatic enzyme activities of this group were unexpected since it was thought that the histidine-deficient

TABLE 5

Effect on the restoration of pancreatic nitrogen and pancreatic enzyme activities in rats fed diets deficient in certain essential amino acids, 23 hours after depletion of enzyme activity with soybean trypsin inhibitor

Diet	No. rats	Pancreatic nitrogen	Pancreas activity/100 gm body weight		
			Lipase	Protease	Amylase
		mg/100 gm body weight	*mEq acid/hr*	*mEq tyrosine/10 min*	*units*
Control	7	15.0 ± 0.4[1]	79.0 ± 9.6	0.371 ± 0.038	5650 ± 551
Less histidine	6	10.3 ± 1.2[2]	47.3 ± 9.6	0.257 ± 0.037	1450 ± 271[3]
Less methionine	4	12.9 ± 0.4[3]	39.7 ± 8.0[2]	0.197 ± 0.030[2]	2340 ± 321[3]
Less phenylalanine	7	11.7 ± 0.5[3]	38.6 ± 4.8[3]	0.110 ± 0.013[2]	1660 ± 348[3]
Less threonine	7	12.8 ± 0.7[3]	28.5 ± 5.5[3]	0.176 ± 0.038[2]	1660 ± 289[3]

[1] SE.
[2] Difference from control significant ($P < 0.05$).
[3] Difference from control significant ($P < 0.01$).

rats would show the least increase in enzyme activity. The nitrogen content of the pancreases from the deficient animals increased, by 23 hours after feeding SBTI, to substantially above the level present two hours after feeding the inhibitor, although it still remained significantly below that of the control group.

DISCUSSION

Removal of the food from the stomach two hours after feeding, was apparently unaffected by the acute amino acid deficiencies. The values of 32 to 45% of the administered solids that had been removed from the stomach after two hours compared well with the 38 to 40% obtained by Rodgers et al. ('60) with a 30% amino acid diet.

The proportion of water, fat and protein observed in the carcasses of the amino acid-deficient animals that had been force-fed was remarkably similar to the values reported by Cohn and Joseph ('59) for rats force-fed 80% of their normal daily food intake (water, 64.4%; fat, 7.9%; protein, 20%). The decrease in weight in the deficient animals apparently resulted from loss of entire cell structure rather than from any individual component such as fat or protein.

The results of these experiments indicate that the ability of the pancreas to supply or synthesize active digestive enzyme protein was impaired by the acute deficiencies of histidine, methionine, phenylalanine or threonine. The capacity for the pancreases of the deficient animals to respond to a secretory stimulus seemed to be relatively unaffected since pancreatic enzyme activities were depleted and intestinal activities increased when the organ was stimulated to secrete by feeding. However, the deficiencies did not necessarily have the same effect on each of the enzymes. Histidine, for instance, appeared to be especially important for providing optimal levels of pancreatic lipase, protease and amylase activities in the stimulated organ. A lack of dietary methionine, although reducing all activities of the pancreatic digestive enzymes, had much less effect on amylase activity than did the other deficiencies. There is a possibility that amylase synthesis in the pancreas has a low requirement for methionine, since Sidransky and Farber ('58a) reported an apparent increase in amylase activity in the pancreases of methionine-deficient rats. In addition, Hokin ('51) demonstrated that methionine is the only essential amino acid not required for optimal amylase synthesis in slices of pigeon pancreas.

The results of the present experiment also suggested that threonine may be less important for maintaining protease activity than were the other amino acids. This is interesting in view of observations made by Hong and Magee ('57) that complete removal of threonine from the diet increased the protease activity of the resting rat pancreas. Since, in their study, fairly large doses of threonine (1%) added to a low casein diet reduced protease activity, it was suggested that removal of threonine may have overcome some amino acid antagonism that may have existed. Since the control diet in the present experiment was not imbalanced with respect to threonine, as was the case in the study reported by Hong and Magee, threonine apparently is needed to maintain optimal levels of pancreatic protease. However, it may not be so important as some of the other amino acids for the rapid synthesis of pancreatic proteases.

Uram et al. ('60) showed that the rat pancreas has considerable reserve enzyme and that normal digestion was unimpaired when as little as 1% of the exocrine function had been left intact. Although, in the present study, a large part of the potential enzyme reserve was depleted by the amino acid deficiencies imposed, enough apparently remained to allow for a relatively normal secretion of at least lipase and protease. Even when forced to secrete abnormally large quantities of pancreatic enzymes over a short period of time (as when SBTI was fed), the deficient animals were able to produce many times the enzyme activity evoked when a complete diet was fed without SBTI. Whether the decrease in active enzyme reserve resulted from an inability to activate an inactive enzyme precursor or was due to an impairment in the synthesis of new enzyme protein was not unequivocally answered by these experiments. The formation of new

enzyme protein was strongly suggested, however, by the relative increase in pancreas nitrogen and enzyme activities between two hours and 23 hours after depletion with SBTI.

The missing amino acid necessary for maintaining the activity of the digestive enzymes in the pancreases of the deficient animals would have to come from the tissues in these experiments. Sidransky and Farber ('58b) demonstrated a reduction in muscle nitrogen in rats force-fed methionine- or threonine-free diets. In the same animals, no decrease in liver nitrogen was apparent. The breakdown of muscle protein may therefore be an important source of the missing amino acids during acute amino acid deficiencies. Histidine could, perhaps, be supplied from the breakdown of hemoglobin. Nasset ('56) has calculated that this source would provide, if needed, as much as 3 mg of histidine per day for a 250-gm rat. However, hemoglobin analyses in the animals made histidine-deficient failed to reveal any significant decrease in this substance.[4]

Van Pilsum et al. ('57) postulated that certain enzymes have a "priority" over other enzymes for amino acids needed to maintain their function during periods of protein or amino acid deficiency. If this is the case, the pancreatic enzymes apparently have a relatively high "priority" in this respect.

SUMMARY

Groups of rats were force-fed diets free of either histidine, methionine, phenylalanine or threonine for 10 days. Following a single feeding of their respective diets, with or without the pancreatic stimulant, soybean trypsin inhibitor, the animals were killed two hours or 23 hours later. Pancreases and intestinal contents were analyzed for lipase, protease and amylase activities. Stomach contents were weighed as an indication of digestion. The carcasses were analyzed for water, fat and protein.

Although the deficient animals lost weight when fed the same amount of diet that allowed the controls to gain, the relative proportions of water, fat and protein in the carcasses of the deficient groups did not differ from those of the control animals.

The amount of solids removed from the stomach two hours after feeding was not affected by the acute deficiencies.

Analyses of pancreatic and intestinal enzyme activities two and 23 hours after feeding indicated that acute deficiencies of any one of the essential amino acids reduced the level of enzyme activity in the secreting pancreas and impaired the ability of that organ to restore normal activities after a fast. Omission of histidine from the diet caused the greatest decrease in the enzyme activities of the secreting pancreas. A lack of methionine also depressed pancreatic lipase and protease activities, but had much less effect on amylase activity. Threonine-deficient rats appeared to be more able to support a higher level of pancreatic and intestinal protease activity than did animals fed diets devoid of the other amino acids.

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Effect of Acute Amino Acid Deficiencies on Carcass Composition and Pancreatic Function in the Force-fed Rat

II. DEFICIENCIES OF VALINE, LYSINE, TRYPTOPHAN, LEUCINE AND ISOLEUCINE[1]

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A preceding publication (Lyman and Wilcox, '62) reported the effects of acute deficiencies of histidine, methionine, phenylalanine and threonine on growth, digestion, carcass composition and pancreatic enzyme secretion in the rat. One of the most striking results was a significant reduction below control values in the level of activity of the pancreatic digestive enzymes while the organ was secreting in response to a meal.

Evidence also indicated that, depending upon the amino acid missing, the activities of the enzymes were independently influenced. Thus the omission of histidine from the diet depleted enzyme activities of the secreting pancreas more than did omission of any of the other amino acids under study. The absence of dietary methionine depressed pancreatic lipase and protease activities, but had much less effect on the activity of amylase than did the other deficiencies. On the other hand, threonine deficiency appeared to influence the level of pancreatic protease least.

As a result of these observations, similar experiments were performed on rats force-fed diets from which the essential amino acids valine, lysine, tryptophan, leucine and isoleucine were omitted. In these experiments, an additional period, 5 hours post-feeding, was investigated to see whether the level of enzyme activities observed at two hours after feeding were sustained over longer periods of digestion. Pertinent references are given in the preceding paper.

EXPERIMENTAL

The basal diet fed was the same as described previously (Lyman and Wilcox, '62). The amino acid deficiency desired was achieved by omitting that amino acid from the amino acid mixture and replacing it with an equal amount of sucrose. The rats in these experiments were slightly smaller than those used previously (155 to 170 gm). In general control animals were chosen to be about 5 gm smaller than the experimental animals at the start of the experiment so that by the end of the feeding period, not too large a disparity would exist between the two groups.

Details for care of the animals, force-feeding, general experimental procedures, use of soybean trypsin inhibitor (SBTI), preparation of samples and chemical and enzyme analyses have been presented (Lyman and Wilcox, '62; Lyman and Lepkovsky, '57). Plasma amylase was determined as described by Smith and Roe ('49). Statistical comparison of the results was made by t test as described in Snedecor ('56). Values having a $P < 0.05$ were considered to be significant.

RESULTS

Table 1 shows data on food intake, weight changes and the percentage of solids removed from the stomach. The quantity of diet fed (table 1) was sufficient to produce a weight gain in the control groups, but did not support the deficient animals. The isoleucine-deficient group suffered the severest weight loss and was the most emaciated by the end of the experiment. However, the animals fed the

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valine-free diet had the highest mortality of any of the groups.

Digestion, as measured by removal of food from the stomach at two and 5 hours after feeding, was not significantly influenced by the lack of any of the essential amino acids. The absence of dietary leucine and isoleucine appeared to have delayed stomach emptying at two hours, but by 5 hours, the percentage of food removed did not differ from that of the other groups. The amount of solids in the intestinal tract of all but the lysine-deficient animals was significantly higher ($P < 0.05$) at two hours after feeding than it was in the animals fed the control diet. At 5 hours, however, none of the groups differed significantly from the controls.

These results were similar to those obtained previously (Lyman and Wilcox, '62), and indicated that in rats fed diets devoid of a single essential amino acid for 10 days, gastric and intestinal handling of food is relatively unimpaired.

Table 2 shows the carcass composition and relative weights of adrenal, kidney and spleen from rats deprived of a single essential amino acid. The proportions of water, fat, and protein were similar to those of the controls in all groups except

TABLE 1

Weight change and rate of stomach emptying in rats fed diets devoid of certain essential amino acids

Diet	Diet fed/day[1]	Wt change in 10 days	Solids removed from stomach[2]		Wt of dry solids in small intestine[2]	
			2 hr	5 hr	2 hr	5 hr
	gm	*gm*	*%*	*%*	*mg*	*mg*
Control	9.6	+ 7 ± 1[3]	31 ± 4	61 ± 3	217 ± 36	311 ± 38
Less valine	8.3	− 14 ± 1	33 ± 7	59 ± 4	454 ± 49	344 ± 39
Less lysine	9.9	− 11 ± 1[4]	40 ± 3	69 ± 4	335 ± 37	308 ± 29
Less tryptophan	9.7	− 13 ± 1	36 ± 5	57 ± 4	519 ± 41	359 ± 75
Less leucine	10.4	− 11 ± 1	24 ± 4	63 ± 3	342 ± 23	468 ± 51
Less isoleucine	9.4	− 24 ± 1	26 ± 3	57 ± 5	540 ± 114	348 ± 44

[1] Valine group is average of 19 rats; less food was fed this group in an effort to reduce the mortality. Other groups are average of 20 rats.
[2] Control group had 6 rats at each period; others had 5 rats. Values are the percentage of solids removed following a single feeding of the diet (5 gm).
[3] Means ± SE.
[4] Weight loss for 11 days.

TABLE 2

Carcass composition and organ weights of rats fed diets devoid of certain essential amino acids

Diet	No. rats	Avg final body wt	Carcass composition[1]			Adrenal[2]	Kidney[2]	Spleen[2]
			Water	Fat	Protein			
		gm	*%*	*%*	*%*	*mg/100 gm body wt*	*gm/100 gm body wt*	*gm/100 gm body wt*
Control	20	165 ± 3[3]	65.2 ± 0.5	9.7 ± 0.5	18.4 ± 0.4	24.1 ± 0.5	0.93 ± 0.02	0.36 ± 0.04
Less valine	19	147 ± 3	63.0 ± 0.6	8.9 ± 0.8	19.2 ± 0.4	36.0 ± 1.5	1.00 ± 0.02	0.20 ± 0.01
Less lysine	20	156 ± 3	62.6 ± 0.2	10.0 ± 0.5	18.2 ± 0.5	27.5 ± 0.7	0.99 ± 0.01	0.43 ± 0.03
Less tryptophan	20	159 ± 3	61.2 ± 5.7	11.5 ± 0.6	19.2 ± 0.2	23.7 ± 0.7	0.98 ± 0.03	0.19 ± 0.01
Less leucine	20	165 ± 2	64.0 ± 0.4	8.1 ± 0.6	21.8 ± 0.9	29.5 ± 1.3	0.96 ± 0.02	0.31 ± 0.02
Less isoleucine	20	134 ± 2	64.5 ± 0.5	7.4 ± 0.5	20.5 ± 0.3	36.3 ± 0.9	1.13 ± 0.02	0.16 ± 0.01

[1] Analyses on 10 randomly picked carcasses/group. Liver, pancreas, 3 to 4 ml of blood, and stomach and intestinal contents removed prior to analyses.
[2] Wet weights of organs.
[3] Means ± SE.

the animals fed the isoleucine-free diet. In this group, the percentage of fat was significantly less than in the controls ($P < 0.01$). Since the decrease in fat was not accompanied by an increase in water content, there appeared to be a significant increase in the percentage of protein ($P < 0.01$). Adrenal weights relative to body size were significantly increased over those of the force-fed controls only in the rats fed diets lacking in valine, leucine or isoleucine ($P < 0.01$). Kidney enlargement occurred in all the groups, but was significant only in the isoleucine-deficient animals ($P < 0.01$). In the groups fed diets free of valine, tryptophan or isoleucine, spleen size was significantly smaller than in control animals ($P < 0.01$).

The functional ability of the pancreas, as measured by residual enzyme activity after dietary secretory stimulation, is shown in table 3. The absence of dietary valine, tryptophan, leucine or isoleucine caused a significant reduction in the activities of pancreatic lipase, protease, and amylase at both two and 5 hours after feeding, as compared with enzyme activities in the control animals. Enzyme activities of lysine-deficient animals were nearly normal for the first two hours, and dropped significantly below control values only at 5 hours post-feeding. Although pancreatic amylase activity in the leucine-deficient animals was significantly higher than in the groups fed diets free of valine, tryptophan or isoleucine, the level of activity was still much less than that in the controls. The much greater reduction in the enzyme activities of the valine, tryptophan and isoleucine-deficient animals at two and 5 hours after feeding was reflected

TABLE 3

Pancreatic and intestinal enzyme activities in rats two, 5 and 23 hours after being fed diets devoid of certain essential amino acids

Hr after feeding	Control[1]	Less valine	Less lysine	Less tryptophan	Less leucine	Less isoleucine
			Pancreas activity/100 gm body weight			
			Lipase (mEq acid/hr)			
2	69.7 ± 9.4	1.2 ± 0.4[2]	57.8 ± 5.5	4.1 ± 1.4[2]	10.6 ± 4.0[2]	3.5 ± 0.8[2]
5	44.5 ± 4.4	0.9 ± 0.4[2]	13.9 ± 2.1[2]	1.5 ± 0.4[2]	7.04 ± 1.3[2]	3.6 ± 1.4[2]
23	73.6 ± 3.6	—	82.8 ± 8.2	35.1 ± 4.3[2]	29.4 ± 4.1[2]	27.6 ± 5.0[2]
			Protease (mEq tyrosine/10 min)			
2	0.194 ± 0.028	0.005 ± 0.001[2]	0.126 ± 0.007	0.031 ± 0.017[2]	0.081 ± 0.018[3]	0.008 ± 0.001[2]
5	0.159 ± 0.029	0.004 ± 0.001[2]	0.060 ± 0.010[3]	0.006 ± 0.002[2]	0.030 ± 0.009[2]	0.010 ± 0.003[2]
23	0.283 ± 0.027	—	0.316 ± 0.055	0.131 ± 0.007[2]	0.209 ± 0.020	0.095 ± 0.01[2]
			Amylase (amylase units)			
2	8374 ± 663	329 ± 47[2]	3187 ± 418[2]	349 ± 109[2]	2177 ± 485[2]	292 ± 52[2]
5	5128 ± 981	587 ± 24[2]	1384 ± 155[2]	173 ± 29[2]	2051 ± 159[4]	535 ± 124[3]
23	9558 ± 721	—	4027 ± 463[2]	1195 ± 237[2]	4907 ± 170[2]	1076 ± 135[2]
			Total intestinal contents activity			
			Lipase (mEq acid/hr)			
2	3.1 ± 0.8	0.5 ± 0.1[4]	2.7 ± 1.0	1.8 ± 0.6	4.1 ± 1.6	2.0 ± 0.8
5	8.4 ± 2.0	0.4 ± 0.1[2]	0.8 ± 0.2[2]	1.1 ± 0.4[2]	3.9 ± 1.6	0.6 ± 0.2[2]
			Protease (mEq tyrosine/10 min)			
2	0.136 ± 0.014	0.090 ± 0.012[4]	0.160 ± 0.015	0.166 ± 0.020	0.096 ± 0.010[4]	0.11 ± 0.015[4]
5	0.138 ± 0.014	0.080 ± 0.011[3]	0.122 ± 0.014	0.110 ± 0.022	0.137 ± 0.015	0.09 ± 0.014
			Amylase (amylase units)			
2	750 ± 54	522 ± 69[4]	673 ± 113	239 ± 56[2]	644 ± 31	292 ± 79[2]
5	1031 ± 202	566 ± 132	412 ± 81[4]	119 ± 26[2]	615 ± 93	173 ± 27[2]

[1] Values are means ± SE from 5 rats/group at each time period. Control group contained 6 rats at each period.
[2] Difference from control significant ($P < 0.01$).
[3] Difference from control significant ($P < 0.02$).
[4] Difference from control significant ($P < 0.05$).

in the low nitrogen content of the pancreases from these groups (table 4).

The enzyme activities secreted into the small intestine in response to the diets free of a single essential amino acid were variable. Activities in the groups fed the diets free of valine, tryptophan and isoleucine were consistently lower, however, than were those in the controls. In general, intestinal enzyme activities of deficient animals were lower at 5 hours after feeding than at two hours, whereas the enzyme levels of the control animals were higher at 5 hours. Therefore, it is doubtful whether normal digestive enzyme secretion was maintained throughout the entire digestion period in these animals. Lysine- and leucine-deficient animals showed the least reduction in intestinal enzyme activities under these conditions.

When the animals were fasted 23 hours after they had been fed 5 gm of diet, all deficient groups showed appreciable increases in pancreatic enzyme activity over that present at 5 hours after feeding. With the exception of amylase, the activities of the lysine-deficient group were restored essentially to normal. Leucine-, tryptophan- and isoleucine-deficient rats were able to restore only about one-half the activities present in the controls, yet the increase was still many times the level at 5 hours after feeding. Soybean trypsin inhibitor (SBTI) was not used in these 23-hour experiments to deplete the pancreas of its enzyme activity as it had in the previous experiments, because it was thought possible that the activity increase in the earlier experiments could have been due to amino acids contributed by the digested inhibitor. However, the results of this experiment were similar to those obtained with the other acute amino acid deficiencies when the SBTI was used. Therefore, the increased enzyme activities as well as increased pancreas nitrogen (table 4) at 23 hours, as compared with values at 5 hours, indicated that digestive enzyme protein was synthesized. The amino acids necessary for the synthesis must have been supplied from tissue breakdown.

The results in table 5 show the effect of the amino acid deficiencies on the ability of the pancreas to secrete active enzymes when an exaggerated secretion is produced by feeding a single dose of SBTI with the diet.

Soybean trypsin inhibitor, as expected, depleted control pancreatic enzyme activities considerably below the levels present when diet alone was fed. Animals fed SBTI with diets devoid of valine, tryptophan and isoleucine, however, showed a much more severe depletion of pancreatic enzyme activity than did the control animals. Although the concomitant increase in intestinal enzyme activity was not as much as in the controls, it was still many times more than that produced when only the diet was fed. The results indicated, therefore, that these acute amino acid deficiencies did not interfere with the ability of the pancreas to secrete in response to a stimulus, but did reduce the rate at which the organ could synthesize active enzyme protein. Interpretation of the intestinal protease results was not attempted because of a variable quantity of trypsin that undoubtedly was inactivated by the trypsin inhibitor fed.

As in the case when diet alone was fed, pancreatic and intestinal enzyme activities of animals fed diets devoid of lysine showed the least deviation from control

TABLE 4

Pancreatic nitrogen per 100 gm of body weight in rats two, 5 and 23 hours after being fed diets devoid of certain essential amino acids

Hr after feeding	Control[1]	Less valine	Less lysine	Less tryptophan	Less leucine	Less isoleucine
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
2	11.3 ± 0.6	7.8 ± 0.6[2]	12.7 ± 1.3	7.3 ± 0.5[2]	10.9 ± 0.6	7.8 ± 0.4[2]
5	10.7 ± 0.6	7.2 ± 0.7[3]	9.8 ± 0.3	6.0 ± 0.5[2]	10.5 ± 0.7	8.0 ± 0.4[3]
23	12.3 ± 0.4	—	14.1 ± 0.4	9.5 ± 0.5	10.8 ± 0.6	9.7 ± 0.7

[1] Values are means ± SE from 5 rats/group at each time period. Control group contained 6 rats at each period.
[2] Difference from control significant ($P < 0.01$).
[3] Difference from control significant ($P < 0.02$).

values of any of the groups. In this group, enzyme activities secreted into the intestine under the extreme condition of SBTI feeding were essentially no different from those of the control group. In the animals fed diets lacking leucine, only intestinal lipase activity was affected and was significantly lower than that of the control animals.

Plasma amylase determinations were made on all rats subjected to diets deficient in a single essential amino acid to learn whether they would correlate with the ability of the pancreas to synthesize and secrete this enzyme. The results are shown in table 6. Comparison of the corresponding pancreas activities (tables 3 and 5) with the plasma amylase activities

TABLE 5

Pancreatic enzyme activity per 100 gm of body weight and total intestinal enzyme activities in rats two and 5 hours after a single feeding of diets devoid of certain essential amino acids but which contained 160 mg of soybean trypsin inhibitor

Hr after feeding	Control[1]	Less valine	Less lysine	Less tryptophan	Less leucine	Less isoleucine
			Pancreas			
			Lipase (mEq acid/hr)			
2	15.1 ± 4.4	0.3 ± 0.1	15.9 ± 1.3	0.9 ± 0.6	1.9 ± 0.6	0.3 ± 0.1
5	4.3 ± 3.0	0.6 ± 0.1	1.4 ± 0.7	0.7 ± 0.1	1.1 ± 0.3	0.7 ± 0.1
			Protease (mEq tyrosine/10 min)			
2	0.031 ± 0.006	0.001 ± 0.0	0.039 ± 0.004	< ± 0.001	0.016 ± 0.004	0.001 ± 0.0
5	0.031 ± 0.005	0.006 ± 0.003	0.010 ± 0.015	0.003 ± 0.0	0.008 ± 0.001	0.003 ± 0.0
			Amylase (amylase units)			
2	2980 ± 359	321 ± 74	1606 ± 336	245 ± 100	926 ± 163	135 ± 10
5	1440 ± 195	302 ± 38	804 ± 90	157 ± 16	1000 ± 147	214 ± 42
			Intestinal contents			
			Lipase (mEq acid/hr)			
2	52.2 ± 8.2	11.8 ± 3.4[2]	19.5 ± 3.6[2]	5.9 ± 1.5[2]	9.3 ± 1.6[2]	10.8 ± 3.3[2]
5	33.8 ± 2.7	11.2 ± 2.8[2]	26.2 ± 3.3	7.7 ± 1.2[2]	13.7 ± 2.2[2]	9.3 ± 2.7[2]
			Protease (mEq tyrosine/10 min)			
2	0.242 ± 0.015	0.042 ± 0.021[2]	0.245 ± 0.041	0.088 ± 0.008[3]	0.124 ± 0.024	0.183 ± 0.030
5	0.189 ± 0.011	0.173 ± 0.054[4]	0.180 ± 0.009	0.100 ± 0.017[2]	0.180 ± 0.019	0.147 ± 0.036
			Amylase (amylase units)			
2	8280 ± 1820	1790 ± 299[3]	5130 ± 1180	1097 ± 169[2]	4960 ± 693	1628 ± 305[2]
5	4610 ± 1050	2280 ± 657	5250 ± 1130	729 ± 199[2]	7550 ± 1190	1449 ± 451[4]

[1] Values are means ± SE from 5 rats/group at each time period.
[2] Difference from control significant ($P < 0.01$).
[3] Difference from control significant ($P < 0.02$).
[4] Difference from control significant ($P < 0.05$).

TABLE 6

Plasma amylase activity in rats two, 5 and 23 hours after being fed diets devoid of certain essential amino acids

Hr after feeding	Control[1]	Less valine	Less lysine	Less tryptophan	Less leucine	Less isoleucine
	Amylase units/100 ml		*Amylase units/100 ml*		*Amylase units/100 ml*	
			Amino acid diet			
2	1754 ± 129	1618 ± 79	1577 ± 104	2112 ± 101	1800 ± 58	1739 ± 64
5	1647 ± 35	1361 ± 323	1115 ± 134[2]	1466 ± 73	1390 ± 79[3]	1355 ± 57
23	1001 ± 111	—	1010 ± 54	1984 ± 140[2]	1402 ± 58[3]	1566 ± 257
			Amino acid diet plus 160 mg SBTI[4]			
2	1767 ± 47	—	1971 ± 129	2555 ± 144[2]	2017 ± 111	1912 ± 68
5	1623 ± 99	1887 ± 125	1768 ± 127	1681 ± 146	1706 ± 34	1448 ± 175

[1] Values are means ± SE from 5 rats/group at each time period. Control group contained 6 rats at each period.
[2] Difference from control significant ($P < 0.01$).
[3] Difference from control significant ($P < 0.02$).
[4] Soybean trypsin inhibitor.

at two, 5 and 23 hours after feeding shows that there was no consistent correlation between amylase activity in the pancreas and its activity in the plasma.

DISCUSSION

The experiments in this paper were designed to investigate certain physiological effects in rats produced by force-feeding, for 10 days, diets free of a single essential amino acid. Under the conditions, acute deficiencies of any one of 5 essential amino acids (valine, lysine, tryptophan, leucine, and isoleucine) had very little effect upon the ability of the rat to digest the diets (as measured by the percentage of food removed from stomach). Although some accumulation of solids in the small intestine occurred in certain deficient groups, there was no evidence that intestinal function or motility was impaired.

Adrenal gland size relative to body weight was measured as an indication of the stress imposed by the force-feeding as well as by the amino acid deficiency. The groups that exhibited a significant increase in adrenal size attributable to the amino acid deficiency were the animals fed diets devoid of valine, isoleucine or leucine. Deficiency symptoms such as weight loss, hair condition and mortality were most pronounced in the animals deficient in valine and isoleucine. Therefore, the adrenal enlargement in these groups may have resulted from the severity of the deficiency, which appeared to develop more rapidly than in the other groups. Ousterhout ('60) suggested that valine- or isoleucine-free diets produced an imbalance of tissue amino acids, which interfered with the synthesis of essential enzyme protein.

The atrophic spleen in rats made deficient in certain essential amino acids has been reported by others. Cole and Scott ('54) noted it in rats fed a tryptophan-deficient diet ad libitum and attributed it to the accompanying inanition. In the present experiments, however, inadequate food intake was avoided by means of force-feeding. Sidransky and Baba ('60) also reported that the average weights of spleens from young rats force-fed a valine-free diet for 7 days were significantly less than those from the force-fed controls. Gunderson et al. ('62) demonstrated that the rat spleen produced antibodies and increased in size in response to antigen injections. The decreased spleen size in the amino acid-deficient rats may be related to an inability to maintain normal antibody protein synthesis.

In all the deficiencies except that of the isoleucine-free group, although large losses of weight occurred as compared with controls fed isocalorically, the relative proportions of water, fat and protein in the carcasses were not altered. These results may be contrasted with the effects produced by food restriction or starvation in which, initially, depot fat is rapidly lost (Lee and Lucia, '61). The results of the present experiments indicate that, under conditions of an acute amino acid deficiency in which voluntary inanition has been avoided by force-feeding, entire body tissue must be catabolized, with no single component utilized preferentially.

Pathological changes and functional impairment of the pancreas during protein malnutrition have been reported by a number of investigators. Davies ('48) described atrophic changes in the acinar cells and diminution in the number of secretory granules in pancreases from infants suffering from kwashiorkor. Waterlow ('59) cited observations by others which showed that the duodenal enzyme level or functional capacity of the pancreas was affected in this protein malnutritional disease. Sidransky and Farber ('58a, b) established that acute deficiencies of certain single essential amino acids produced pathological lesions in many organs in the rat, including the pancreas. The lesions closely resembled those reported in kwashiorkor.

The results of the present experiment indicate that, in the rat, diets free of any one of the essential amino acids interfere with the ability of the pancreas to synthesize and, therefore, to secrete normal amounts of digestive enzymes into the intestine. The main difficulty appears to be with the synthesis of the active enzymes, rather than from any failure by the pancreas to respond to dietary stimulation.

Under the conditions of this experiment, acute deficiencies of valine, tryptophan and isoleucine caused the severest depletion of lipase, protease and amylase ac-

tivities in the secreting pancreas and significantly depressed the amount of these enzymes regenerated after a 23-hour fast. A dietary lack of leucine, although having a similar effect on lipase and protease, produced much less effect on amylase activity. On the other hand, lysine-free diets, when fed a comparable length of time, had a much less deleterious effect on pancreatic enzyme synthesis mechanisms than did the other deficiencies.

Deficiency symptoms were either mild or absent in animals deficient in lysine, although they lost nearly the same amount of weight as the other deficient rats did. Ousterhout ('60) observed that chicks fed diets devoid of lysine were stronger and lived longer than did chicks made deficient in other essential amino acids. He suggested that the breakdown of hemoglobin could contribute the amino acid necessary for enzyme synthesis. Hemoglobin is also rich in histidine (8%); yet, as shown in the preceding paper, an acute deficiency of histidine reduced the functional ability of the rat pancreas considerably. It might be expected that, if breakdown of hemoglobin contributed most of the lysine needs, it should also contribute heavily to the histidine requirements. It appears more likely that breakdown of muscle protein, as suggested by Sidransky and Farber ('58b), is a major source of the missing amino acids. The present study showed that 1 gm or more of muscle tissue was catabolized daily by all of the deficient groups. Breakdown of that amount of tissue, which consists of about 20% protein, could provide a limited supply of any amino acid necessary for the synthesis of hormone and enzyme systems basic to life as well as those needed for pancreatic enzyme synthesis. The amount and ready availability of amino acids from this source may be partially responsible for the differences observed in the severity of the acute deficiencies. According to Block and Weiss ('56), mammal carcasses, including that of the rat, contain relatively large amounts of lysine (5.5 to 9.0%).

Plasma amylase showed no relation to the condition of the pancreas or its ability to synthesize the enzyme in any of the deficiencies. These results indicate that this blood enzyme must be derived primarily from extra-pancreatic sources. Liver has been reported to synthesize significant quantities of amylase (Rutter et al., '61; Elliot and Williams, '61).

Trowell et al. ('54) observed a striking increase in plasma amylase in patients being treated for protein malnutrition. It is conceivable that, in the latter stages of chronic protein deficiency, when severe liver damage is likely to be present, synthesis of amylase by the liver or other nonpancreatic sources is so impaired that blood levels of the enzyme are reduced.

SUMMARY

Groups of rats were force-fed diets free of either valine, lysine, tryptophan, leucine and isoleucine, for 10 days. The animals were killed two, 5 and 23 hours after feeding. The pancreases and intestinal contents were analyzed for lipase, protease, and amylase activity. Amylase activity was also determined in the plasma. Stomach contents were collected, as a measure of digestion, and carcass composition was determined.

All animals made deficient in a single amino acid lost considerable amounts of weight, but only in the group fed a diet free of isoleucine was the composition of the carcass significantly different from that of the control.

Digestion, as measured by the amount of solids removed from the stomach and small intestine two and 5 hours after feeding, was unimpaired in the deficient animals.

Animals fed diets free of valine, tryptophan, leucine or isoleucine had the least ability to maintain pancreatic enzyme activities during dietary stimulation and were least able to recover pancreatic enzyme activities after a 23-hour fast. A deficiency of lysine had less effect on pancreatic enzyme activities in the secreting and fasted pancreas than did a deficiency of the other 4 amino acids.

Plasma amylase activity did not correlate with the functional capacity of the pancreas, thus indicating that extra-pancreatic sources were probably responsible for this blood enzyme.

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Nutrition Studies in the Cold

IV. EFFECT OF COLD ENVIRONMENT ON EXPERIMENTAL ATHEROSCLEROSIS IN THE RABBIT[1]

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Sellers and co-workers ('56, '60) and Wilgram ('59) have reported that rats exposed to cold environments for long periods developed coronary arterial "lipidosis" and increased subendothelial deposits. Little change occurred in either serum lipids or in the levels of serum free and esterified cholesterol of rats exposed to cold for as long as 9 months (Wilgram, '59). However, rats maintained in the cold for a year had significantly higher serum lipid levels than the controls (Sellers and You, '56). Sellers and Baker ('60) also reported that exposure to cold increased the incidence and severity of coronary lesions in the rats fed a high fat-cholesterol diet. Sodeman and Logue ('60) extended these studies to rabbits fed either stock chow or cholesterol-supplemented chow, and maintained at 0°C or room temperature for 42 days. They reported that, despite lower blood cholesterol levels, there was a greater incidence of experimental atheroma in the cold-exposed animals, as determined by staining techniques.

We have reported that a cold environment exhibits a lipotropic effect on blood and liver triglyceride levels of rats fed a high fat diet (Treadwell et al., '57). There was also a significant increase in liver esterified cholesterol in cold-exposed rats fed a high fat-cholesterol diet, compared with animals fed the same diet and maintained at room temperature (Vahouny et al., '59). This latter effect was termed the "antilipotropic" effect of cold on tissue esterified cholesterol levels.

It seemed pertinent to determine whether cold would induce changes in serum and tissue triglycerides and esterified cholesterol in rabbits comparable to the changes observed in rats, and to relate these alterations to the incidence and severity of experimental atherosclerosis. A preliminary report of this study has been presented.[2]

EXPERIMENTAL

Thirty-two male albino rabbits were divided into 4 groups of 8 animals. One control and one experimental group were maintained at 25°C, and comparable groups at 1°C for 10 weeks. The control diet consisted of commercial rabbit pellets[3] enriched with 5 gm of lard/100 gm of pellets. These were prepared by slowly pouring the warm liquified lard over pellets while mechanically stirring the entire mixture until homogeneous. The experimental diet consisted of commercial rabbit pellets enriched with 2 gm of cholesterol and 5 gm of lard/100 gm of pellets, and was prepared like the control diet after dissolving the cholesterol in liquefied lard. The animals at room temperature were given 50 gm/day of their respective diets; the two groups of rabbits in the cold were given 50 gm/day of their respective treated diets, and an additional daily feeding of 50 gm of untreated commercial pellets due to the increased energy requirement. The daily intake of cholesterol in the experimental groups was 1 gm per day per rabbit, and the total lard intake in all groups was 2.5 gm per day per rabbit.

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[2] Vahouny, G. V., A. Moede and C. R. Treadwell 1961 Effect of cold on experimental atherosclerosis. Federation Proc., 20: 367 (abstract).
[3] Gleco Rabbit Pellets, Charlottesville, Virginia.

Blood samples were obtained from 8 animals chosen at random prior to the start of the experiment, and were taken from all animals at two-week intervals throughout the 10-week experimental period. The serum was frozen and stored for subsequent analysis of the lipid fractions. At the termination of the experiment, the animals were killed and the adrenals, sections of liver, abdominal aorta and thoracic aorta were removed. The thoracic aorta was graded for incidence and severity of atheroma.

Extraction of tissue total lipids was as follows: The tissue (0.5 gm to 1.5 gm) was homogenized three times in 20 ml of ethanol-ether (1:1). After each homogenization, the mixture was warmed to boiling, cooled, and the supernate was decanted and filtered. The three extracts were combined and evaporated to dryness under a stream of nitrogen, and the lipid residue taken up in 20 ml of petroleum ether (bp 35° to 45°C). This solution was dried over sodium sulfate overnight and was subsequently filtered into an appropriate volumetric flask. For blood lipid analysis, 1 ml of serum was added by drops to 20 ml of ethanol-ether (3:1) and the mixture was brought to a boil. After cooling, the mixture was filtered, taken to dryness under nitrogen, and further treated as described for tissue lipid extraction.

Total dichromate reduction was determined by a modification of the method of Bragdon ('51). Phospholipid phosphorus was determined by a modification of the method of Fiske and Subbarow ('25), and phospholipid was calculated by the conventional method (lipid phosphorus × 25). Total and free cholesterol were determined by the procedure of Sperry and Webb ('50). The values for the determined lipid fractions were converted to dichromate reduction values (Bragdon, '51), and triglyceride was calculated by difference between the total dichromate reduced and the combined dichromate reduction values for phospholipid, free cholesterol and esterified cholesterol (calculated as oleate). Tissue total lipid was then taken as the sum of the lipid fractions.

Standard error of the mean was calculated for all values, and apparent differences between groups were analyzed for significance by the t test (Fisher, '38).

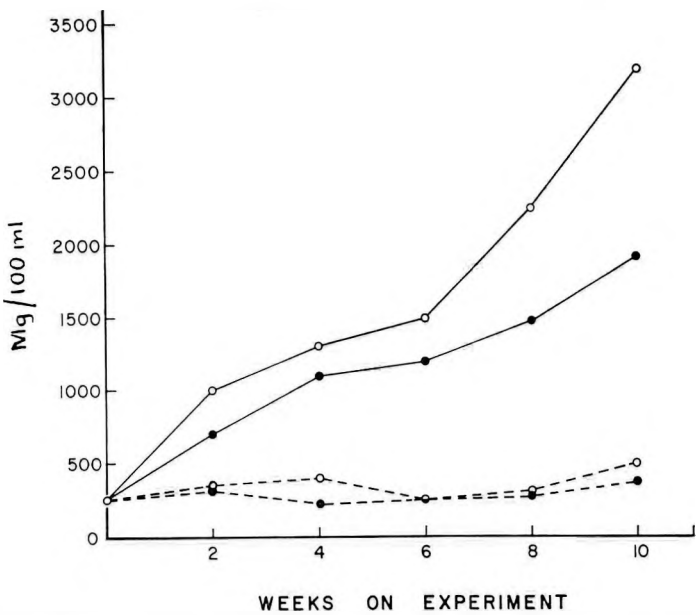

Fig. 1 Total lipids. Changes in total serum lipids in rabbits maintained for 10 weeks at 1° or 25°C with pellets enriched with 5% lard or 5% lard and 2% cholesterol. Open circle = 25°C; solid circle = 1°C; solid line = cholesterol plus lard; broken line = lard.

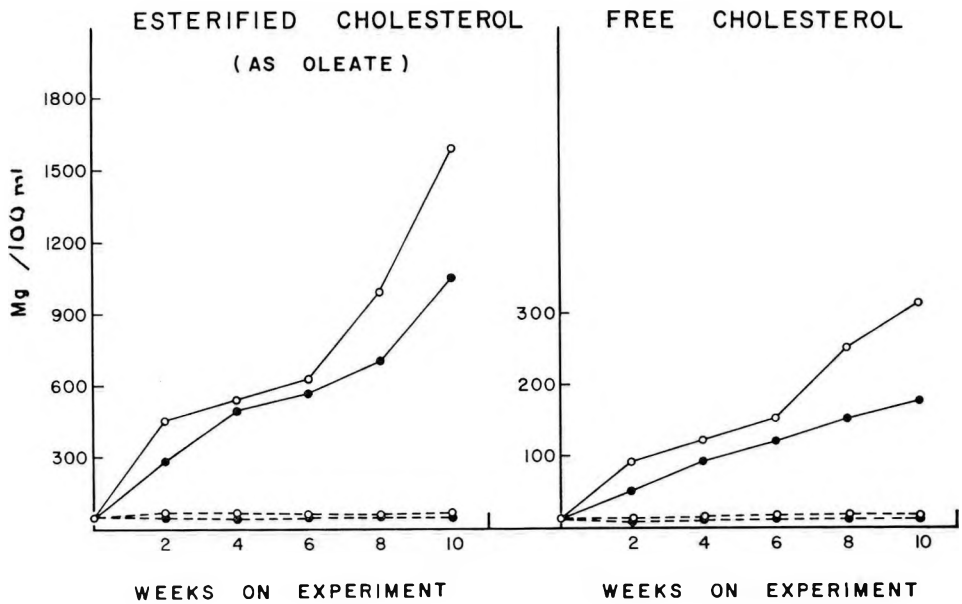

Fig. 2 Changes in serum free and esterified cholesterol in rabbits maintained for 10 weeks at 1° or 25°C with pellets enriched with 5% lard or 5% lard and 2% cholesterol. Open circle = 25°C; solid circle = 1°C; solid line = cholesterol plus lard; broken line = lard.

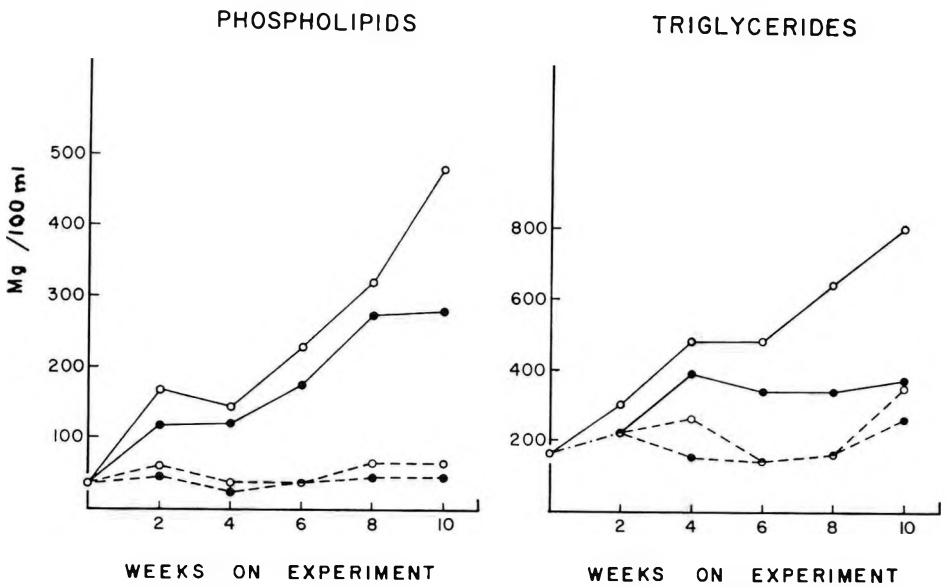

Fig. 3 Changes in serum phospholipids and triglycerides in rabbits maintained for 10 weeks at 1° or 25°C with pellets enriched with 5% lard or 5% lard and 2% cholesterol. Open circle = 25°C; solid circle = 1°C; solid line = cholesterol plus lard; broken line = lard.

RESULTS AND DISCUSSION

Serum lipids. In the control lard-fed groups maintained at 25° and 1°C, serum total lipids and the individual lipid fractions remained unchanged throughout the experimental period (fig. 1–3).

Rabbits maintained at room temperature with the cholesterol-lard diet showed a continuous increase in serum total lipids throughout the experiment. This was comparable to results reported by Wang and coworkers ('54). As in our earlier work with rats, total serum lipids in the experimental group at 1°C were also increased but less than in the comparable group maintained at room temperature, so that after 10 weeks, serum lipid levels at 1° were 40% lower than at 25°C.

As shown in figure 2, free and esterified cholesterol fractions of serum were higher throughout the experiment in the cholesterol-fed animals in room temperature than in the comparable group kept at 1°. This difference was most apparent at the end of 10 weeks when serum free cholesterol differed by 135 mg/100 ml, and the esterified fraction by 525 mg/100 ml. These results are in contrast with those obtained previously with rats, in which plasma esterified cholesterol was significantly higher in cold-maintained animals than in those at room temperature (Vahouny et al., '59). Sodeman and Logue ('60) also reported that cold-exposed rabbits exhibit lower plasma cholesterol levels than animals receiving the same dietary regimen at room temperature.

Serum phospholipid levels in the cholesterol-fed groups maintained at both temperatures were comparable during the first 8 weeks of experiment, after which a significant difference occurred (fig. 3). At room temperature, serum phospholipids continued to increase between the eighth and tenth weeks, whereas at 1°C, no further increase occurred.

Of greatest interest were alterations of serum triglycerides in the cholesterol-fed experimental groups (fig. 3). Our previous work with rats had indicated that cold markedly increased neutral fat metabolism. Young rats kept at room temperature had a marked accumulation of hepatic lipids when the fat content of the diet was serially increased from 10 to 40%. In the cold-exposed animals, there was no change in liver fat on the same dietary regimens. Thus, cold was an effective "lipotropic" agent with respect to liver lipid accumulation (Treadwell et al., '57). Subsequently, it was shown that this hepatic effect was due to decreased triglyceride accumulation in the cold (Vahouny et al., '59).

At both temperatures, in control groups fed lard there were no significant changes in serum triglycerides throughout the experiment. Cholesterol-fed rabbits in a temperature of 25°C showed a progressive increase in serum triglycerides, and after 10 weeks, this lipid fraction had increased fivefold over the pre-experimental value. In cold-exposed animals fed cholesterol, serum triglycerides increased twofold during the first 4 weeks; thereafter, this level remained constant for the remainder of the experiment. By 10 weeks the serum triglyceride level in this group was the same as that in the two lard-fed control groups. Since fatty acids and triglycerides are the primary source of the additional energy requirement during cold exposure, the lower serum triglyceride level in rabbits maintained in a temperature of 1°C may reflect increased utilization of this lipid fraction by tissues.

Hepatic lipids. As summarized in table 1, at 25° and 1°C total liver lipids in cholesterol-fed animals were higher than in the respective lard-control groups. Comparing the liver lipid partition between the lard-fed animals at the two temperatures, at a low level of dietary fat (2.5%), temperature had no effect on any of the lipid fractions. At higher levels of dietary fat, significant differences in liver triglyceride and esterified cholesterol occurred in rats maintained at the two temperatures (Treadwell et al., '57).

At 25°C feeding the cholesterol-lard diet resulted in about a threefold increase in liver triglyceride (182%) over the control group. However, at 1°C, the increase in hepatic triglyceride was only 69%. This difference may reflect increased neutral fat metabolism in the cold, resulting in less hepatic accumulation of this lipid fraction. These data are in agreement with the reported "lipotropic" effect of cold on hepatic lipids of the rat (Treadwell et al., '57).

TABLE 1
Hepatic lipids

Diet (Additions to chow)	Temp	No. of samples	Total lipid	Triglycerides	Phospholipid	Cholesterol	
						Free	Ester[1]
	°C		*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*
5% Lard	25	8	39.5±1.8[2]	5.1±0.2	32.2±1.6	1.7±0.6	0.5±0.1
5% Lard+2% cholesterol	25	8	64.5±6.3	14.4±3.9	34.4±1.2	3.4±0.3	12.8±3.0
5% Lard	1	7	42.0±1.2	6.1±0.6	35.3±1.4	1.9±0.8	0.3±0.1
5% Lard+2% cholesterol	1	7	59.5±3.6	10.3±1.9	38.3±1.3	3.2±0.3	7.9±1.5

[1] Calculated as cholesterol oleate.
[2] Figures represent the mean values ± standard error.

TABLE 2
Adrenal lipids

Diet (Additions to chow)	Temp	No. of samples	Adrenal weight	Total lipid	Triglycerides	Phospholipid	Cholesterol	
							Free	Ester[1]
	°C		*gm*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*
5% Lard	25	8	0.341	283±17[2]	130±19	24.5±1.7	3.2±0.2	125±6
5% Lard+2% cholesterol	25	8	0.526	403±16	134±15	19.4±0.6	8.0±1.0	242±18
5% Lard	1	8	0.392	161±13	50±8	28.7±1.4	3.0±0.3	79±14
5% Lard+2% cholesterol	1	6	0.589	282±28	73±10	23.7±2.1	5.4±0.9	180±19

[1] Calculated as cholesterol oleate.
[2] Figures represent the mean values ± standard error.

Free cholesterol levels, which did not change in the two lard-fed control groups during the experiment, increased only two-fold in the cholesterol-fed groups. Hepatic esterified cholesterol, on the other hand, increased 25-fold in the cholesterol-fed groups at both temperatures. Despite large variations between animals in these groups, the esterified cholesterol levels more closely reflect differences in plasma esterified cholesterol between the experimental groups than had been previously noted in rats (Vahouny et al., '59).

Adrenal lipids. The data on adrenal lipids are summarized in table 2. Exposure to cold had little effect on adrenal size in either the control or experimental animals. In both cholesterol-fed groups, however, the adrenals were larger than in the corresponding lard-fed controls.

Total adrenal lipids were significantly higher in the cholesterol-fed animals at both temperatures than those in the control groups; total lipids were also higher in the two groups at room temperature than those in the comparable cold-exposed groups. The results suggest a higher rate of adrenal metabolism in the cold and therefore less accumulation of tissue total lipids.

The adrenal lipid partition indicates that decreased accumulation of adrenal lipids in the cold was due in part to a reduction of adrenal triglyceride levels, which were about one-half those in the groups at 25°C. In comparing the control and experimental animals at either 25° or at 1°C, it appears that dietary cholesterol has no significant effect on the level of adrenal triglyceride.

Although there were no differences in the free cholesterol levels between the two lard-fed control groups, or between the two cholesterol-fed experimental groups, significant differences were noted in the levels

TABLE 3

Thoracic aorta lipids

Diet (Additions to chow)	Temp	No. of samples	Atheroma		Total lipid	Triglycerides	Phospholipid	Cholesterol	
			Incidence	Grade 0–4				Free	Ester[1]
	°C		%		*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*
5% Lard	25	8	0	0	141 ± 16[2]	136 ± 16	4.1 ± 0.4	1.0 ± 0.1	0.1 ± 0.08
5% Lard + 2% cholesterol	25	8	100	2.1	140 ± 20	129 ± 18	5.7 ± 0.9	1.9 ± 0.2	3.1 ± 1.0
5% Lard	1	7	25	0.3	85 ± 5	78 ± 5	5.8 ± 0.6	1.1 ± 0.1	0.2 ± 0.04
5% Lard + 2% cholesterol	1	7	100	2.1	108 ± 9	94 ± 10	7.5 ± 1.5	2.2 ± 0.4	5.0 ± 2.1

[1] Calculated as cholesterol oleate.
[2] Figures represent the mean values ± standard error.

of esterified cholesterol. First, cholesterol feeding at either temperature resulted in a twofold increase in both free and esterified adrenal cholesterol above the levels in the corresponding control group. At both temperatures, the increase in esterified cholesterol represented 96 to 97% of the total accumulation of cholesterol in the adrenals. However, the esterified cholesterol level in the cold-exposed groups was significantly lower than that in the groups at 25°C.

Aorta lipids. Data in table 3 summarize total lipids and lipid fractions of thoracic aorta sections. At the termination of the experiment and prior to homogenization and extraction of lipids, thoracic aorta was visually graded for incidence and severity of atherosclerotic lesions.

At room temperature, all animals fed cholesterol for 10 weeks had minor-to-extensive aortic involvement by atheromatous lesions with an average grade of 2.1; control animals fed the lard-pellet diet had no arterial lesions. In these two groups at 25°C, aortic total lipid, triglyceride and phospholipid were comparable; in the cholesterol-fed group, aortic free cholesterol was increased twofold, and esterified cholesterol 30-fold over the control level.

At 1°C, the control-lard group contained two animals that showed slight aortic involvement after 10 weeks (graded +1). Analysis of aortic lipids in this group showed a significantly lower total lipid level compared with the comparable control group at 25°C. This effect was due entirely to a lower triglyceride level. All cholesterol-fed rabbits in the cold had varying degrees of aortic lesions ranging from +1 to +4, with an average grade of 2.1. This is the same grade as noted for the cholesterol-fed rabbits maintained at 25°C. Total aortic lipids and triglycerides were lower in the cold-exposed group than in the animals maintained at 25°C, which may also reflect increased tissue metabolism of neutral fat in the cold. Only the phospholipid, and free and esterified cholesterol levels of aorta were comparable in the two cholesterol-fed groups.

Similar differences were noted in the abdominal aorta sections of the cholesterol-fed groups at the two temperatures (table

TABLE 4
Abdominal aorta lipids

Diet (Additions to chow)	Temp	No. of samples	Total lipid	Triglycerides	Phospholipid	Cholesterol	
						Free	Ester[1]
	°C		*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*	*mg/gm tissue*
5% Lard	25	8	127 ± 19[2]	121 ± 19	4.3 ± 0.4	1.1 ± 0.05	0.7 ± 0.3
5% Lard + 2% cholesterol	25	7	137 ± 37	125 ± 37	5.8 ± 0.6	2.1 ± 0.3	4.3 ± 1.1
5% Lard	1	8	53 ± 11	48 ± 12	4.0 ± 0.3	1.0 ± 0.1	0.8 ± 0.4
5% Lard + 2% cholesterol	1	4	79 ± 12	69 ± 12	6.3 ± 0.9	1.9 ± 0.2	1.8 ± 0.3

[1] Calculated as cholesterol oleate.
[2] Figures represent the mean values ± standard error.

4). However, the esterified cholesterol level in the experimental group at 1°C was lower than in the comparable group at 25°C (table 4). This may be due to a gradient of cholesterol deposition in aorta beginning proximal to the heart and decreasing in the descending aorta.

It has been shown that experimental stress or administration of ACTH (Mann and White, '53) lowers plasma cholesterol levels in dogs. At least a part of this effect is due presumably to accelerated conversion of cholesterol into adrenal steroid hormones. In addition, there is a simultaneous decrease in the level of adrenal cholesterol in many species. This depression involves mainly the esterified fraction, whereas free cholesterol remains constant (Popjak, '44). Wang et al. ('55) and Dury ('59) have shown that administration of cortisone, however, will elevate all plasma lipid levels in rabbits, including plasma cholesterol.

In the present work, it is probable that the lower plasma cholesterol level in cold-exposed rabbits is due in part to an effect of stress. This cold stress also causes a decrease in adrenal esterified cholesterol. However, lower serum triglyceride in animals maintained at 1°C may reflect increased triglyceride metabolism for energy and heat production; this effect can also be observed in the triglyceride levels of adrenal, thoracic and abdominal aorta, and, to a lesser extent, the liver.

Albrink and Man ('59) have suggested that human coronary lipid disease is related to elevated serum triglyceride levels and not directly to the serum level of cholesterol. In the present report, serum triglycerides in cholesterol-fed, cold-exposed animals remained at or near the control level, whereas serum esterified cholesterol was markedly elevated. Still, the incidence and severity of experimental atherosclerosis was identical to that in cholesterol-fed animals maintained at 25°C, whose serum triglycerides and esterified cholesterol were both markedly elevated. Thus, it appears that the dietary production of experimental atheroma in rabbits by cholesterol feeding is directly related to elevated serum esterified cholesterol and is not necessarily associated with high serum triglycerides.

SUMMARY

Young male albino rabbits were maintained for 10 weeks at 1°C or at 25°C with either commercial rabbit pellets enriched with lard, or pellets containing lard and cholesterol.

No differences were apparent in the serum lipid levels or in the lipid fractions between the lard-fed control groups maintained at the two temperatures. In cholesterol-fed animals, all serum lipid fractions were lower in the cold-exposed group, particularly serum triglycerides, which remained at control levels.

Despite these serum lipid alterations in cold-exposed rabbits, the incidence and severity of experimental atheroma were the same as in the animals maintained at room temperature. Analysis of tissue lipids showed that aortic triglyceride levels were considerably lower in animals kept at 1°C.

Free and esterified cholesterol, and phospholipid levels of the thoracic aorta were comparable in the cholesterol-fed groups at both temperatures.

Cholesterol feeding resulted in marked accumulation of cholesterol esters in the adrenal gland. However, this fraction, as well as the triglyceride level, was lower in the group maintained at 1°C than in the comparable group at 25°C.

Changes in the liver lipid partition due to cold-exposure were not as marked as in other tissues. Control groups at both temperatures had similar hepatic lipid levels, whereas in the cholesterol-fed groups, there was less accumulation of triglyceride and esterified cholesterol in the animals maintained in the cold.

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Effect of Age on the Response of Chickens to Dietary Protein and Fat[1,2]

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The influence of dietary protein level on the incorporation of sodium acetate-1-C^{14} into liver cholesterol and fatty acids has been studied by Nishida et al. ('60). They noted that the incorporation of acetate into fatty acids and possibly into cholesterol was increased by feeding a low protein diet. It has been shown that low protein feeding in rats (Benton et al., '56; Litwack et al., '52) and in chicks (Nishida et al., '60) increases the lipid content of the liver. More recently (Marion and Edwards, '62a) showed that the protein level of the diet influences the response of fat-deficient chicks to corn oil supplementation. It was also shown in this study that the levels of linoleic and arachidonic acids in the liver are dependent upon age of the birds as well as protein and fat level of the diet.

The present study was undertaken to determine the response of female chickens to dietary protein and fat from early age to maturity. From our previous observations (Marion and Edwards, '62a, b) it appears that at least two critical periods occur during the development of the chicken when dietary fatty acids as supplied by corn oil are essential for optimal performance. These periods are during early growth and during early egg production. The essentiality of linoleic acid in the diet of the young chick has been established by Machlin and Gordon ('61), whereas the role of essential fatty acids in the diet of the laying hen has not been as clearly defined. The criteria used during this study were growth, feed consumption, size and lipid character of the liver, and the incorporation of acetate-1-C^{14} into liver lipid components. After the chickens reached sexual maturity, observations were also made on the number and size of eggs produced, and on the fatty acid composition of eggs and various tissues of the hen. It was possible to further clarify the role of dietary fat and the influence of protein level on the metabolism of fat during the stages of growth and egg production in female chickens as a result of this study.

PROCEDURE

One hundred and sixty female Athens-Canadian Randombred chicks were divided into 4 groups of 40 each. Each group received ad libitum one of the experimental diets shown in table 1 from one day of age. The only alteration of the diets throughout the 28-week experiment was to increase the levels of vitamin A, vitamin D_3 and calcium when the chickens were 16 weeks of age to provide adequate levels of these nutrients for egg production. All diets contained the same level of metabolizable energy to minimize differences in caloric intake. The method of keeping the diets isocaloric is similar to the one reported by Rand et al. ('58) and consisted of lowering the level of glucose monohydrate and adding additional cellulose when corn oil was added to the diet. The diets supplied by analysis 14.4 or 24.1% of protein (N × 6.25) and contained two levels of fat (zero and 5% of added corn oil) at each protein level.

The chicks were housed in heated battery brooders until 4 weeks of age. They were then transferred to rearing batteries for an additional 6 weeks. From 10 weeks of age until the termination of the experiment all birds were housed in individual

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TABLE 1

Composition of experimental diets

Diet no.	1	2	3	4
	%	%	%	%
Isolated soybean protein[1]	16.00	26.67	16.00	26.67
Methionine hydroxy analogue (90%)	0.44	0.74	0.44	0.74
Glycine	0.20	0.33	0.20	0.33
Corn oil	—	—	5.00	5.00
Cellulose	4.36	5.00	12.46	13.10
Glucose monohydrate	73.18	61.44	60.08	48.34
Choline chloride (70%)	0.29	0.29	0.29	0.29
Defluorinated phosphate	3.33	3.33	3.33	3.33
Limestone	0.18	0.18	0.18	0.18
Antioxidant[2]	0.015	0.015	0.015	0.015
Mineral mixture[3]	+	+	+	+
Vitamin mixture[4]	+	+	+	+

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

[2] 1,2-Dihydro-6,ethoxy-2,2,4-trimethyl quinoline. Monsanto Chemical Co., St. Louis.

[3] Mineral mixture supplies per 100 gm of feed: (in gm) NaCl, 0.75; KCl, 0.60; $MgSO_4 \cdot 7H_2O$, 0.26; and the following expressed as milligrams per 100 gm of feed: $FeSO_4 \cdot 7H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 1.1; $CoCl_2 \cdot 6H_2O$, 1.1; KI, 1.1; $Na_2MoO_4 \cdot 2H_2O$, 0.11; $ZnSO_4 \cdot 7H_2O$, 16.5; $MnSO_4 \cdot H_2O$, 17.0.

[4] Vitamin mixture supplies per 100 gm of feed: (in mg) vitamin A (325,000 USP units/gm), 2.44; vitamin E (275 IU/gm), 26.1; vitamin D_3 (325,000 USP units/gm), 0.20; thiamine·HCl, 1.98; riboflavin, 1.98; Ca pantothenate, 3.30; pyridoxine·HCl, 0.99; niacin, 3.96; folic acid, 0.66; biotin, 0.07; vitamin B_{12} (1 mg/gm), 3.30; *p*-aminobenzoic acid, 16.5; inositol, 165.0; menadione (63%), 0.53.

cages. While in the rearing batteries and cages the birds were held at a room temperature of approximately 25°C. Throughout the experimental period the birds were kept on raised wire floors.

At one, 5, 12 and 28 weeks of age 4 birds from each diet treatment were chosen at random and dosed intraperitoneally with sodium acetate-1-C^{14}. Thirty minutes after dosing the birds were killed and the livers were excised, weighed, frozen in liquid air and stored at −10°C until laboratory analysis. The livers were prepared individually for analysis by partially thawing, homogenizing and freeze-drying. Aliquots were then weighed into Teflon-capped bottles and cold-extracted for 24 hours with 30 to 60° bp petroleum ether by agitation on a mechanical shaker. The petroleum ether extract was separated by filtration and dried under a stream of nitrogen. The lipid extract was weighed and then fractionated on silicic acid columns into major lipid fractions by the method of Hirsch and Ahrens ('58). Aliquots of each fraction were transferred to stainless steel planchets and the radioactivity determined by counting in a low background beta counter. Corrections were made for mass absorption and the results were calculated for each lipid component of the liver as a percentage of dose.

The birds were weighed at intervals throughout the experiment and feed consumption was recorded. After sexual maturity all eggs laid were recorded and weighed. During the last three weeks of the trial, hatchability of eggs was determined as previously reported (Marion and Edwards, 1962b). During the last two weeks of the experiment part of the eggs were saved for quality measurements and for lipid analysis. Interior egg quality was determined by breaking out the eggs and measuring the height of the thick albumen after the eggs had been held at 25°C for three weeks. This value was used to calculate Haugh Units (Haugh, '37). Egg shell thickness was determined with a micrometer. The eggs collected for laboratory analysis were treated in the same manner as the excised livers with respect to drying, extraction and fractionation of the lipid material. Aliquots of lipids from egg, liver, plasma, heart and adipose tisuse of mature birds were subjected to gas-liquid chromatography (GLC). These lipids were prepared individually for GLC by forming methyl esters of the fatty acids with a dry HCl-methanol solution.

Statistical significance was determined for most measurements by an analysis of variance in conjunction with the multiple range test of Duncan ('55). Differences

at the 5% probability level were considered significant.

RESULTS

Mean body weights of birds taken during the experiment are given in figure 1. Birds receiving the high protein and corn oil diets grew at a significantly faster rate until 16 weeks of age at which time the response only to corn oil was significant. No significant differences in body weight were noted at 28 weeks of age.

Liver weight as a percentage of body weight (fig. 2) and liver lipids as a percentage of dry matter (fig. 3) each decreased during early life of the birds. No significant treatment effect on liver weights was noted at one week of age, whereas at 5 and 12 weeks the group receiving the high protein, corn oil diet had significantly lower liver weights than those receiving either of the low fat diets. At 28 weeks the liver weights of the high protein, low fat group differed significantly only from those of the high protein, corn oil group. In general, liver lipids were decreased by raising the level of dietary protein or adding corn oil, or both. However, these differences were not significant at 12 and 28 weeks of age.

Liver lipid fractions as separated by silicic acid chromatography are shown in table 2. At one week of age the lipids for each treatment were combined and fractionated. Although a statistical analysis of this data could not be made, the values for cholesterol, cholesterol esters, glycerides and phospholipids were generally lowered by raising the protein level and by adding corn oil to the diet. Exceptions to this were the levels of free cholesterol and phospholipids which were not influenced by protein level in the presence of corn oil. At all other time intervals the

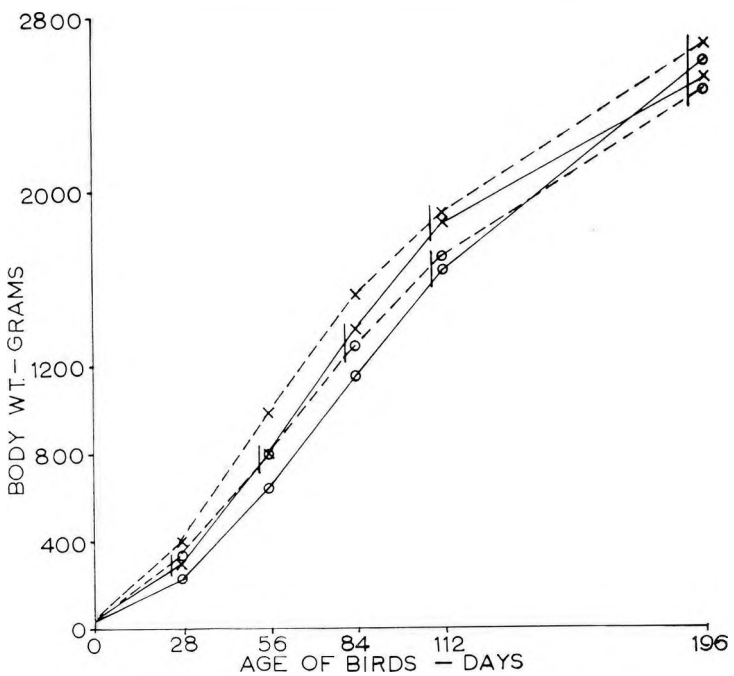

Fig. 1 Influence of dietary protein and fat on growth rate of birds. Means bordered by same adjacent vertical line are not significantly different ($P < 0.05$).

Legend:

% *Protein*	% *Corn oil*	
14.4	0	o ——— o
24.1	0	o - - - - o
14.4	5	x ——— x
24.1	5	x - - - - x

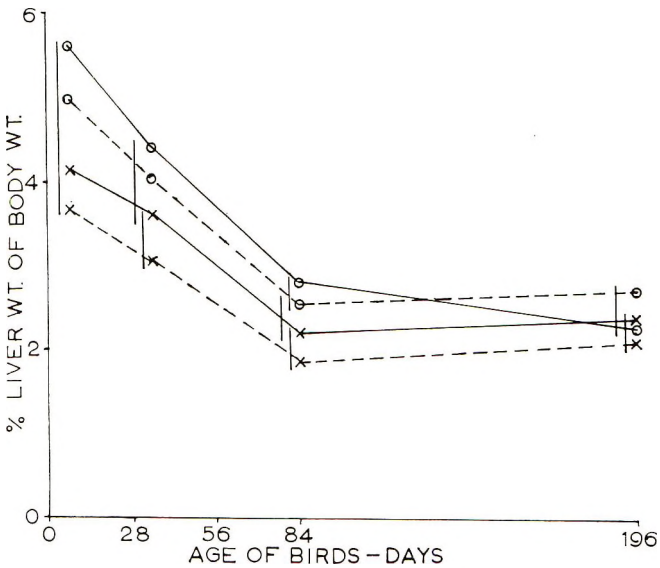

Fig. 2 Effect of diet and age on liver weights of birds. Means bordered by the same adjacent vertical line are not significantly different ($P < 0.05$).

Legend:

% Protein	% Corn oil	
14.4	0	o ——— o
24.1	0	o - - - - o
14.4	5	x ——— x
24.1	5	x - - - - x

values are means of 4 determinations and have been analyzed statistically for treatment differences. The only significance noted was in the cholesterol ester fraction at 5 and 12 weeks, and the glyceride fraction at 5 weeks. At 5 weeks cholesterol esters were significantly lowered by feeding corn oil and by elevating the protein level in the absence of corn oil. This same cholesterol ester pattern was noted at 12 weeks except that the mean value noted for the high protein, low fat diet was much higher than expected. At 5 weeks the glyceride levels were influenced by diet in the same manner as the cholesterol ester level. However, the only significance noted was between the low protein, low fat diet and the diets supplemented with corn oil.

The incorporation of acetate-1-C^{14} into liver lipid fractions (table 2) was less variable within treatment at 5 weeks than at the other time intervals. At 5 weeks mean values for each fraction were lower when the protein level was raised and when corn oil was fed. However, statistical significance was noted only in the cholesterol ester, glyceride and phospholipid fractions between the low protein, low fat diet and the other diets. At 12 and 28 weeks the trend of acetate incorporation into the glyceride fraction was the same as noted at 5 weeks even though differences due to diet treatment were not significant. At 12 weeks the incorporation of acetate into the phospholipid fraction was significantly different between the low protein, low fat diet and the high protein diets.

The silicic acid fractions of egg lipids are shown in table 3. Free cholesterol, cholesterol esters, total glycerides and phospholipids were not influenced by diet. However, more of the glycerides appeared as the mono- and di- forms rather than as triglycerides when the low protein, corn oil diet was fed.

The fatty acid compositions of depot fat and egg fat are shown in table 4. The fatty acid content of the liver and heart is shown in table 5 and the fatty acids of the plasma in table 6. When considered together these results show that the fatty acid pattern of each of these sites tended

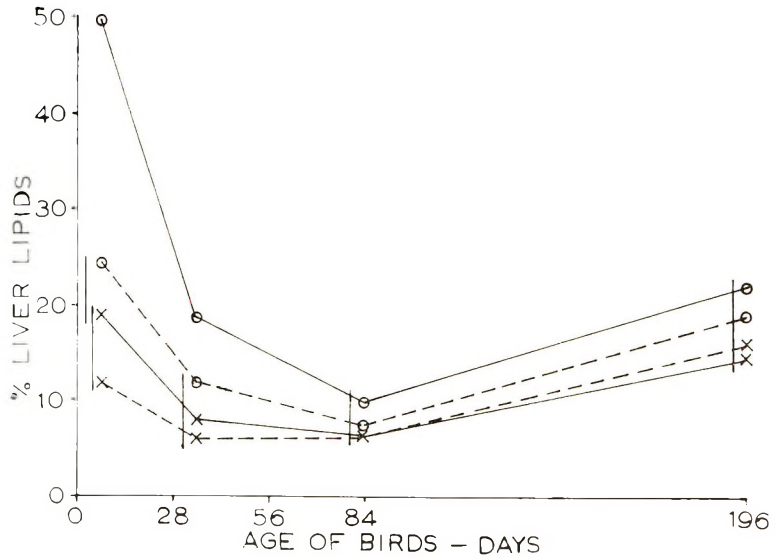

Fig. 3 The effect of diet and age on liver lipids in birds. Means bordered by the same adjacent vertical line are not significantly different ($P < 0.05$).

Legend:

% Protein	% Corn oil	
14.4	0	o ——— o
24.1	0	o - - - - o
14.4	5	x ——— x
24.1	5	x - - - - x

TABLE 2

Influence of diet and age on liver weight and acetate incorporation into liver lipid fractions

Fraction	Diet		Age of birds in weeks						
			1	5		12		28	
	Protein	Corn oil	Liver wt	Liver wt	Acetate incorporation[1]	Liver wt	Acetate incorporation	Liver wt	Acetate incorporation
	%	%	mg[2]	mg		mg		mg	
Cholesterol	14.4	0	46	54	4.06	85	2.49	340	2.12
	24.1	0	25	65	2.43	67	0.16	193	1.96
	14.4	5	13	58	4.79	69	0.45	245	2.62
	24.1	5	14	38	3.10	90	0.51	550	1.62
Cholesterol esters	14.4	0	98	102[a 3]	10.55[a]	166[ab]	3.15	336	1.45
	24.1	0	34	41[b]	2.10[b]	220[a]	3.14	278	2.35
	14.4	5	28	19[c]	0.73[b]	112[bc]	2.91	130	1.21
	24.1	5	16	7[c]	0.78[b]	52[c]	0.86	74	0.91
Glycerides	14.4	0	636	485[a]	46.48[a]	441	14.09	3090	19.61
	24.1	0	250	310[ab]	21.27[b]	274	7.57	2187	16.00
	14.4	5	138	161[b]	21.88[b]	278	12.31	1746	17.01
	24.1	5	73	122[b]	12.88[b]	308	3.52	1177	6.43
Phospholipids	14.4	0	33	88	3.43[a]	192	2.48[a]	453	2.37
	24.1	0	17	62	1.52[b]	95	0.51[b]	829	2.37
	14.4	5	9	93	1.87[b]	156	1.47[ab]	456	3.82
	24.1	5	10	60	1.08[b]	140	0.50[b]	450	2.99

[1] Expressed as a percentage of total intraperitoneal dose of sodium acetate-1-C^{14} per liver fraction.
[2] Expressed as total milligrams of fraction per liver.
[3] Values having different superscript letters are significantly different ($P < 0.05$).

TABLE 3

Effect of diet on egg lipid fractions

Protein, %	14.4	24.1	14.4	24.1
Corn oil, %	0	0	5	5
Fraction	%	%	%	%
Hydrocarbons	1.0	0.9	2.5	1.2
Cholesterol	7.1	7.2	8.6	7.4
Cholesterol esters	1.6	1.6	1.6	2.4
Triglycerides	76.9	76.0	49.1[1]	71.9
Diglycerides	2.5	2.1	16.2[1]	4.6
Monoglycerides	1.1	0.9	12.6[1]	4.6
Phospholipids	10.0	11.4	9.4	7.9

[1] Significantly different from values obtained with other diets ($P < 0.01$).

TABLE 4

Fatty acid composition of eggs and depot fat

Protein, %	14.4	24.1	14.4	24.1	14.4	24.1	14.4	24.1
Corn oil, %	0	0	5	5	0	0	5	5
	Egg fat				Depot fat			
	%	%	%	%	%	%	%	%
Fatty acid[1]								
14:0	0.4±0.1[2]	0.3±0.1	0.3±0.1	0.3±0.1	0.4±0.1	0.4±0.2	0.2±0.1	0.2±0.1
14:1	0.2±0.1	0.1±0.1	0.3±0.1	0.0	0.0	0.0	0.0	0.0
16:0	27.6±2.8	26.8±0.4	27.0±1.4	25.7±0.9	28.9±1.6	30.8±0.7	22.7±0.9	23.6±1.3
16:1	9.1±2.0	6.9±0.9	4.3±0.1	4.5±0.3	12.5±1.8	11.1±0.9	3.5±0.4	4.3±0.9
18:0	4.9±0.7	5.6±0.7	8.1±1.5	6.0±0.9	3.2±0.8	4.2±0.4	3.4±0.5	3.6±0.5
18:1	57.0±5.1	58.5±1.1	40.3±2.6	47.1±1.2	55.0±2.9	51.8±1.4	43.2±1.2	33.8±3.2
18:2	0.7±0.2	1.7±0.1	19.2±0.1	16.4±2.5	0.0	1.6±0.3	26.9±1.7	34.3±1.9
20:4	0.0	0.0	0.4±0.3	0.0	0.0	0.0	0.0	0.0

[1] First number is carbon chain length, second the number of double bonds.
[2] Each value is a mean of 4 determinations shown with the standard error of the mean.

TABLE 5

Fatty acid composition of liver and heart fat

Protein, %	14.4	24.1	14.4	24.1	14.4	24.1	14.4	24.1
Corn oil, %	0	0	5	5	0	0	5	5
Tissue	Liver				Heart			
	%	%	%	%	%	%	%	%
Fatty acid[1]								
14:0	0.5±0.1[2]	0.6±0.1	0.3±0.1	0.2±0.1	0.6±0.1	0.7±0.2	0.4±0.1	0.4±0.0
14:1	0.1±0.1	0.1±0.1	0.0	0.0	0.1±0.1	0.5±0.2	0.2±0.1	0.4±0.2
16:0	29.6±1.9	31.4±2.4	26.8±0.7	26.1±1.0	28.1±0.9	26.5±1.3	22.8±1.1	21.7±0.6
16:1	8.1±1.4	8.6±2.0	2.5±0.3	3.1±0.5	9.4±0.9	10.6±2.4	4.5±0.4	3.2±0.3
18:0	7.0±1.0	7.7±1.3	11.6±1.0	10.4±1.0	8.2±0.8	7.2±1.3	8.1±1.2	7.7±1.0
18:1	54.1±2.7	51.2±3.3	41.8±2.5	42.4±1.4	48.2±2.0	48.5±1.7	37.5±1.7	32.1±1.9
18:2	0.6±0.2	0.6±0.3	15.9±2.7	17.0±3.1	2.4±0.2	3.3±0.4	23.3±2.9	30.0±1.4
20:3	0.0	0.0	0.0	0.0	0.6±0.2	0.3±0.3	0.0	0.0
20:4	0.0	0.0	1.0±0.4	0.9±0.4	2.4±0.8	2.5±0.9	3.3±1.6	4.3±1.5

[1] First number is carbon chain length, second the number of double bonds.
[2] Each value is a mean of 3 or 4 determinations shown with the standard error of the mean.

TABLE 6

Fatty acid composition of plasma fat

Protein, %	14.4	24.1	14.4	24.1
Corn oil, %	0	0	5	5
	Plasma			
Fatty acid[1]	%	%	%	%
14:0	0.4±0.1[2]	0.3±0.2	0.3±0.1	0.3±0.2
16:0	25.7±1.0	32.4±4.1	29.8±4.0	31.5±1.5
16:1	10.1±1.0	9.3±3.3	4.3±0.5	4.5±0.8
16:2	0.0	0.0	0.3±0.3	1.0±0.4
18:0	4.2±0.2	4.5±0.7	9.9±0.8	6.2±1.5
18:1	58.2±2.1	52.2±6.3	49.6±3.1	48.7±2.7
18:2	1.5±0.4	1.2±0.6	5.8±1.8	7.2±4.1
18:3	0.0	0.0	0.0	0.1±0.1
20:4	0.0	0.0	0.0	0.3±0.3

[1] First number is carbon chain length, second the number of double bonds.
[2] Each value is a mean of 3 or 4 determinations shown with the standard error of the mean.

to reflect the composition of the dietary fat when corn oil was fed. In the absence of corn oil the content of linoleic acid (18:2) was reduced, whereas that of palmitoleic acid (16:1) and oleic acid (18:1) was increased. The level of stearic acid (18:0) appeared to be elevated slightly by corn oil feeding only in the liver and plasma, whereas palmitic (16:0) was slightly reduced by corn oil feeding in the liver, heart and depot fat. Linolenic acid (18:3) was found in traces only in the plasma when both high protein and corn oil were present in the diet. Arachidonic acid (20:4) was found in small quantities in the egg when corn oil was fed at the low protein level and in the plasma with the high protein, corn oil diet. Also, arachidonic acid appeared in heart lipids of birds with all diets and in the liver only when corn oil was fed. Eicosatrienoic acid (20:3) appeared only in small quantities in heart lipids with low fat feeding and was not present in any of the tissues sampled from birds fed corn oil.

The data on feed consumption, egg quality observation and hatchability that were collected during the trial are not presented in tabular form; however, the following observations were made. The feed conversion (unit of feed consumed per unit of weight gain) was improved by both high protein and corn oil feeding during the growth period. The dry matter and lipid content of eggs, interior egg quality, and shell thickness were not influenced by diet treatment. Hatchability of fertile eggs was highest when corn oil was fed although the differences were not statistically significant.

The data on egg production and egg weights are not presented since the level of protein in the diet did not influence these measurements. Also, the increase in egg production resulting from corn oil feeding and egg size that was noted in the present trial has been previously reported (Marion and Edwards, '62b).

DISCUSSION

The slow growth rate of chicks reared with the low fat diets in this experiment is typical of chicks having an essential fatty acid deficiency (Machlin and Gordon, '61). The growth response to added corn oil was very pronounced by 4 weeks of age and remained so during the first 16 weeks. The decreased growth rate accompanying the low level of dietary protein during the early growing period appeared to be corrected with age more quickly than the slow growth due to low fat feeding. These data show clearly that birds deficient in either corn oil or protein cannot readily compensate for these deficiencies during the early growing period, and additional time is required for full body weight to be attained.

A deficiency of dietary fat and a low protein level have each been shown to produce an enlarged and fatty liver during the early life of the chick (Marion and

Edwards, '62a). The data here substantiate these observations and show further that this general relationship extends throughout the growing period of the chicken. In the present experiment, the influence of dietary fat on liver size and lipid content was not significant at 28 weeks. This was probably due to the small number of observations, because it has been demonstrated that both of these measurements are significantly lowered when corn oil is added to the diet of fat-deficient hens (Marion and Edwards, '62b).

A general trend toward a lowering of the glyceride and cholesterol content of the liver by corn oil and high protein feeding was noted throughout the present experiment. Lorenz et al. ('38) showed that adding fat to the diet of chickens decreased the neutral fat but not the cholesterol ester and phospholipid fractions of the liver. Our data substantiate the results of Nishida et al. ('60) which showed that an accumulation of liver cholesterol is accompanied by an increased synthesis of fatty acids. The present data also show that the accumulated cholesterol is primarily in the esterified, rather than the free form.

The synthesis of cholesterol from acetate in the liver was not greatly influenced by diet treatment. However, the uptake of acetate into cholesterol did not appear to be inhibited by a high level of cholesterol in the liver as has been reported by Mukherjee and Alfin-Slater ('58). The acceleration of acetate incorporation into glycerides by feeding the low protein or low fat diets appeared to be positively correlated with acetate uptake into cholesterol esters. Labeling of the fatty acids of phospholipids probably accounts for the influence of diet on the incorporation of acetate into the phospholipid fractions. This has support since the data show no statistically significant influence of diet on the phospholipid content of the liver.

Total glycerides, free and combined cholesterol and phospholipid fractions of the egg were not influenced by diet. The lack of diet effect on lipid content, lipid fractions and dry matter of the egg were discussed previously (Marion and Edwards, '62b). No explanation can be offered at present for the increased amounts of mono- and di- forms of glycerides when the low protein, corn oil diet was fed. Eggs from this same diet, however, had the highest level of linoleic acid.

The increase in linoleic acid content of the various sites sampled from hens fed corn oil showed essentially the same pattern as noted in the livers of chicks (Marion and Edwards, '62a). Also, the present data indicate that a high dietary protein level is beneficial in maintaining body stores of linoleic acid in the absence of corn oil in the diet.

The presence of eicosatrienoic acid in the heart lipids of birds fed the low fat diets indicates that these birds had a borderline deficiency of essential fatty acids, if this measure has the same significance in hens as in chicks (Machlin and Gordon, '61). This was expected since the quantities of linoleic acid supplied by the low fat diets were by analysis 0.16% for the low protein level and 0.27% for the high protein level. Since linoleic acid is not synthesized by the hen (Murty et al., '60) and since only small quantities of this acid were supplied at the low protein level in this experiment, this may explain the effect of protein level on the linoleic acid levels of the egg and depot fat. Because significant differences in rate of egg production did not occur, no conclusion may be made as to the essentiality of linoleic acid for egg production. Yet, it was noted that the low protein, low fat diet supported the lowest production rate and that this was the only diet which allowed linoleic acid to disappear from the depot fat. These data support previous results (Jensen et al., '58; Marion and Edwards, '62b) in which it was shown that the addition of corn oil to the diet increased egg size. The former workers attributed the response in egg size to the presence of an unknown fat-soluble factor in crude corn oil. The present data on the fatty acid composition of eggs, plasma, livers and depot fat indicate that the factor responsible for the increased egg size is the linoleic acid which is supplied by corn oil.

SUMMARY

A high level of protein and the presence of corn oil in the diet of growing female chickens each increased body weights, de-

creased the size and lipid content of the liver, and decreased the incorporation of sodium acetate-1-C^{14} into liver lipids. The changes in liver lipid content were largely a reflection of changes in the level of glycerides and cholesterol esters in this organ. Generally, these measures were not significantly affected by diet after the birds reached sexual maturity.

The analysis of eggs, plasma, heart, liver and depot fat from mature birds showed that the fatty acid content of these sites was altered little by protein level. Adding corn oil to the diet increased the level of linoleic acid in these sites and decreased the palmitoleic and oleic acid content.

Egg weights and egg production were not significantly influenced by dietary protein level. However, higher values for these measurements were noted when corn oil was added to the diet.

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Metabolism of Labeled Linoleic-1-C^{14} Acid in the Sheep Rumen[1]

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The fate of lipids which make up approximately 5% of the dry matter consumed by herbivorous animals has not been well defined. About 75% of the fatty acid content of grass is composed of 18 carbon unsaturated fatty acids as reported by Hilditch ('56). Domesticated herbivores also consume lipids in feedstuffs such as hay, silage, and concentrates of both vegetable and animal origin.

Depot fats of simple-stomach animals are modified to resemble dietary fats when the diet is rich in unsaturated fats; however, the depot fats of ruminants are relatively unaltered. Mattson and Lutton ('58) reported that the iodine number of ruminant depot fat averaged only one-half the value of nonruminants. Hilditch and Stainshy ('35) suggested that the peculiarities of the glyceride structure of ruminant fat could be due to hydrogenation of preformed oleoglycerides. Reiser[5] incubated linseed oil with rumen contents and observed a decrease in linoleic acid content which was attributed to hydrogenation by rumen microorganisms. Reiser and Reddy ('56), Shorland et al. ('55), Hartman et al. ('54), and Shorland et al. ('57), have obtained results to support Reiser's findings.

It is generally accepted that lipids are absorbed through the lymphatic system and get into the blood stream via the thoracic duct. McAnally and Phillipson ('42), Danielli et al. ('45), Kiddle et al. ('51), Johnson ('51), Masson and Phillipson ('51) and others have shown that acetic, propionic, and butyric acids are absorbed from the rumen via the portal blood system. However, no data were found on absorption of longer chain fatty acids from the rumen, either via the lymphatics or portal blood system.

Pennington ('51, '54), Pennington and Pfander ('57) and Knox et al. ('60), using an *in vitro* technique, reported that rumen epithelium has the ability to metabolize acetic, propionic, and butyric acids. They also found that butyric acid was metabolized more readily than propionic or acetic acids. Literature on metabolism of longer chain fatty acids in the rumen is sparse.

This investigation was carried out to determine the extent of absorption, *in vivo* hydrogenation, and metabolism of linoleic acid in the rumen.

EXPERIMENTAL

Western wethers of mixed breeding were surgically prepared for linoleic-1-C^{14} acid absorption studies (table 1). After a 24-hour fast the sheep were anesthetized with sodium pentobarbital. Using aseptic techniques, the reticulo-omasular orifice of two wethers was ligated twice with double braided umbilical tape, and two additional wethers were ligated with Ferguson angiotribe tongue and grooved forceps. After ligation, the abdominal cavity and muscle edges were dusted with sulfanilamide powder and closed with silk sutures. The sheep were then placed in a small pen and injected intramuscularly with 1,000,000

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[5] Reiser, R. 1951 Hydrogenation of poly-unsaturated fatty acids by ruminants. Federation Proc., *10*: 236 (abstract).

TABLE 1

Experimental animal and dosing data

Sheep no.	Weight	Time dose in rumen	Total DPM[1]/dose
	pounds	*hours*	
1011	119	48	6.30×10^8
1221	115	50	6.55×10^8
399	57	41[2]	4.56×10^8
383	73	34[2]	5.29×10^8

[1] Disintegrations per minute.
[2] Died.

units of penicillin G and 1.25 gm of streptomycin sulfate. After the wethers regained consciousness, they were dosed by injecting through the abdominal wall directly into the rumen approximately 0.25 mc of linoleic-1-C^{14} acid[6] (1 mc/17.6 mg) in 2.5 ml of ethanol which had been brought to 50 ml with oleic acid. After dosing with the entire 50 ml, the sheep were held in a metabolism crate as described by Briggs and Gallup ('49).

Fifteen milliliters of blood were collected in heparinized blood tubes from the jugular vein at zero, 1, 2, 4, 8, 12, 16, 24, 36 and 48 hours after dosing. Tissue samples were taken at the time the animals were killed or immediately after death if the animal did not survive. Liver, lung, spleen, kidney, rumen-reticulum, and contents of the rumen-reticulum were excised, weighed, and sampled. Rumen tissue samples were taken from the dorsal sac extending across the right longitudinal groove and into the ventral sac. Omasum contents were taken to check on possible leakage from the rumen. The squamous epithelia and serosa were removed from the rumen and reticulum tissues and discarded. The muscularis was removed from the mucosa and both were finely divided and treated as the other tissues. All organs were homogenized in a blender to facilitate the extraction of lipids. Weighed aliquots of tissue and 5 ml of blood were extracted twice with 40-ml portions of chloroform:methanol (2:1 v/v) for 30 minutes each in a continuous-type extractor;[7] then the extract was filtered and evaporated to near dryness. Twenty drops of 30% aqueous hydrogen peroxide were added to oxidize extracted chromogens, then 10 ml of xylene were added and mixed well. Two milliliters of the xylene layer were removed and added to 15 ml of (xylene)[8] scintillator. Samples were cooled, counted, in a liquid scintillation spectometer equipped with a coincidence counter of high efficiency, internal standard added, recounted, and efficiencies determined.

Steam distillation of blood. At intervals from 4 to 24 hours post-dosing, approximately 50 ml of blood were taken from each sheep and steam distilled until 400 ml of distillate were collected from each sample. Potassium hydroxide was added to the distillate until basic to phenophthalein and evaporated to near dryness in an oven at 90°C. The residue was acidified with 15 ml of concentrated phosphoric acid and extracted 10 hours with 35 ml of petroleum ether in a liquid-liquid extractor. The ether was then evaporated, using a stream of dry nitrogen gas in a 5-dram counting vial, then 15 ml of scintillator (toluene)[9] were added and counted as previously described. Fifty grams of rumen contents acidified with 50 ml of 50% sulfuric acid, to which 75 ml of acetone and 75 ml of ethanol were added, were steam distilled until 400 ml of distillate were collected. The distillate was divided into two portions which were saponified with KOH, evaporated, liquid-liquid extracted, and counted as described above. One portion was counted to determine amount of activity while the other was used to determine what compounds contained the activity.

Partition chromatography. Steam volatile fatty acids from the rumen contents were separated on partition chromatograms as described by Elsden ('46). The acids from the rumen contents were not sufficiently concentrated to give distinct yellow bands; therefore, it was necessary to add 30, 60 and 60 μliters of acetic, butyric, and valeric acids, respectively. The effluent was divided into 5 fractions; that of valeric or faster flowrate, butyric, propionic, acetic, and slower than acetic. Due to the color of each fraction, it was neces-

[6] Volk Chemical Company, Chicago, Illinois.
[7] Goldfisch.
[8] Consisting of 385 ml xylene, 385 ml dioxane, 230 ml ethanol, 80 gm naphthalene, 5 gm 2,5 diphenyloxazole and 0.5 gm 1,4-bis (5 phenyl-2-oxazolyl)-benzene.
[9] Consisting of 4 gm of 2,5 diphenyloxazole and 0.1 gm 1,4-bis (5-phenyl-2-oxazolyl)-benzene in one liter of toluene.

sary to saponify, evaporate, acidify, and liquid-liquid extract before counting each fraction in the toluene scintillator.

Paper chromatography. A procedure using a hydroxamic acid derivative as described by Block et al. ('52) was used for chromatographing the steam volatile fatty acids. Methyl esters were prepared by a procedure that will be described later. Hydroximates were spotted on Whatman 3-mm paper in duplicate with one of the duplicates cut from the paper before development. After development, sections from the undeveloped duplicate corresponding to the spots on the developed one were counted for activity. Several modifications of the technique as described by Inouye et al. ('55) were used for paper chromatography of additional compounds of unsaturated fatty acid methyl esters. Ten microliters of the ether layer containing the mercuric acetate compounds were spotted in duplicates on Whatman 3-mm paper previously dipped in kerosene. Standards and unknowns were developed for 24 hours with 90% methanol, acetic acid, and kerosene (30:1:1 v/v) using the ascending technique. After drying, one of the duplicates was removed with a sharp knife while the other was developed by spraying with 0.2% solution of diphenylcarbazone in ethanol. The R_F values of the unknowns were compared with known standards. Sections from the undeveloped paper, corresponding to the spots on the developed one, were placed in 10 ml of toluene scintillator and counted.

Liquid-liquid extraction of rumen contents. Approximately 25 gm of acidified rumen contents was liquid-liquid extracted for 24 hours with 125 ml of petroleum ether. The extract was hydrolyzed with 50 ml of 80% ethanol containing 1 N sulfuric acid. The free fatty acids were extracted with petroleum ether and then evaporated in a 22 × 125 mm culture tube with a screw cap fitted with a polyethylene center and subsequently lyophilized to remove any traces of water.

Methylation. The fatty acids were refluxed 4 hours with 5 ml of absolute methanol containing 4% concentrated sulfuric acid at 85°C. After the tubes were removed from the water bath, 5 ml of water were added and then the contents were extracted three times with 5-ml portions of petroleum ether. All washings were evaporated with nitrogen gas and the volume rebuilt to 5 ml with absolute methanol. Methyl esters of stearic, palmitic, and myristic acids were separated by crystallization at − 35° to − 40°C. Standards of methyl linoleate, oleate and methyl esters of laurate and shorter chain acids remained in solution while the saturated methyl esters of stearic, palmitic and myristic acids were precipitated. The solutions were filtered through Whatman no. 31 filter paper and washed with cold absolute methanol at reduced temperatures. Filter papers containing the saturated methyl esters were extracted with petroleum ether, evaporated, and restored to the 5-ml volumes with absolute methanol. The filtrates evaporated to 5-ml and 100-μliter duplicates of the saturated and unsaturated fractions were counted.

RESULTS AND DISCUSSION

Experimental animal performance. The 4 sheep with reticulo-omasular orifice ligatures refused to eat or drink during the entire period, which suggests that this area may control the appetite of the sheep. Other sheep treated in a similar manner but without reticulo-omasular orifice ligatures ate and drank soon after regaining consciousness.

Absorption of labeled compounds from the sheep rumen. Total activity of labeled linoleic-1-C^{14} acid in the dose administered to each sheep is shown in table 1. The validity of the absorption studies depends on the reticulo-omasular orifice ligature to prevent activity from passing into the lower digestive tract. Data from sheep 1011 and 1221 were not used in the absorption studies because activity appeared in omasal contents as shown in table 3. However, absorption data from 399 and 383 are considered to be valid. Activity per milliliter of blood at intervals after dosing indicates a limited amount of absorption from the rumen (fig. 1).

The results in table 2 show that most of the absorbed activity appeared in the non-steam distillable fraction, indicating activity in compounds 10 or more carbon atoms long. The steam distillable fraction contained activity indicating degradation

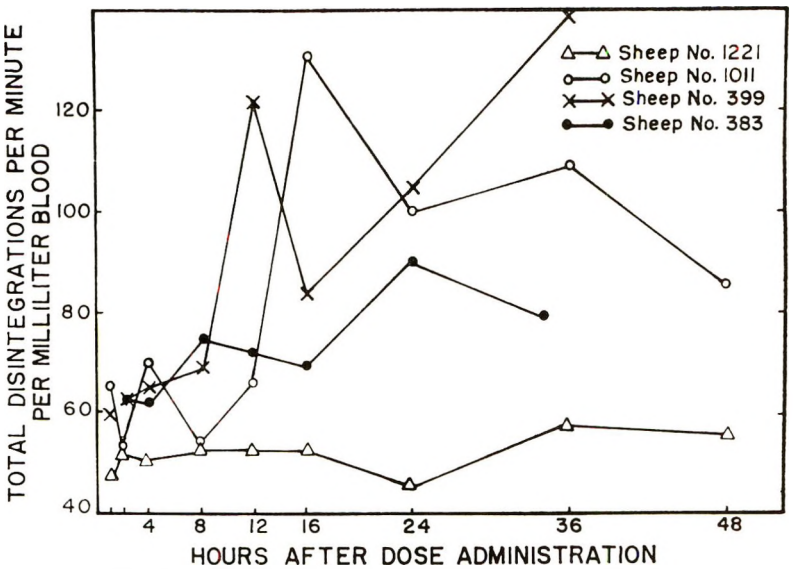

Fig. 1 Absorption of C-14 activity into sheep blood.

TABLE 2

C^{14} activity in steam volatile and nonvolatile fractions of blood

Animal no.	Quantity distilled	Steam distillable	Non-steam distillable
	ml	*DPM*[1]	*DPM*
399	48	46	837
383	49	50	779

[1] Disintegrations per minute.

of linoleic acid to compounds of less than 10 carbon atoms.

Total disintegrations per minute per gram of tissue and percentage of dose in specific tissues and organs are shown in table 3. About 0.3 to 0.5% of the total dose appeared in the rumen mucosa and muscularis which had to be absorbed through the epithelia. The percentage of dose in the rumen mucosa and muscularis was based on the total weight of the rumen and reticulum, and should be considered valid since the reticulum contained as much activity as the rumen, although more variable.

Gray et al. ('52), Pennington ('51, '54), Pennington and Pfander ('57), Knox et al.[10] and others have shown that rumen epithelia has the ability to metabolize butyric and shorter chain acids. The rumen epithelium may also have the ability to metabolize long-chain unsaturated fatty acids, which could give rise to metabolic products that could be absorbed into the mucosa and muscularis. On the other hand, linoleic acid may be absorbed through the epithelium into the mucosa and muscularis without change. The specific activity of the fat extracted from the muscularis was too low to be related to any particular fatty acid on a paper chromatogram.

Total activity which had to be absorbed from the rumen, ranged from 0.1 to 0.001% of the total dose in the liver, lung, kidney, and spleen, with the highest activity appearing in the liver.

Previous work by Wood[11] on normal sheep showed that activity from labeled linoleic-1-C^{14} appeared in portal blood one hour after oral dosing with 0.5 mc of activity. Little activity was found until 12 hours, then it increased rapidly and reached a peak at 36 hours at the same level in both jugular and portal blood. At 51 hours the relative concentration of radioactivity was: > kidney fat > rumen contents, > liver > kidney > feces > lung > urine > brain.

[10] Knox, K., E. E. Flambae, R. M. Johnston and G. W. Ward 1960 *In vitro* metabolism of short chain fatty acids in bovine rumen epithelial tissue. Federation Proc., 19: 323 (abstract).

[11] Wood, R. D. 1961 *In vivo* and *in vitro* studies in metabolism of fat in the rumen using C^{14}-labeled linoleic acid. M. S. Thesis, University of Kentucky, Lexington, Kentucky.

TABLE 3

Activity and percentage of dose in various tissues from sheep dosed directly into the rumen with approximately 0.25 mc of linoleic-1-C^{14} acid

Tissue		Animal numbers			
		1011	1221	399	383
Rumen mucosa	DPM/gm	5,072	54,325	4,222	6,768
	% dose[1]	0.30	3.00	0.16	0.28
Rumen muscularis	DPM/gm	1,543	1,124	1,298	1,381
	% dose	0.18	0.13	0.11	0.14
Rumen contents	DPM/gm	189,553	170,892	152,615	143,425
	% dose	86	86	97	92
Liver	DPM/gm	636	704	496	885
	% dose	0.04	0.12	0.06	0.09
Lung	DPM/gm	142	378	422	128
	% dose	0.01	0.01	0.04	0.01
Kidney	DPM/gm	112	243	267	294
	% dose	0.004	0.004	0.005	0.005
Spleen	DPM/gm	378	186	386	201
	% dose	0.003	0.003	0.002	0.001
Reticulum mucosa[2]	DPM/gm	5,835	17,074	1,067	22,529
Reticulum muscularis[2]	DPM/gm	641	596	1,333	1,322
Omasum contents[2]	DPM/gm	1,933	1,525	148	20
Kidney fat[2]	DPM/gm	213	26	258	26

[1] Percentage of dose carried to two significant figures with exception of kidney and spleen.
[2] Total weight unknown for determining percentage of dose.

Degradation of labeled linoleic-1-C^{14} acid in the rumen. It has already been noted that steam distillation of blood revealed the presence of some volatile material with activity. The percentage of dose in the steam volatile fraction of rumen contents is shown in table 4. To assure that the steam volatile fraction was not present in the dosing solution, labeled linoleic acid was steam distilled but no activity appeared in the volatile fraction. Fractional separation on silica gel partition column revealed that the activity of the steam volatile fraction appeared in compounds of 5 carbon atoms or longer. Using paper chromatography of hydroximates, the R_F value was found to be greater than that of pelargonic acid, which is the last steam volatile member of the homologous series. This suggests that the steam volatile compound has a hydroxyl group, double bond, or some radical that caused the higher R_F value. This degrada-

TABLE 4

C^{14} activity in steam-volatile fraction of rumen contents

Animal no.			DPM/gm in fraction R[2]
	DPM[1]/gm	*% of dose*	
1011	1,365	0.62	965
1221	1,349	0.68	979
399	1,498	0.95	973
383	1,372	0.88	1,188

[1] Disintegrations per minute.
[2] Fraction R = eluent containing valeric and higher fatty acids.

TABLE 5

Activity of rumen fatty acids from linoleic-1-C^{14} acid

	Animal number				Methyl linoleate-C^{14}
	383	399	1011	1221	
Saturated,[1] %	38.94	51.03	50.02	39.85	0.02
Oleic or elaidic acid, %	50.68	34.85	32.73	39.18	0.80
Linoleic acid, %	5.69	2.88	5.19	4.07	99.18
Miscellaneous[2]	4.69	11.24	12.06	16.90	0

[1] Percentage saturated includes methyl stearate, methyl palmitate, and methyl myristate.
[2] Includes everything from R_F zero to R_F 0.242.

tion product of linoleic acid was steam volatile, saponifiable, ether extractable, had a larger R_F value than that of pelargonic acid, was 5 or more carbons long, and amounted to 0.6 to 1% of the total dose.

Hydrogenation of labeled linoleic-1-C^{14} acid in the sheep rumen. From 85 to 96% of the dose remained in the rumen (table 3). The lipids were separated to determine whether the labeled linoleic acid had undergone any change while in the rumen. An aliquot of the dosing solution was methylated and treated in the same manner as the samples, to show the validity of the methods used. On the average, 45% of the original labeled linoleic-1-C^{14} acid was completely saturated in the rumen (table 5). This is somewhat higher than values reported by Shorland et al. ('57). The unsaturated fatty acids were separated on paper chromatograms and the chromatograms were cut into sections at the following R_F values: zero, 0.055, 0.242, 0.688, 0.860, and one. These sections were cut into small pieces, placed in 10 ml of (toluene) scintillator, and counted. The R_F values of the standards were: methyl oleate and methyl elaidate, 0.37 to 0.55; methyl linoleate, 0.78; and methyl linolenate, 0.92. These R_F values corresponded well with the R_F values on the chromatograms from the rumen lipids. Methyl oleate and elaidate were not completely separated and, therefore, were counted together.

Only three to 6% of the original labeled linoleic acid appeared as such, whereas 33 to 50% were hydrogenated to oleic or elaidic acids. This is in good agreement with the work of Shorland et al. ('57). The 5 to 17% listed as miscellaneous in table 5 came from R_F value zero to R_F value 0.242. This is made up of unidentified acids, probably corresponding to those of low R_F values found in the rumen muscularis and may include the steam volatile degradation product described previously.

SUMMARY

Four western wethers with ligated reticulo-omasular orifices were used to conduct rumenal fat studies using labeled linoleic-1-C^{14} acid. Tagged compounds appeared in the jugular blood about 4 hours after dosing and increased rapidly between the eighth and twelfth hours. Most of the activity appeared in the non-steam distillable fraction, indicating that the compound absorbed from the rumen contained 10 or more carbons. About 0.3 to 0.5% of the total dose appeared in the rumen mucosa and muscularis. Activity also appeared in the liver, lung, kidneys, spleen, and kidney fat. Of the total dose, from 0.6 to 1.0% was degradated in the rumen. Eighty-five to 96% of the dose was recovered from the rumen of which only 3 to 6% was the original linoleic acid. Approximately 45% was hydrogenated to saturated acids, whereas 33 to 50% had been hydrogenated to oleic or elaidic acids. Unidentified acids accounted for only 5 to 17% of the recovered dose.

ACKNOWLEDGMENTS

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Vitamin E Deficiency in the Monkey

V. ESTIMATED REQUIREMENTS AND THE INFLUENCE OF FAT DEFICIENCY AND ANTIOXIDANTS ON THE SYNDROME[1]

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Muscular dystrophy and anemia, both of which can be prevented or cured by α-tocopherol therapy, regularly develop in young rhesus monkeys supplied with a purified diet deficient in vitamin E (Dinning and Day, '57a). The muscular dystrophy resembles nutritional muscular dystrophy in other animals,[3] but severe anemia due to vitamin E deficiency has been found only in the rhesus monkey. The anemia appears to be due primarily to inadequate erythropoiesis and is associated with abnormal multinucleated erythroid precursors in the bone marrow (Porter et al., '62). The response of the anemia to α-tocopherol is dramatic and similar to the response of other nutritional anemias to a specific nutrient (Porter et al., '62; Dinning and Day, '57a).

Several biochemical abnormalities occur in vitamin E-deficient monkeys but the exact role of vitamin E in the metabolism of bone marrow and skeletal muscle has not been delineated (Dinning and Day, '57a, b, '58). The vitamin undoubtedly functions as an antioxidant, *in vivo*, and it has been suggested that all of the signs of vitamin E deficiency may be explained by the lack of this antioxidant function which might allow oxidative deterioration of unsaturated cellular lipids (Zalkin and Tappel, '60). An antioxidant role of vitamin E is supported by the observations that dietary unsaturated fatty acids modify the requirement for vitamin E (Dam, '57; Century and Horwitt, '60; Bieri et al., '60) and that certain antioxidants such as N,N′-diphenyl-*p*-phenylenediamine (DPPD) have a beneficial effect in vitamin E-deficient animals (Dam, '57; Scott, '62; Draper and Csallany, '58). It is of importance that one of the manifestations of vitamin E deficiency in the chicken, encephalomalacia, can be prevented completely by various antioxidants (Scott, '62), and that reducing the dietary intake of unsaturated fatty acids greatly reduces or eliminates the requirement for vitamin E in the chicken (Bieri et al., '60).

The diets that were previously used to induce vitamin E deficiency in the monkey contained significant amounts of unsaturated fatty acids (Dinning and Day, '57a), which could have been partially responsible for the syndrome that was produced. It therefore seemed important to determine the effect of unsaturated fatty acids and antioxidants on the deficiency syndrome, especially since anemia is an unusual sign of vitamin E deficiency and may indicate a specific function of the vitamin. The present report describes experiments in which the effects of a fat-deficient diet and DPPD on vitamin E deficiency in the monkey were tested. The fat-deficient diet did not prevent the anemia or any of the other signs of vitamin E deficiency, and although DPPD had a beneficial effect, it did not produce a complete recovery of the vitamin E-deficient monkeys.

METHODS

Immature rhesus monkeys initially weighing between 1.5 and 2.5 kg were

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[2] Studies carried out during the tenure of a National Vitamin Foundation—Russell M. Wilder Fellowship.
[3] Fitch, C. D., J. S. Dinning, R. S. Young, P. L. Day and H. N. Marvin 1960 Some histological changes in the vitamin E-deficient monkey. Federation Proc., *19*: 420 (abstract).

supplied with a purified vitamin E-deficient diet (Dinning and Day, '57a) or the same diet in which the fat was replaced by a similar weight of starch (Fitch et al., '61). The diets were supplied ad libitum and the amount consumed by each monkey varied from 100 gm/day initially to about 500 gm/day during the final year of the experiment. Three milliliters of cod liver oil per day were given to each of the monkeys receiving the diet containing fat, and each of the monkeys that received the fat-deficient diet were given 400 IU of vitamin D_3 and 2 mg of vitamin A acetate in ethanol orally daily. Each monkey was given 0.05 to 0.1 μg of vitamin B_{12} daily. Six monkeys received the diet containing fat and 4 received the fat-deficient diet.

In an attempt to keep the serum vitamin E concentration near the initial values, three control monkeys (animals no. 214 and 217 received fat in the diet and no. 220 received the fat-deficient diet) were each given DL-α-tocopherol in doses ranging from 20 mg/week initially to 240 mg weekly divided into three doses during the last year of the experiment. The tocopherol was given either orally as the acetate dissolved in ethanol or intramuscularly as the disodium salt of the phosphate ester dissolved in water. When DPPD was used, 5 ml of a suspension in glycerol containing 20 mg of DPPD and 0.01 ml of polyoxyethylene sorbitan monooleate (Tween 80) per ml were given orally each day.

After the animals had received the purified diets for 11 months a monkey in another experiment developed tuberculosis. None of the monkeys in the present experiments had a positive skin test for tuberculosis or abnormal chest x-ray examination, but because of the exposure to tuberculosis, each monkey was given 100 mg of isoniazid in the diet daily for one month followed by 66 mg daily (10 to 22 mg/kg of body weight) during the remainder of the experiment. None of the animals in these experiments developed tuberculosis. The basal diet contained pyridoxine, and there were no signs of pyridoxine deficiency as a result of the isoniazid therapy during the course of these experiments.

Serum vitamin E concentrations (Rindi, '58), hemoglobin concentrations, reticulocyte counts, creatine-to-creatinine ratios of urine collected without contamination by food (Folin, '14) and body weight were measured frequently. A biopsy was obtained from the triceps, gastrocnemius or a paraspinous muscle as each monkey became vitamin E-deficient. Biopsies at similar times were also obtained from the control monkeys.

Because of the possibility that abnormalities of the electrocardiogram may appear early in vitamin E-deficient monkeys (Filer et al., '49), a Sanborn twin beam Cardiette was used to obtain electrocardiograms of each monkey without anesthesia at the beginning of the experiment, after one year, and when the severe signs of vitamin E deficiency were present. The three standard limb leads and the augmented unipolar limb leads were routinely obtained and the three chest leads described by Atta and Vanace ('60) were obtained in most instances. The records were standardized so that 1 mv caused a deflection of 1 cm, and the paper was run at the speed of 75 mm/sec.

RESULTS

The serum vitamin E concentrations are shown in figure 1. The rate at which the serum concentration of vitamin E decreased was similar in both groups of deficient animals and reached the limit of reliability of the method in about 400 days. The one fat-deficient monkey that was supplemented with vitamin E showed a consistent decrease in the serum concentration of the vitamin after one year. This might be due to a decreased intestinal absorption of vitamin E similar to the decreased absorption of carotenoids in humans consuming low-fat diets (Roels et al., '58).

The rate of growth of the various groups of animals are shown in figure 2. The animals accepted the purified diet well and selected it in preference to a chow diet if both were offered at the same time. The diet supported rapid growth when it was supplemented by vitamin E, but in the absence of this supplement, growth ceased and the animals began to lose weight as an early manifestation of the deficiency

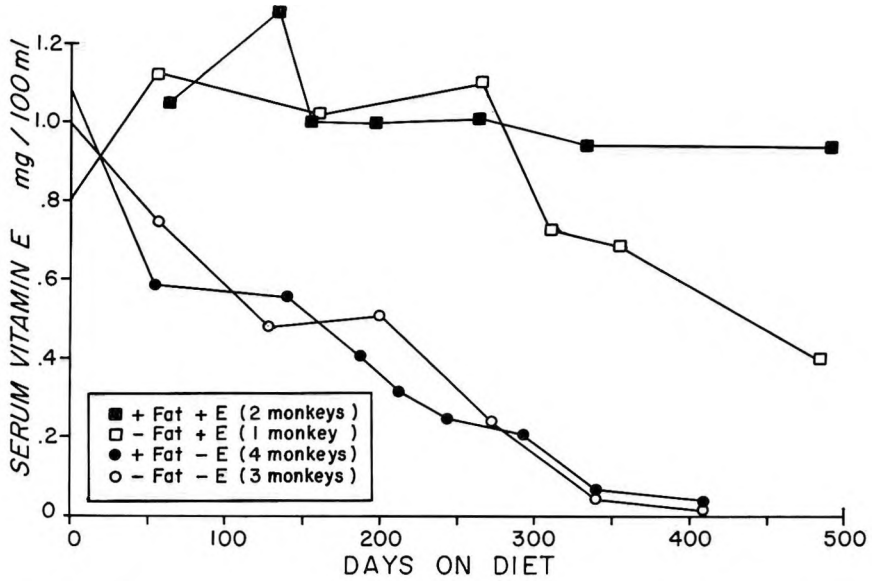

Fig. 1 Rate of decrease of serum vitamin E concentration in vitamin E-deficient monkeys.

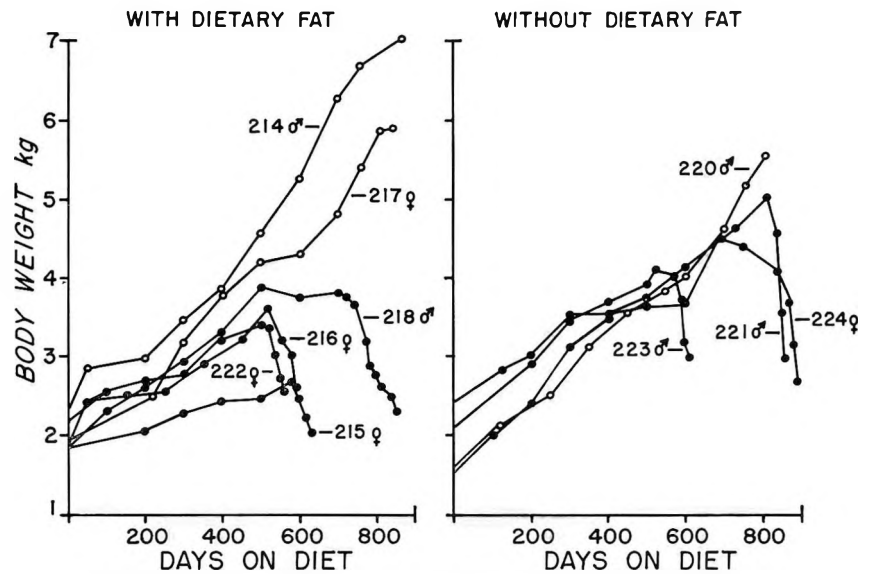

Fig. 2 Effect of vitamin E deficiency on growth. Open circles = with vitamin E; closed circles = without vitamin E.

syndrome. Despite progressive debility the monkeys continued to eat well.

As the muscular weakness developed the ratio of urinary creatine-to-creatinine increased rapidly (fig. 3). A ratio of 1.5 or above indicates a severe deficiency state which if not corrected would lead to death in a few days. The creatinuria responded promptly to α-tocopherol therapy, usually within 48 to 72 hours. The creatine-to-

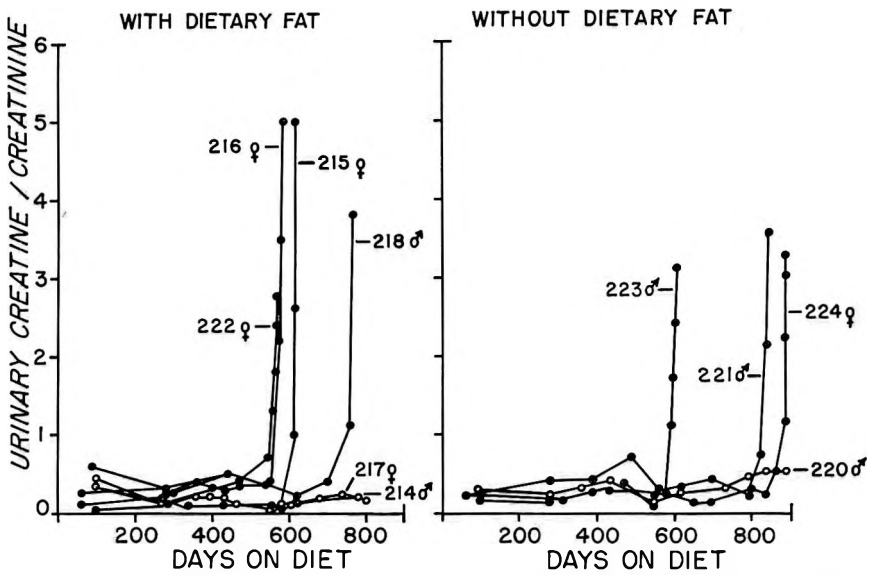

Fig. 3 Effect of vitamin E deficiency on creatine excretion. Open circles = with vitamin E; closed circles = without vitamin E.

creatinine ratio of the control monkeys was never greater than 0.5 and seldom greater than 0.3.

Muscle biopsies were obtained as the monkeys in each group developed significant creatinuria. Examples of the histological lesions are shown in figure 4. Degeneration of the muscle fibers associated with increased numbers of sarcolemma nuclei and rowing of nuclei within muscle fibers was present in the muscle from each vitamin E-deficient monkey. The absence of dietary fat did not appreciably alter the histological appearance of the muscle in the control monkey nor did it affect the lesions caused by the lack of vitamin E.

The anemia of vitamin E deficiency also developed regardless of the fat content of the diet (fig. 5) and progressed rapidly until the animal expired or received appropriate treatment. The anemia, as well as the other signs of vitamin E deficiency, tended to develop later in the animals receiving the fat-deficient diet which suggests that the requirement for vitamin E may have been reduced in this group.

Vitamin E requirements were estimated for some of the monkeys by a method that gives reasonable results for the rabbit (Mackenzie and McCollum, '40). An example of a recovery experiment that allows an estimate of the vitamin E requirement is shown in figure 6. The average daily requirement was determined after a single dose of α-tocopherol by dividing the amount of tocopherol by the number of days that the urinary creatine-to-creatinine ratio remained below one. The results of this calculation are shown in table 1. A combination of DL-α-tocopheryl acetate orally and the disodium salt of DL-α-tocopheryl phosphate intramuscularly was used to recover this animal which was supplied with the fat-deficient diet. The recovery was prompt and complete with the estimated requirement being 0.7 mg of DL-α-tocopherol per kg of body weight per day.

TABLE 1

Vitamin E requirement of monkeys

Monkey no.	Diet	DL-α-Tocopherol required
		mg/kg body wt/day
222	With fat	2.9
216	With fat	2.8
222	With fat	2.0
223	Without fat	0.7

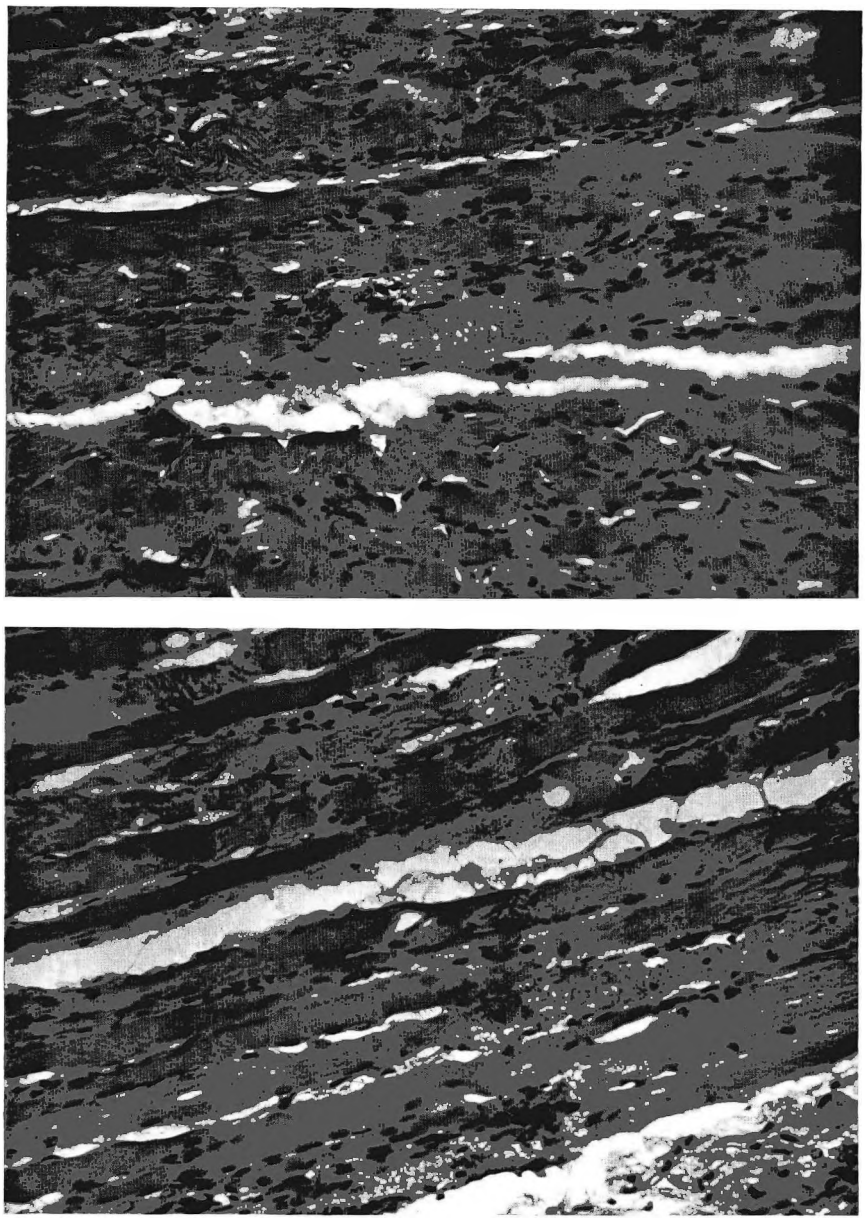

Fig. 4 Skeletal muscle lesions in vitamin E-deficient monkeys. The upper photomicrograph is from a monkey that developed vitamin E deficiency while receiving a fat-deficient diet. The other photomicrograph is from a monkey that received the diet containing fat.

This is in contrast to requirements of over 2 mg per kg of body weight per day for two of the animals that received fat in the diet. The same method gives estimated requirements of about 1 mg of α-tocopherol/kg of body weight for the rabbit (Mackenzie and McCollum, '40).

The effect of DPPD was studied in two vitamin E-deficient monkeys that received the diet containing fat. In both instances the administration of DPPD was begun when creatinuria, mild anemia, and obvious muscle wasting were present, a time at which the animals would not be ex-

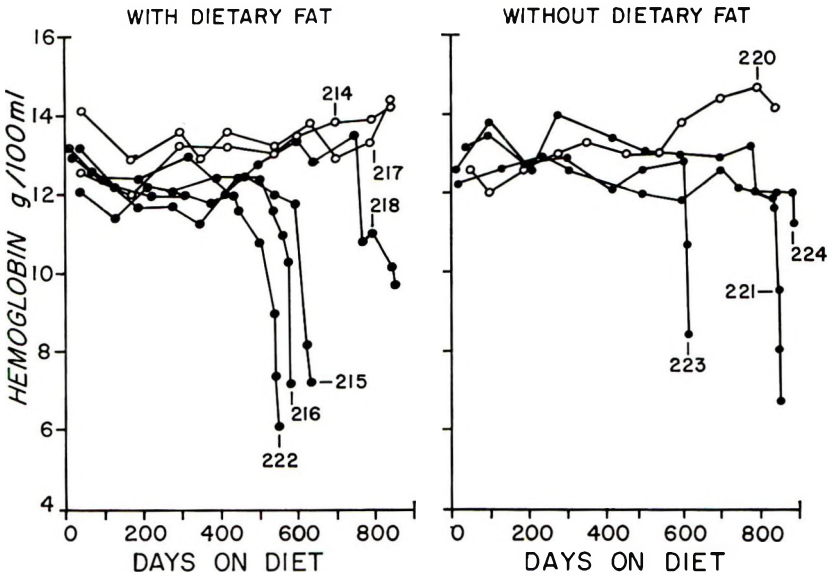

Fig. 5 The anemia of vitamin E deficiency. Open circles = with vitamin E; closed circles = without vitamin E.

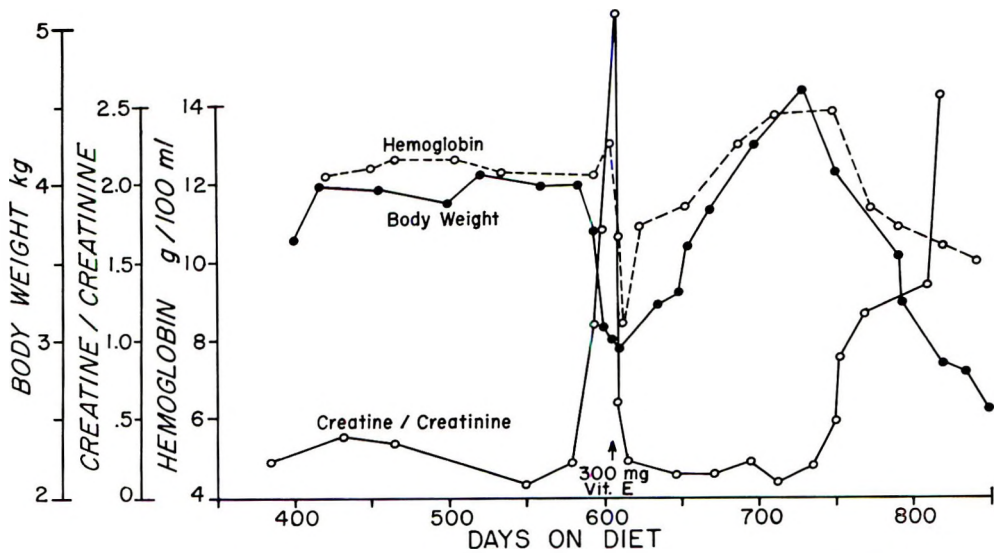

Fig. 6 Monkey 223, fed vitamin E-deficient diet without dietary fat, and recovery with vitamin E.

pected to live more than two weeks without vitamin E therapy. Both monkeys responded similarly. The creatinuria decreased but did not reach normal levels in either animal, and the hemoglobin concentration returned toward normal in both without a very dramatic increase in reticulocyte count. This is in contrast to the reticulocyte response to α-tocopherol, an example of which is shown in figure 7. A single oral dose of 150 mg of DL-α-tocopheryl acetate was given to obtain this response, and the animal was again clearly vitamin E-deficient one month later when DPPD was started.

One monkey was maintained for 44 days with DPPD and was clinically in about the same condition as when DPPD

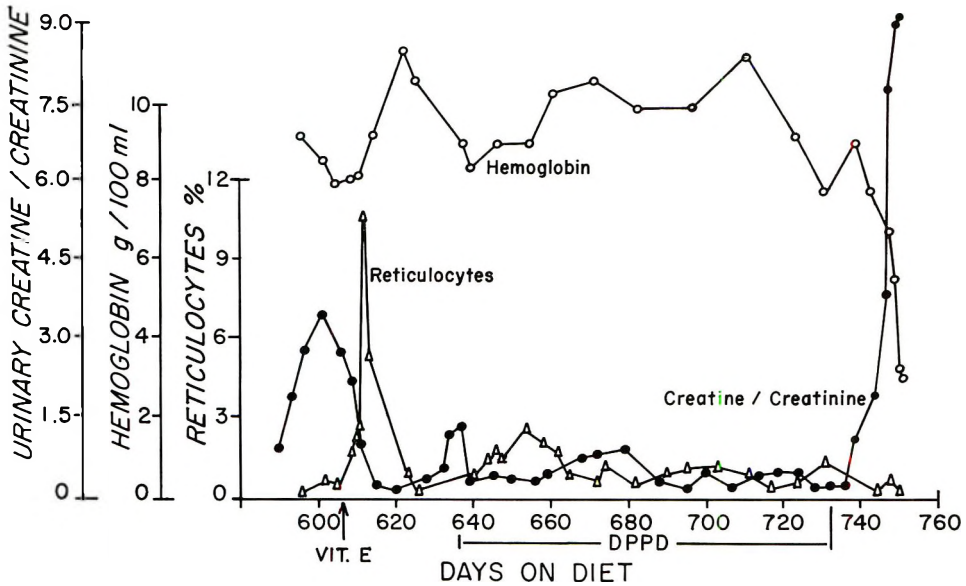

Fig. 7 Monkey 222, fed vitamin E-deficient diet with dietary fat; effect of N,N′-diphenyl-*p*-phenylenediamine.

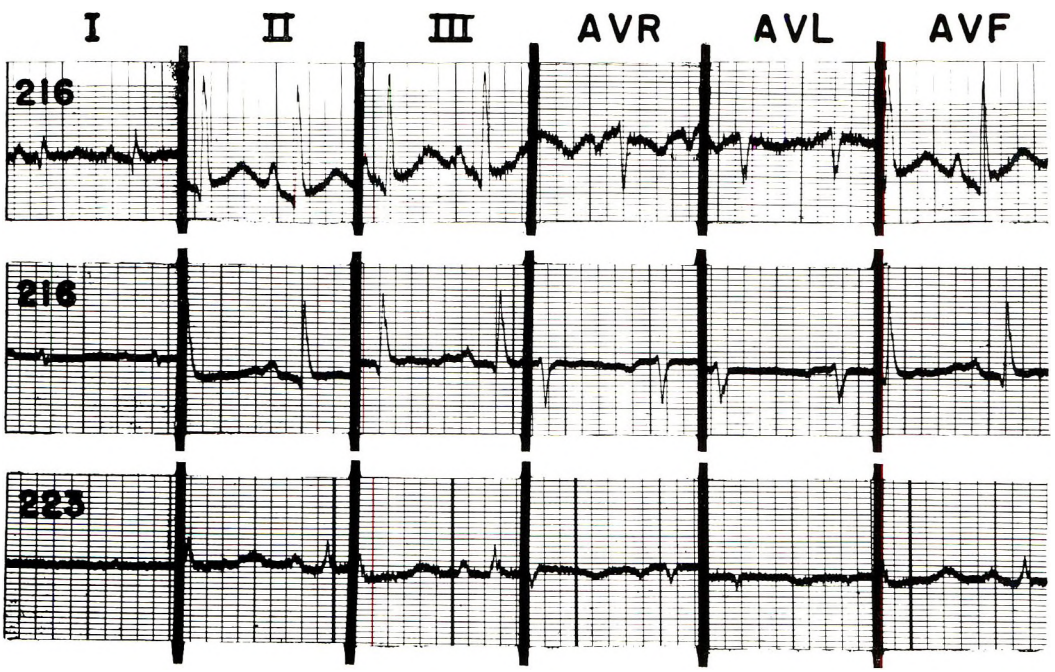

Fig. 8 Effect of vitamin E deficiency on the electrocardiogram of the monkey. The top tracing was obtained at the beginning of the experiment. The standardization of this tracing was slightly in error but note the ST segment elevation in leads II, III, and AVF. The second tracing is from the same monkey almost two years later when it was severely vitamin E-deficient. The third electrocardiogram was obtained when monkey 223 was severely dystrophic. Note that there are no T wave or other abnormalities.

was started. It had not gained a significant amount of weight but the hemoglobin was 13 gm/100 ml of blood and the urinary creatine-to-creatinine ratio was consistently between 0.5 and one. The response of the other monkey is shown in figure 7. It was maintained for 95 days with DPPD and likewise did not have a significant weight gain. Despite a low urinary creatine-to-creatinine ratio and no deterioration of its clinical appearance after DPPD was started, this animal was becoming anemic when DPPD was discontinued. It then showed rapid progression of the muscular dystrophy and anemia and died within three weeks. The beneficial effect of DPPD is in agreement with the results in other animals (Dam, '57; Scott, '62; Draper and Csallany, '58).

A high incidence of electrocardiographic abnormalities was observed in these experiments. Significant elevation of the ST segments was present in several leads of the electrocardiograms of two monkeys at the beginning of the experiments when respiratory infections were prevalent. The ST segments subsequently returned to the baseline and flattening of the T wave occurred. These two animals became vitamin E-deficient several months later and at autopsy each of the monkeys had a thickened adherent pericardium. The electrocardiograms of one of these monkeys is contrasted with that of another vitamin E-deficient monkey, in figure 8. Except for the two animals that had pericarditis the electrocardiograms from the vitamin E-deficient monkeys showed no abnormalities (table 2). Likewise there were no changes that could be attributed to fat deficiency. The electrocardiogram of one of the control monkeys showed significant prolongation of the QRS complex (table 2) with the pattern of a right bundle branch block.

The failure to find electrocardiographic abnormalities in the vitamin E-deficient monkey confirms the relative resistance of the heart to vitamin E-deficiency (Mason and Telford, '47). The high incidence of pericarditis presumably of viral etiology emphasizes the need for carefully controlled studies before ST segment and T wave abnormalities can be attributed to

TABLE 2

Summary of electrocardiographic data

Condition	Monkey no.	Rhythm	Rate	P–R interval	QRS duration	Q–T interval	QRS axis
Control[1]	—	Sinus	235 (167–273)	0.070 (0.050–0.080)	0.031 (0.024–0.038)	0.151 (0.140–0.170)	+75° (+65° +90°)
Vitamin E-deficient	215	Sinus	238	0.068	0.028	0.170	+90°
Vitamin E-deficient	216	Sinus	171	0.080	0.039	0.180	+90°
Vitamin E-deficient	222	Sinus	240	0.080	0.035	0.180	+75°
Vitamin E-deficient	223	Sinus	171	0.080	0.028	0.210	+90°
Control	214	Sinus	260	0.072	0.048	0.190	+150°

[1] The average with the range found in 9 control monkeys is shown. The heart rate is given as beats per minute. The time intervals are in seconds.

the deficiency of a nutrient such as vitamin E.

DISCUSSION

The results of these experiments support the observation in other animals that unsaturated fatty acids in the diet affect the requirement for vitamin E. However, since the fat-deficient diet readily induces fat deficiency (Fitch et al., '61) and does not prevent any of the signs of vitamin E deficiency, the latter syndrome is not directly related to the amounts of unsaturated fatty acids in the diet. Similar results have been obtained in the rabbit (Mackenzie et al., '41).

The requirements for vitamin E as we have measured them are of the same order of magnitude for the rhesus monkey as for other animals. Our estimates are probably too high since more of the vitamin might be required to recover a deficient animal than to maintain one in optimal nutrition, and since all of the vitamin that is administered as one large dose may not be stored. Thus the estimated requirement of 2 to 3 mg of DL-α-tocopherol per kg of body weight per day should be ample for the rhesus monkey.

Amounts much larger than the estimated requirements of α-tocopherol were required to keep the serum concentration near the initial levels in the three control monkeys. This implies poor utilization of the large doses of vitamin E that were used. On the other hand either the small amounts of vitamin E that are present in the purified diets or the vitamin E that is present in the tissues must be efficiently conserved to account for the prolonged period required for the monkey to develop signs of vitamin E deficiency.

The beneficial effect of DPPD, although unquestionable, is subject to at least two explanations. This and other antioxidants might function either by replacing all the functions of vitamin E or by replacing its antioxidant function and thereby reducing the requirement to minute amounts comparable to the requirement for certain other vitamins. Since the purified diets that were used in the present experiments contained appreciable amounts of vitamin E in the starch and cod liver oil (Fitch et al., '61), it is not possible to choose between these explanations. Until more evidence is at hand it would seem unwise to assume that vitamin E functions solely as an antioxidant.

Perhaps the most significant histological change in the skeletal muscle of vitamin E-deficient animals is the increased numbers of sarcolemma nuclei. Since multinucleated cells are also present in the testis of the vitamin E-deficient rat that develops sterility (Mason, '33), and in the bone marrow of the vitamin E-deficient monkey that develops anemia (Porter et al., '62), it is possible that the same metabolic defect may be responsible for many of the manifestations of vitamin E deficiency. The nature of this metabolic defect remains obscure.

SUMMARY

The influence of a fat-deficient diet and N,N′-diphenyl-*p*-phenylenediamine (DPPD) on vitamin E deficiency was studied in the rhesus monkey. Fat deficiency did not prevent the monkeys from developing the full syndrome of vitamin E deficiency including the anemia, but it appeared to reduce the requirement for vitamin E. The estimated requirement for monkeys receiving the diet containing fat was 2 to 3 mg of DL-α-tocopherol per kg body weight per day, whereas a single estimate of the requirement in a monkey supplied with the fat-deficient diet was 0.7 mg per kg of body weight per day. Also the average length of time required to develop vitamin E deficiency was longer in the fat-deficient monkeys. The DPPD had a beneficial effect in the vitamin E-deficient monkeys but the response was not complete.

Several electrocardiograms were obtained for each of the monkeys in these experiments and a relatively high incidence of abnormalities due to pericarditis was noted. No abnormalities were present in the electrocardiograms that could be attributed either to vitamin E deficiency or fat deficiency.

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Influence of Variations in Dietary Calcium: Phosphorus Ratio on Performance and Blood Constituents of Calves[1]

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An interrelationship in the metabolism of calcium and phosphorus has long been recognized and the proper ratios of the two have been established for many species of simple-stomach animals. For these species it is generally agreed that the optimal ratio of Ca:P lies between 1:1 and 2:1.

Although levels of the two elements involved influence the optimal ratio, research with rats has indicated that ratios ranging from 1:1 to 2:1 are desirable (Bethke et al., '32; Cox and Imboden, '36; Boutwell et al., '46). In growing chicks it appears that Ca:P ratios from 1:1 up to 2.5:1 may be used with satisfactory results (Bethke et al., '29; Wilgus, '31). Growing swine exhibit optimal performance when fed diets containing ratios from 1:1 to 2:1 (Bethke, '33) with the optimum being about 1.4:1 for the 100-pound pig (Chapman et al., '55).

Strangely enough, optimal ratios of Ca:P for ruminants have received little attention in the past. Huffman et al. ('33) observed satisfactory growth among dairy calves fed a ration based on alfalfa hay and containing calcium and phosphorus at a ratio between 4:1 and 5:1. Theiler et al. ('37) stated that a Ca:P ratio of 4:1 did not significantly affect performance of growing beef heifers and steers when an adequate amount of phosphorus was supplied in the ration. The National Research Council ('49) summed up the situation with respect to ruminants by the statement, "It is recognized that not all are agreed on the question of the requirements for calcium and phosphorus nor on the optimum calcium-phosphorus ratio."

The research reported herein was designed to determine the optimal Ca:P ratio or the range over which the ratio may vary without harmful effects on growing cattle, and to study the influence of variations in dietary calcium and phosphorus on the metabolism of certain micronutrients.

EXPERIMENTAL PROCEDURES

Forty-five Hereford calves averaging 114 kg were used in a factorial experiment (Snedecor, '56) with three levels of calcium and three levels of phosphorus resulting in 9 Ca:P ratios ranging from 0.4:1 to 14.3:1. The study consisted of 5 replications (four of steers and one of heifers) assigned to blocks on the basis of body weight within sexes. Two replications of the design were completed during one period and three other replications were conducted during a second period. The 5 replications were sufficiently similar to allow pooling of the data and consideration of the combined results.

A period of 4 weeks was used to accustom the young calves to a dry diet after separating them from their dams at 3 to 4 months of age. During the terminal week of this transition period the diet was changed gradually to a low-calcium, low-phosphorus semi-purified basal one, the composition of which is shown in table 1.

The basal diet analyzed 0.17% phosphorus (Koenig and Johnson, '42) and 0.27% calcium (McCrudden, '10, with modifications). Levels and ratios of cal-

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cium and phosphorus were obtained by addition of varying amounts of calcium carbonate, defluorinated rock phosphate and dibasic sodium phosphate to the basal diet. These additions resulted in the design represented in table 2.

The animals were housed in individual tie stalls with concrete floors. Wood shavings were used for bedding. The calves were individually fed, ad libitum, throughout a test period of 98 days for the first two replications and 112 days for the last three replications.

Daily feed intakes were recorded and body weights were measured at two-week intervals. Blood was sampled by jugular puncture, initially and at 14-day intervals thereafter. Serum calcium was determined by the method of Weybrew et al. ('48) and serum inorganic phosphorus was assayed by the Simonsen et al. ('46) method. The analytical procedure of Simonsen et al. ('47) was employed for serum magnesium determinations, and serum alkaline phosphatase activity was assayed by the method of Bessey et al. ('46).

TABLE 1

Composition of basal diet[1]

	%
Degerminated corn meal	40.0
Beet pulp and dried molasses	15.0
Glucose·H_2O[2]	15.0
Corn oil	5.0
Corn starch	14.5
Blood meal	9.0
Urea	0.5
NaCl	1.0
Trace minerals[3]	—

[1] Vitamin A and D concentrates were added to supply 1,531 IU of vitamin A activity and 295 IU of vitamin D/kg of feed.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] A trace mineral mixture supplied 9.07 mg of iron, 4.54 mg of copper, 4.54 mg of manganese and 0.45 mg of cobalt/kg of feed.

TABLE 2

Experimental design including calcium levels, phosphorus levels and Ca:P ratios

	Phosphorus, %		
	0.17	0.34	0.68
Calcium, 0.27%	1.6[1]	0.8	0.4
Calcium, 0.81%	4.8	2.4	1.2
Calcium, 2.43%	14.3	7.2	3.6

[1] The tabular values represent the units of calcium per unit of phosphorus. Five calves received each treatment combination (Ca:P ratio).

Data for the criteria used in the growth and blood studies were statistically analyzed using the analysis of variance technique. Linear and quadratic components were isolated to evaluate treatment effects. A covariance analysis was applied when its use appeared logical.

RESULTS

Based on the data reported herein, the semi-purified basal rations used in these studies apparently supplied the nutrients required for normal responses in calves. Average responses of the calves fed the 9 different Ca:P ratios are presented in table 3. A summary of the mean squares from the analyses of variance is shown in table 4.

Growth study. Body weight gains were markedly influenced by the treatments. Growth was more rapid with the intermediate level of dietary calcium (0.81%). This resulted in a curvilinear growth response due to calcium levels. Response to phosphorus levels was similar at the two lower levels, but growth was reduced at the 0.68% phosphorus level. This reduction in growth due to elevated dietary phosphorus was more dramatic at the lowest level of calcium (0.27%) than at higher levels.

Depression of growth attributable to Ca:P ratio aberrations was apparent at ratios below 1:1 (0.4:1 and 0.8:1) and also at the highest ratio (14.3:1). The depression was much more severe at the low than at the high ratio. These statements are illustrated in figure 1 which also shows that ratios varying from 1:1 to 7:1 did not cause significant differences in calf growth. This plot of performance by Ca:P ratios (fig. 1) revealed a growth rate curve that was remarkably smooth with optimal growth performance at a ratio of 4.8:1. The only point which did not fall directly on the curve represented the diet that contained both elements at the highest level. The depression below the expected value in this case may be due, at least in part, to the high mineral content (over 7% of added minerals) of this particular diet.

The daily feed intake ranged from 2.72 to 3.63 kg/animal with the various rations and did not prove to be different in the analysis of variance. When body weight

TABLE 3

Average responses of calves receiving various dietary calcium:phosphorus ratios[1,2]

Ca level, % P level, % Ca:P ratio	0.27 0.17 1.6	0.27 0.34 0.8	0.27 0.68 0.4	0.81 0.17 4.8	0.81 0.34 2.4	0.81 0.68 1.2	2.43 0.17 14.3	2.43 0.34 7.2	2.43 0.68 3.6
Initial weight, kg	117.5	115.7	110.7	108.9	118.8	109.3	114.3	113.9	116.1
Final weight, kg	188.7	169.2	140.6	184.6	192.8	176.4	166.9	184.6	169.6
Avg total gain, kg	71.2	53.5	29.9	75.7	74.0	67.1	52.6	70.7	53.5
Avg daily gain, gm	669	503	281	712	694	631	494	664	503
Avg daily feed, kg	3.63	3.36	2.72	3.40	3.49	3.54	3.18	3.63	3.36
Feed/gain[3]	5.6	6.7	14.6	4.8	5.1	5.9	6.5	5.5	6.9
Serum Ca, mg/100 ml									
Change[4]	+0.8	+0.3	−0.9	+1.9	+0.9	+0.2	+1.2	+0.7	+0.7
Terminal[5]	15.3	15.0	13.3	16.6	14.8	14.0	16.1	14.9	14.6
Serum P, mg/100 ml									
Change[4]	+1.5	+1.2	+1.9	−0.9	+2.0	+1.4	−1.3	+1.5	+3.3
Terminal[5]	9.4	9.7	10.2	7.7	10.2	10.3	7.2	9.8	11.3
Serum Mg, mg/100 ml									
Change[4]	+0.16	+0.31	−0.55	−0.11	+0.06	−0.29	+0.23	+0.06	+0.25
Terminal[5]	2.04	2.00	1.43	1.76	2.02	1.66	2.06	1.90	2.05
Serum phosphatase, μM units									
Change[4]	+3.1	+2.7	+0.2	+5.8	+3.0	+1.3	+3.8	+3.8	+3.2
Terminal[5]	5.6	5.4	2.6	8.4	4.9	3.5	5.4	6.1	5.5

[1] Each tabular value represents an average of 5 individuals.
[2] Average number of days on experiment was 106.4. First two replications, 98 days; last three replications, 112 days.
[3] Calculated as an average of individual feed/gain ratios.
[4] Represents difference in first and average of last three determinations on each individual.
[5] Average of last three determinations for each individual.

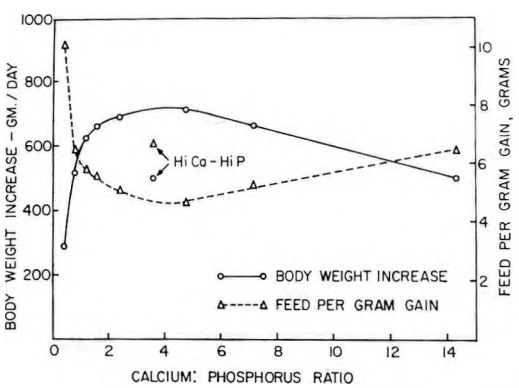

Fig. 1 Growth and feed conversion of calves fed Ca:P ratios varying from 0.4:1 to 14.3:1.

gains were adjusted for feed intake by covariance analysis, the differences in gain were still significant ($P < 0.01$). Feed intake differences attributable to treatment remained insignificant ($P > 0.05$) after covariance adjustment for average body weight during the experiment.

Efficiency of conversion of the diet to body weight gains (table 3) was most favorable with the 4.8:1 Ca:P ratio and poorest with the 0.4:1 ratio. Individual variation for this criterion (tables 4 and 5) was greater than is normally experienced in similar experiments due primarily to variation in response of calves fed the 0.4:1 ratio. A significant linear trend ($P < 0.05$) was noted in the statistical analysis indicating a decrease in feed efficiency as dietary phosphorus level increased. This, also, was attributable primarily to a low feed intake among calves fed the lowest calcium with the highest phosphorus level. Feed conversion ratios are graphically presented in figure 1 along with values for growth. Notable in this presentation is that the feed/gain ratio curve forms an almost exact inversion of the growth rate curve.

Blood studies. Serum calcium was lowest with the lowest Ca:P ratio and elevated at the higher ratios in the presence of low

TABLE 4

Summary of mean squares from the analysis of variance of growth, feed and blood data

Source of variation	df	Avg daily gain	Feed intake	Feed / gain	Serum Ca[1]	Serum P[1]	Serum Mg[1]	Serum phosphatase[1]
Replication	4	0.02	3.51*	20.06	4.90**	3.55	0.02	28.97**
Treatments	8	0.45**	2.02	45.24*	5.09**	8.28**	0.24**	12.76*
Ca levels[2]	2	0.66**	1.22	54.49	1.97	0.61	0.18	5.72
Ca linear	1	0.16	1.01	52.99	3.31	0.77	0.25*	8.69
Ca quadratic	1	1.16**	1.44	56.00	0.64	0.44	0.11	2.74
P levels	2	0.56**	1.57	59.85*	16.19**	24.15**	0.30**	24.86**
P linear	1	0.84**	1.47	93.32*	32.30**	45.51**	0.42**	48.82**
P quadratic	1	0.29	1.68	26.38	0.08	2.78	0.19	0.90
Ca × P	4	0.29	2.64	33.31	1.10	4.18*	0.24**	10.23
Ca × P linear × linear	1	0.87**	7.32*	92.49*	0.22	14.18**	0.45**	11.49
Ca × P linear × quadratic	1	0.13	0.25	8.64	2.35	0.47	0.29*	0.76
Ca × P quadratic × linear	1	0.08	2.27	22.31	1.02	0.03	0.07	19.47*
Ca × P quadratic × quadratic	1	0.07	0.71	9.78	0.80	2.04	0.15	9.20
Experimental error	32	0.095	1.318	17.78	0.85	1.48	0.054	4.61

[1] Analysis of average of last three determinations on each animal.
[2] Isolation of individual degrees of freedom.
* $P < 0.05$.
** $P < 0.01$.

TABLE 5

Coefficients of variation of response criteria used in these studies[1]

Criteria	%
Average daily gain	24
Feed intake	15
Feed/gain	62
Serum calcium	6
Serum phosphorus	13
Serum magnesium	12
Serum phosphatase	41

[1] Computed from values on 45 calves.

dietary phosphorus; however, in no case were serum calcium levels markedly changed. Dietary calcium level did not influence serum levels of this element. On the other hand, elevation of dietary phosphorus effected a distinct linear decrease in serum calcium at all dietary calcium levels.

Inorganic phosphorus levels in the serum (table 3) were not significantly influenced by dietary calcium but were markedly increased by higher levels of dietary phosphorus. The response of blood phosphorus to increases of this element is rapid and directly related to the amount in the feed (Wise et al., '58, '61).

Mean values for serum magnesium (table 3) were lowest among calves fed the 0.4:1 Ca:P ratio (1.43 mg/100 ml) and highest with the 14.3:1 ratio (2.06 mg/100 ml). Increasing dietary phosphorus levels caused a concurrent decrease in serum magnesium as indicated by the significant linear component in the statistical analysis (table 4). The influence of the high level of dietary phosphorus on serum magnesium was not in evidence in the presence of high dietary calcium. This observation is supported by the comparatively large Ca × P interaction term (table 4). Covariance adjustments of magnesium values for variations in serum inorganic

phosphorus accentuated treatment differences in serum magnesium.

Activity of the enzyme serum alkaline phosphatase was inversely related to serum inorganic phosphorus with values of 2.64 μM units at the 0.4:1 Ca:P ratio ranging up to value of 8.37 μM units at the 4.8:1 Ca:P ratio. Increasing levels of dietary phosphorus resulted in a linear decrease in serum alkaline phosphatase at the two lower dietary calcium levels but in no difference at the highest (2.43%) dietary calcium level.

DISCUSSION

The results presented herein strongly indicate that wide Ca:P ratios are tolerated by the ruminant animal. This is in agreement with previous observations by Huffman et al. ('33) and Theiler et al. ('37). The data suggest that Ca:P ratios as high as 7 or 8:1 may be fed to the ruminant without serious effects. This is in sharp contrast with the growing chicken, pig and rat which do not thrive on ratios wider than approximately 3:1 (Wilgus, '31; Bethke et al., '32, '33; Boutwell et al., '46; Chapman et al., '55).

In this research, Ca:P ratios lower than unity (0.4:1 and 0.8:1) resulted in reduced growth and poor nutrient conversion. The 0.4:1 ratio markedly reduced serum magnesium. These observations also contrast with results involving the simple-stomach animal in that Combs et al. ('62) reported an optimal Ca:P ratio of 0.9:1 for the young growing pig.

Hence, the optimal Ca:P ratio for the ruminant is higher than that for the nonruminant animal and that the ruminant will tolerate wide ratios of Ca:P (7:1) but ratios lower than unity are deleterious. This is in contrast with the nonruminant which tolerates a Ca:P ratio of less than 1:1 with ratios wider than 3:1 resulting in undesirable effects. Teleological reasoning, though admittedly hazardous, would suggest that the ruminant has subsisted and developed on forages which normally have a high Ca:P ratio, and has more highly developed physiological mechanism(s) than the nonruminant for handling higher levels of calcium. This does not, however, give insight into the mechanism(s) involved. Research is needed to elucidate this question.

Depression of serum magnesium by high levels of dietary phosphorus as observed in this study has been reported previously by O'Dell et al. ('60) working with guinea pigs and rats, and by Bunce et al. ('62) working with dogs. This depression of serum magnesium in the presence of high dietary phosphorus and low calcium perhaps reflects an excretory pattern in which magnesium furnished the cation which combined with the phosphate anion for elimination through the urinary pathway. The fact that serum calcium was also lowest at this dietary calcium-phosphorus combination supports this hypothesis. It is probable that other monovalent and divalent cations would have followed this pattern had they been measured. Hawkins et al. ('55) reported that high levels of calcium and phosphorus caused a depression of serum manganese; however, in their experiment, it was not possible to determine whether the depression resulted from high calcium or phosphorus or both.

SUMMARY

Forty-five Hereford calves were used in a factorial experiment with three levels each of calcium (0.27, 0.81 and 2.43% of the diet) and of phosphorus (0.17, 0.34 and 0.68% of the diet). The 9 resulting calcium: phosphorus ratios ranged from 0.4:1 to 14.3:1. Levels and ratios of Ca and P were obtained by addition of varying amounts of calcium carbonate, defluorinated rock phosphate and dibasic sodium phosphate to a semi-purified diet based on degerminated corn meal, starch, glucose, urea, blood meal, beet pulp, corn oil, minerals and vitamins. The calves were individually fed, ad libitum, over a period of 98 days for the first two replications and 112 days for the last three replications.

Performance and nutrient conversion were markedly decreased with Ca:P ratios lower than 1:1. Ratios between 1:1 and 7:1 gave similar and satisfactory results. Ca:P ratios above 7:1 resulted in decreased performance and nutrient conversion values, but adverse effects were not as marked as with the ratios below 1:1. Several significant trends were evi-

denced in serum inorganic phosphorus, magnesium and phosphatase activity attributable to levels and ratios of dietary calcium and phosphorus. Notable among these observations was that serum magnesium levels were decreased with high levels of dietary phosphorus when dietary calcium levels were low. Diets with high calcium levels resulted in a "normal" serum magnesium level even at the highest phosphorus level.

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Effects of a Diet High in Polyunsaturated Fat on the Plasma Lipids of Normal Young Females[1]

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The lowering of plasma lipids by diets containing adequate amounts of polyunsaturated fat has been demonstrated by Kinsell et al. ('52), Ahrens et al. ('54), Beveridge et al. ('56), and Bronte-Stewart, ('56). Most of the original studies were made with formula diets, but more recently specific mixed diets have been used to achieve similar results (Gordon et al., '58; Olson et al., '58; Jolliffe et al., '61).

The "prudent diet" used by Jolliffe and his associates ('61) had a maximal polyunsaturated/saturated (P/S) ratio of 2.0. This diet produced some reduction of the plasma cholesterol in the males studied. Those men whose total plasma cholesterol was in the lowest "tertile" at the beginning of the investigation showed less than a 10% decrease in 6 months, those in the middle "tertile," less than 15%. These observations suggest that a diet with a greater P/S ratio may be essential if one is to obtain and maintain a meaningful decrease in plasma lipids in a large percentage of "normal" individuals.

Because of the higher immunity of the female to clinically obvious atherosclerosis, most of the studies were carried out with male subjects. The present study was carried out in a group of normal young women, all "nutritionally oriented" (10 dietitians and one nurse). During the study the effects of a mixed diet with a high P/S ratio and an absolutely high linoleic acid content were evaluated with respect to acceptability, effects upon plasma lipid levels and changes in cholesterol ester fatty acid composition.

EXPERIMENTAL PROCEDURES

Eleven women between the ages of 21 to 31 participated in the study. Their fasting plasma lipids were: total cholesterol, 193 to 246; phospholipids, 200 to 260; and glycerides, 66 to 149 mg/100 ml. Daily dietary records were maintained by each individual during the entire period of investigation. Each subject regulated her own caloric intake in such a way as to maintain constant weight.

The study was divided into two dietary periods, the first extending for 42 days. During this time the subjects consumed the usual hospital diet which provided approximately 42% of the total calories as fat with a P/S ratio of 0.45 (table 1), and

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[1] Costs of the study were defrayed in part by grants from the Wesson Fund for Medical Research and Education, Corn Products Company, the Pacific Vegetable Oil Corporation, and the Alameda County Heart Association and the Nutrition Foundation, Inc.

TABLE 1

Approximate analyses of dietary fat intakes

	Total calories[1]	P/S[2]	Fatty acid composition		
			Saturated	Oleic	Linoleic
	%				
Period 1 Ad libitum	42.0	0.45	34.0	39.0	15.0
Period 2 High polyunsaturated	45.0	4.2[3]	12.0	21.0	50.0

[1] Total calories approximately 2,000/day.
[2] Polyunsaturated-to-saturated ratio.
[3] Since only linoleic acid is used in computation of P/S, the actual value will be significantly higher.

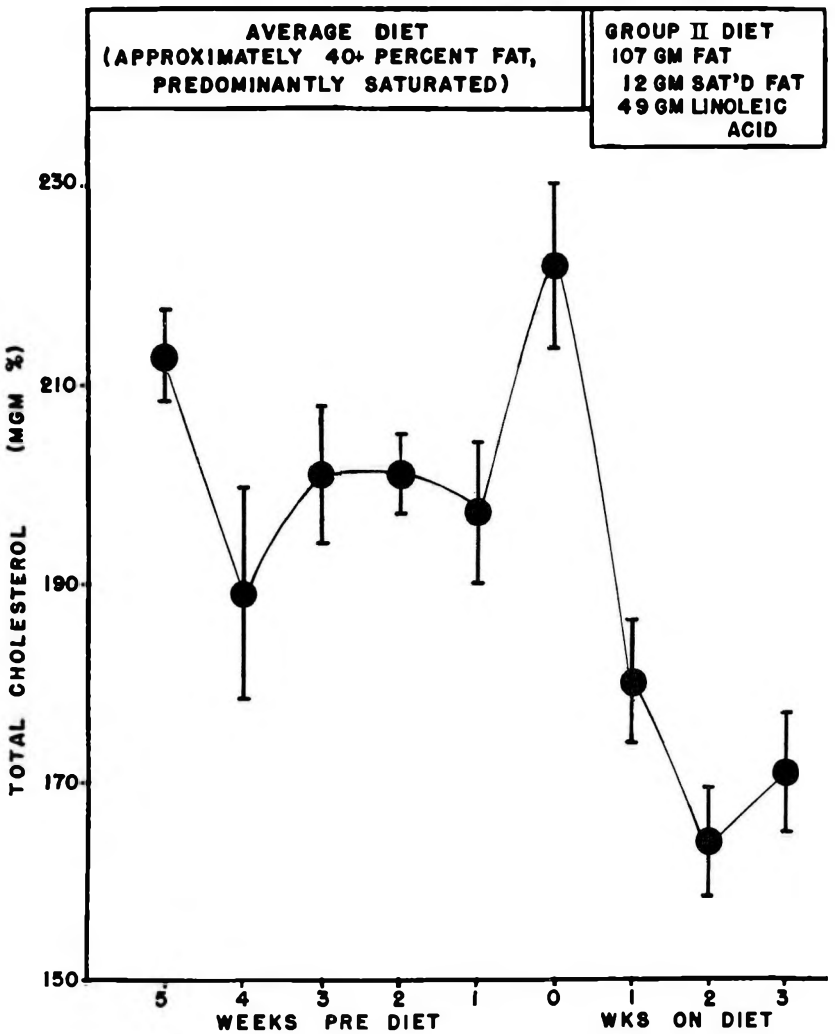

Fig. 1 Differences between cholesterol values during the two dietary periods are highly significant. Bars indicate standard deviation of the mean. The variability of cholesterol levels during consumption of the "average" diet may very well have a relationship to considerable quantitative and qualitative variation in intake.

90 gm of protein. During the second period of 21 days, each subject consumed a diet containing 45% of calories as fat with a P/S ratio of 4.2.[2,3] The make-up of this diet at the 2,000-Cal. level is shown in table 2. All meals were eaten in a common dining room. Preparation of the meals was handled by dietetic interns. All subjects were given vitamin supplements during the last 6 weeks of the study.

Fasting bloods were drawn weekly for lipid analyses. The methods used in lipid analysis included: cholesterol, total and free, by a modification of the Schoenheimer-Sperry technic (Michaels, '58); phospholipids (Youngburg and Youngburg,

[2] This ratio is actually higher because only 18:2 polyunsaturated fatty acids were considered in computing this figure.

[3] Acknowledgment is extended to Frozen Desserts and to Circus Nuts for foods used in the course of this study.

TABLE 2

Polyunsaturated fatty acid diet — 2,000 Calories

Daily intake of nutrients[1]	Quantity	Daily intake of nutrients[1]	Quantity
Milk solids	60 gm	Fruit, 20%	300 gm
Low-calorie cottage cheese	100 gm	or 10%	600 gm
Fish, fowl, liver[2]	120 gm	Vegetable, 20%	100 gm
Safflower oil[3]	60 ml	Vegetable, 7%	200 gm
Nuts, walnuts[3]	65 gm	Vegetable, 3%	ad lib.
Cooked whole grain cereals	100 gm	Low-fat bread	50 gm

[1] Approximate composition: (in grams) protein, 94; carbohydrate, 184; fat, 104.
[2] Liver, once weekly; Fish: fowl = 2:1.
[3] Chinese cooking includes the liberal use of nuts and vegetable oils. Reference to a good Chinese cookbook can result in a combination of business and pleasure.

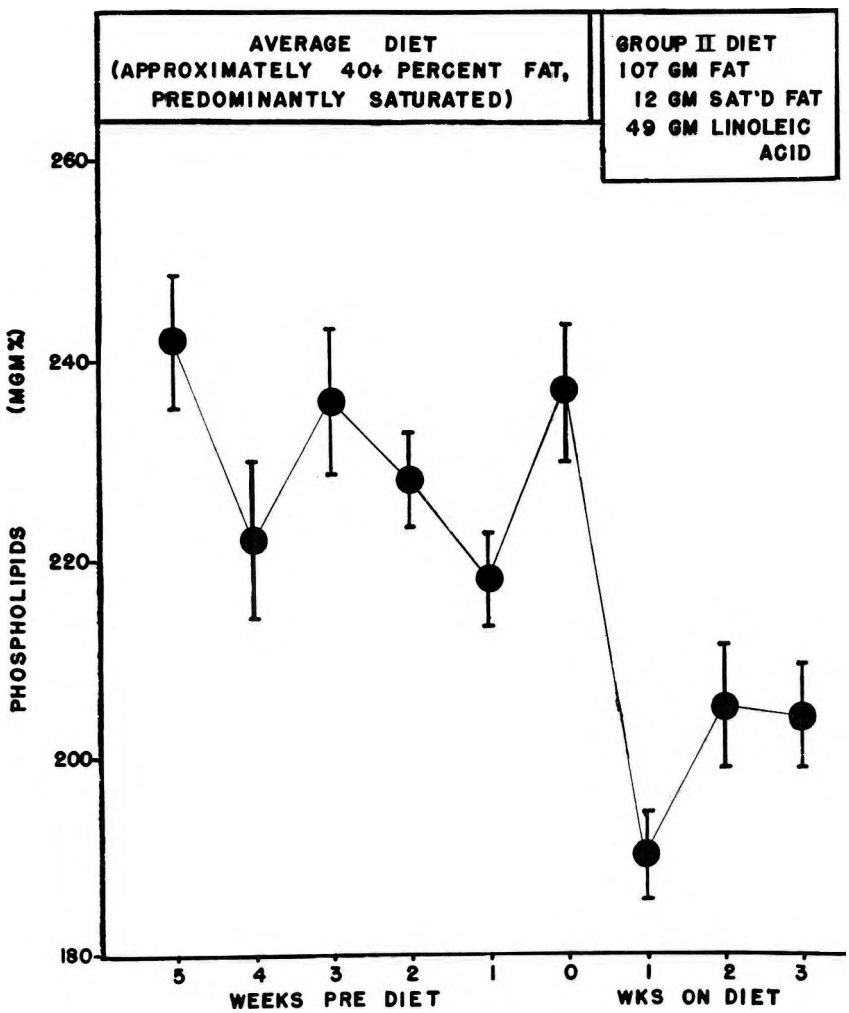

Fig. 2 The reasons for lesser variability in plasma phospholipids than total cholesterol during the "average" diet period are unknown. Bars indicate standard deviation of the mean.

'30); glycerides (Michaels, '62). Cholesterol ester fatty acid composition was determined by gas chromatography after silicic acid chromatography (Michaels, '59). Quantitative methylation of the cholesterol ester fatty acids was carried out by heating the esters in a 2% sulfuric acid-absolute methanol solution for one hour at 70°C in a sealed culture tube. The methylated fatty acids were then taken up in hexane, concentrated to the desired volume, injected into the vaporization chamber of a Barber-Coleman Model 10 gas-liquid chromatograph at 185°C, using a diethylene glycol succinate polyester column.

RESULTS

The high polyunsaturated fat diet used lowered the plasma total cholesterol and phospholipids of young women as shown in figures 1 and 2. The overall total cholesterol decrease attributable to the polyunsaturated diet was 17% (204 as compared with 171 mg/100 ml of plasma). Ester cholesterol (as percentage of total) did not change significantly (71 as compared with 72%). Mean triglyceride values were not significantly different during the two dietary regimens (101 as compared with 98 mg/100 ml of plasma). Mean changes in cholesterol linoleate in relation

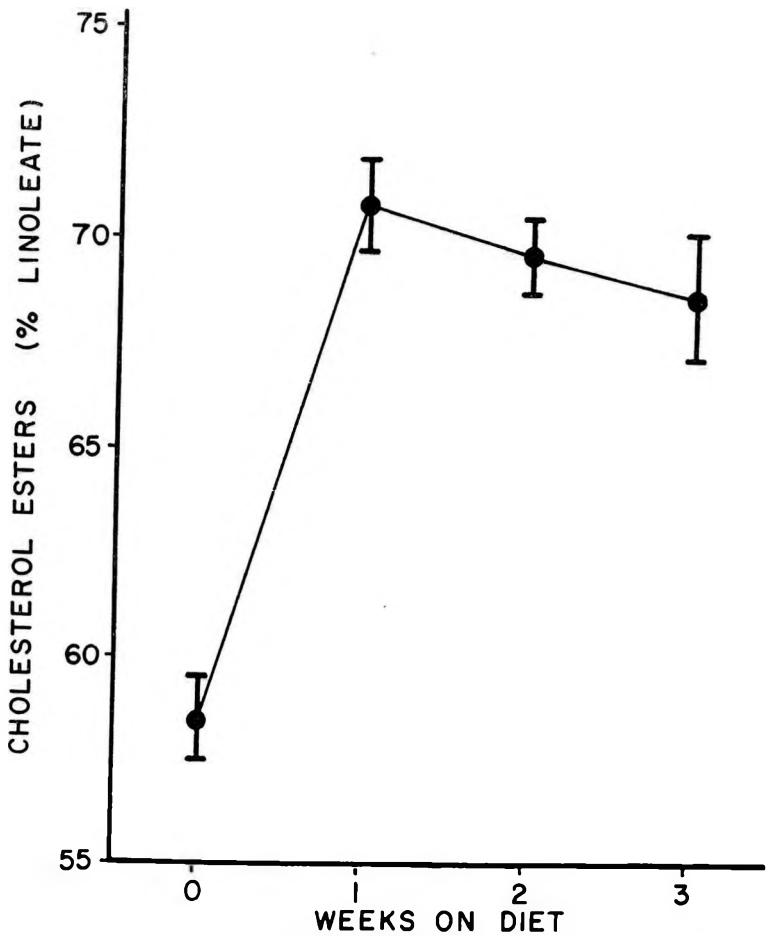

Fig. 3 Cholesterol ester fatty acid composition can be used as a short-term index of dietary adherence. An increase in the cholesterol ester linoleate is observed after one week of supplying a diet high in linoleic acid. Bars indicate standard deviation of the mean.

to diet change are shown in figure 3. In figure 4 are shown the observations with respect to plasma total cholesterol and percentages of linoleic and oleic acids, respectively, in the cholesterol esters of individual subjects. Except for subjects GUD, EME, and MAD, in whom some deviation occurred from the "standard" pattern, a maintained decrease in plasma cholesterol and an increase in cholesterol linoleate occurred in each subject. Subject GUD also had some elevation of plasma glycerides (from 98 to 158 mg/100 ml) during the third week of the study. All subjects had some decrease in cholesterol arachidonate during the period of modified diet. (Averages of 5.5% pre-diet as compared with 2.3% after three weeks of the modified diet.)

Subjects EME and NEU maintained diet records for additional periods of 12 and 22 days, respectively. The former returned to an isocaloric, high saturated fat diet, the latter changed to a low calorie, low fat diet, with a relatively high percentage of polyunsaturated fat. The effects of these dietary changes are shown in figure 5. As might have been anticipated, plasma lipids increased and cholesterol ester linoleate decreased in patient EME on resumption of the initial "saturated" regimen. Not so expected was the increase in plasma lipids when total calories, and total fat as percentage of calories, were reduced in patient NEU after completion of the "unsaturated" phase of the study. The P/S ratio during the low calorie, low fat period was approximately 2.0.

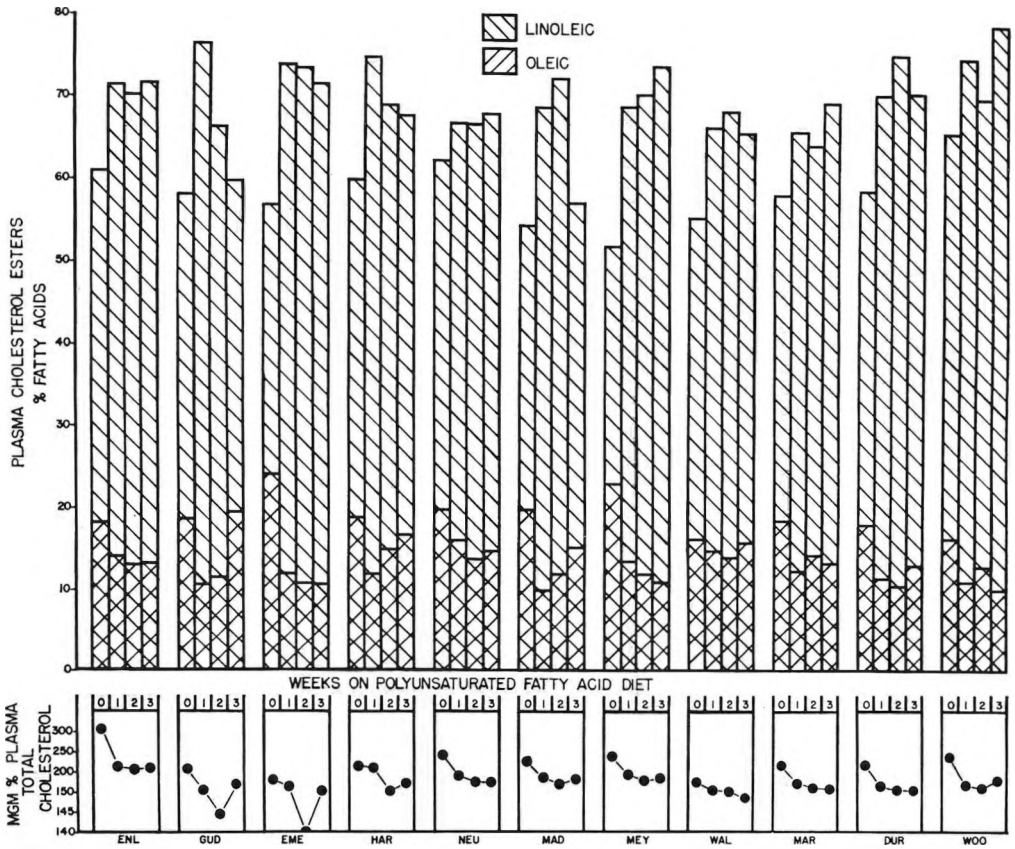

Fig. 4 Plasma cholesterol levels bear a highly significant relationship to plasma cholesterol linoleate content. A high total cholesterol (over 200) is in all cases associated with a low percentage of cholesterol linoleate for any individual subject.

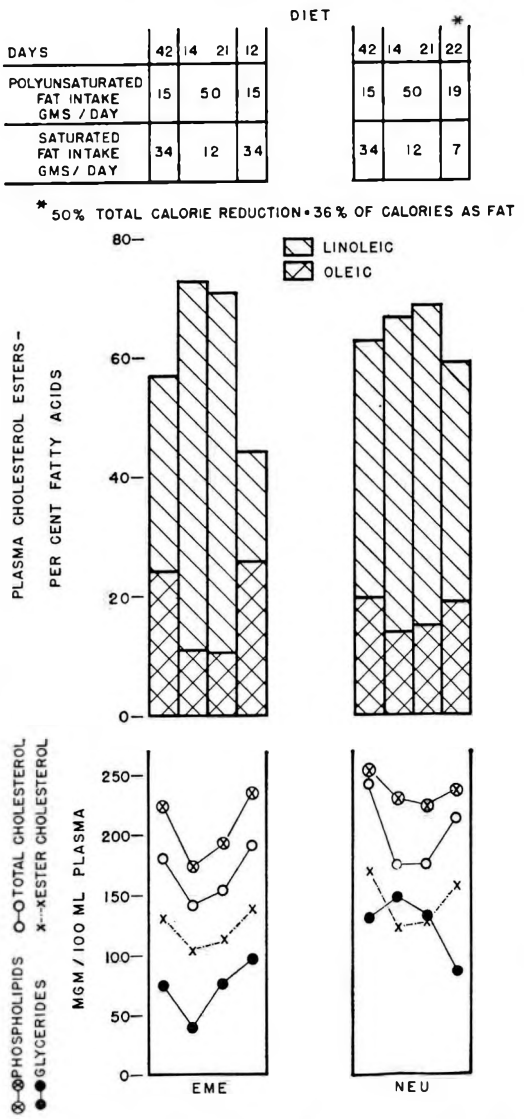

Fig. 5 In patient NEU, in the presence of calorie and fat reduction, despite maintenance of P/S ratio of approximately 2.0, plasma lipids, with the exception of glycerides, increased and cholesterol linoleate decreased (the latter presumably because of greater mobilization of depot fat). In patient EME, lipids increased and cholesterol linoleate decreased upon resumption of the previous "saturated diet."

DISCUSSION AND CONCLUSIONS

The "polyunsaturated" diet consumed during the course of this study was designed for patients either with clinically obvious vascular disease, or significantly elevated plasma lipids, or both. Such subjects have obvious motivation. A typical response pattern in one such subject is shown in figure 6.

The young women who volunteered for this study had no such motivation, and hence, were potentially highly critical of the rather drastic modification of their dietary pattern.

The diet was regarded as "acceptable" by most of those participating. The subjects realized within the first 4 days of diet modification that the most important potential and actual problem was monotony and lack of "sparkle." None of the currently used recipes were given the subjects as guides. As a result, the subjects developed sauces, some low in fat and some containing unsaturated fats, spreads, casseroles, salad dressings, and desserts. The recipes developed by the dietetic interns were accumulated and have since been added to those previously used in instructing patients in the use of high polyunsaturated fat diets. All of the subjects felt the diet would be more acceptable if they were preparing the foods in their own kitchens where a more varied supply of herbs, and greater latitude in the preparation of fish and fowl, would be available than that permitted in an institutional kitchen. After the monotony was partially overcome, enthusiasm increased and remained at a satisfactory level during the balance of the study. Five of the dietetic interns left the hospital at the end of the study, each expressing intentions of continuing some degree of diet modification in her own home. The 6 dietetic interns remaining in the hospital felt the results were convincing enough to warrant modification of the standard high saturated diet of the hospital by selectively using more vegetable oil, fish and fowl, with corresponding decreases in consumption of meat, eggs and dairy products.

Other problems encountered were: (a) difficulties in adhering to the diet when eating elsewhere than in the hospital; and (b) restrictions of meat and dairy products. The latter difficulty obviously can be greatly diminished or eliminated when dairy products and fabricated meat prepa-

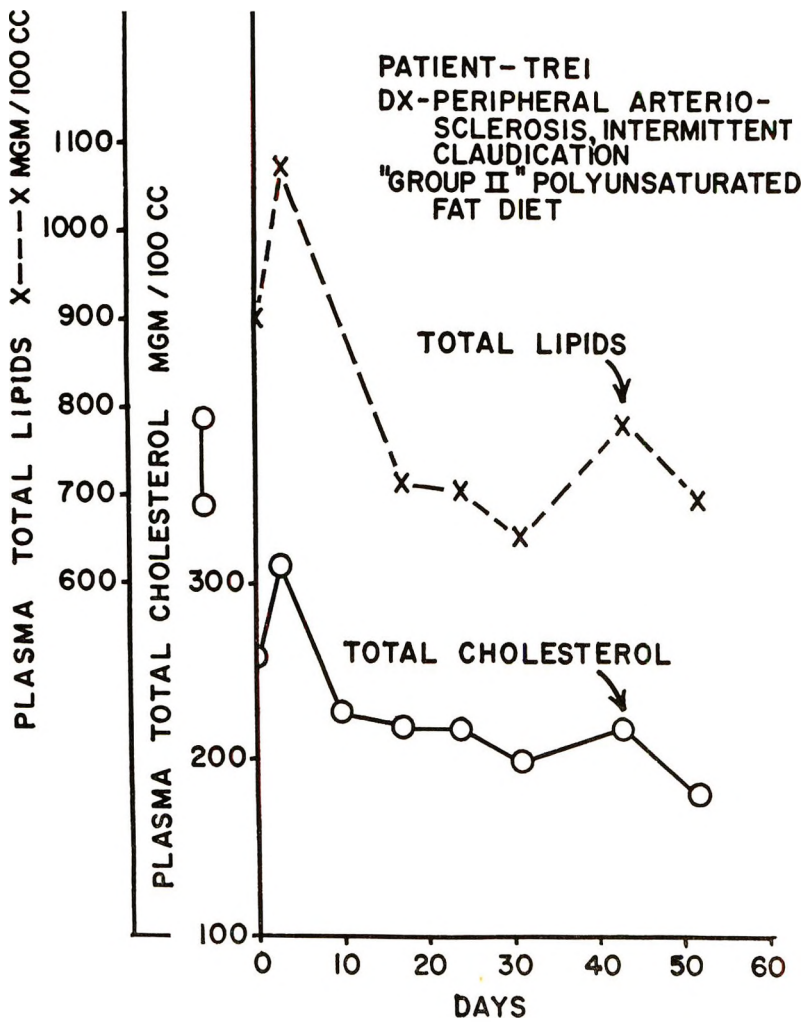

Fig. 6 Response of plasma lipids to "high polyunsaturated fat diet" in a middle-aged male physician, with elevated plasma lipids and clinical and obvious vascular disease.

rations in which much of the intrinsic fat has been substituted by polyunsaturated fat are readily available. The former problem would call for special arrangements with certain restaurants if such a diet were to be continued for long periods of time.

The decrease in plasma cholesterol and and phospholipds during the intake of the polyunsaturated diet in individuals with plasma values which would be considered "low normal" in many laboratories, was quite unequivocal, as was the lack of change in glycerides. These observations might suggest that despite the healthy condition of the volunteers, the cholesterol and phospholipid values were not really normal with the original diet, but that the glycerides were normal. Values of 180 mg/100 ml of plasma for total cholesterol and 200 mg/100 ml of plasma for phospholipids are regarded as the upper limits of "normal" in this laboratory. These figures are based on values observed in a large group of young adult Air Force Cadets. Conceivably the true normal levels are well below this.

Changes in plasma cholesterol fatty acids bear a predictable relationship to the nature of the dietary fat. Since changes occur within one week following change in type of dietary fat, one can use this type of measurement for short-term evaluation of adherence to diet. Previous studies of plasma glyceride fatty acid composition suggest that changes in this fraction occur too rapidly to make this a desirable yardstick for the determination of diet adherence. The changes in plasma cholesterol esters occur more rapidly and are of greater magnitude than those observed in the red blood cell fatty acids.

SUMMARY

1. Substitution of a palatable mixed diet containing 45% of the calories as fat, 50% of this derived from polyunsaturated fatty acids, for an average American diet, produced a significant decrease in the level of plasma cholesterol and phospholipids in normal young women, as tested by *t* values less than the critical level of 15%. (All figures with respect to fat composition are approximate.)

2. No significant change occurred in the plasma glycerides.

3. The increase in cholesterol linoleate which occurred in all subjects can be used as an index of short-term diet adherence.

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Influences of Dietary Carbohydrate-fat Combinations on Various Functions Associated with Glycolysis and Lipogenesis in Rats

I. EFFECTS OF SUBSTITUTING SUCROSE FOR RICE STARCH WITH UNSATURATED AND WITH SATURATED FAT[1]

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Dietary sources of both fat and carbohydrate appear to influence certain phases of their metabolism. Many studies have demonstrated a relationship between the dietary source of fat and the concentration of various lipid metabolites in serum, liver, and other tissues. There are also indications from several laboratories that the relative participation of different pathways in carbohydrate metabolism may depend in part upon the carbohydrate fed. For example, changes in the type of dietary carbohydrate have been shown to induce adaptations in the activities of some of the glycolytic enzyme systems (Freedland and Harper, '59; Fitch and Chaikoff, '60), as well as fluctuations in levels of glycogen in the liver (Landau et al., '58).

Because of the interdependence among many of the reactions associated with the metabolism of lipids and that of carbohydrates (Siperstein, '59; Masoro, '62), it is possible that effects of dietary source of fat on lipogenesis might in turn influence certain phases of glycolysis. Thus the dietary source of fat, through indirect action, might conceivably modify responses of glycolytic enzyme systems to changes in dietary carbohydrates.

The present study was designed to determine whether substitution of hydrogenated coconut oil for corn oil would affect the activities of some of the enzymes involved in the metabolism of carbohydrate — specifically, to note any influence exerted by the dietary fat on the adaptive responses of glucose-6-phosphatase and fructose diphosphatase to substitution of an indirect for a direct source of glucose. Lipid analyses were included to follow any concurrent changes in concentration of liver lipids and serum cholesterol.

EXPERIMENTAL

A total of 135 male, weanling rats[2] of the Sprague-Dawley strain was divided into 8 groups of 17 rats each. Rations for all groups consisted of the following: (in per cent) protein, 20 (casein supplemented with 2% of DL-methionine); fat, 15; carbohydrate, 60.55; choline chloride, 0.2; salts (Wesson, '32),[3] 4; and vitamin mix,[4] 0.25. Variables were the *type* of fat and the *type* of carbohydrate. The 4 fats used were corn oil[5] (CO) and hydrogenated coconut oil[6] (HCO), each with and without added cholesterol.[7] Each of the fats was fed with rice starch[8] (RS), as a direct source of glucose, and with sucrose (S) as a partially indirect source of glucose. For specific combinations for each group see table 1.

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[1] Supported in part by NIH grant A-4854.
[2] Obtained from Hormone Assay Laboratories, Inc., Chicago.
[3] Salt Mixture — W, obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] The vitamin mix provided the following in mg per 100-gm ration: thiamine·HCl, 0.08; riboflavin, 0.6; pyridoxine, 0.4; Ca pantothenate, 4.0; niacin, 5.0; inositol, 20.0; folic acid, 0.04; vitamin B_{12}, 0.004; biotin, 0.02; vitamin A powder, 10.0; (200 units); calciferol, 0.18 (150 units); DL-α-tocopherol powder, 30.0 (7.5 units); and menadione, 0.38.
[5] Mazola, Corn Products Company, Argo, Illinois.
[6] Hydrol, Durkee Famous Foods, Chicago.
[7] Cholesterol added at level of 1% of total ration at expense of dietary fat.
[8] Obtained from Morningstar-Paisley, Inc., New York.

TABLE 1

Liver nitrogen, glycogen, and enzyme activities in rats fed different combinations of fats and carbohydrates

Groups[1]	Liver nitrogen	G-6-Pase activity		FDPase activity		Liver glycogen
		Specific[2]	Total[3]	Specific	Total	
	mg/100 gm rat	*units*[4]	*units*	*units*	*units*	*mg/100 gm rat*
Group 1, CO and RS	116 ± 3[5]	87 ± 3	76 ± 3	55 ± 3	48 ± 2	256 ± 11
Group 2, CO and S	129 ± 4	138 ± 6	109 ± 6	73 ± 3	57 ± 2	357 ± 18
Group 3, HCO and RS	115 ± 3	115 ± 5	101 ± 6	54 ± 2	48 ± 2	220 ± 21
Group 4, HCO and S	131 ± 3	160 ± 5	123 ± 4	60 ± 2	46 ± 2	251 ± 14
Group 5, CO + cholesterol and RS	128 ± 3	93 ± 4	72 ± 3	64 ± 4	50 ± 2	346 ± 22
Group 6, CO + cholesterol and S	149 ± 3	158 ± 10	107 ± 6	98 ± 5	66 ± 3	454 ± 24
Group 7, HCO + cholesterol and RS	123 ± 3	135 ± 8	110 ± 7	72 ± 5	59 ± 4	322 ± 20
Group 8, HCO + cholesterol and S	137 ± 3	182 ± 10	134 ± 7	78 ± 4	57 ± 3	354 ± 28

[1] Abbreviations: CO, corn oil; HCO hydrogenated coconut oil; RS, rice starch; S, sucrose.
[2] Specific activity = units per 100 mg liver nitrogen.
[3] Total activity = units per 100 gm body weight.
[4] One unit = activity catalyzing release of 1 μmole of inorganic phosphate from substrate per minute.
[5] Standard error of the mean.

Criteria for evaluating metabolic responses to fat and carbohydrate combinations were activities of the glucose-6-phosphatase (G-6-Pase) and fructose diphosphatase (FDPase) enzyme systems, and levels of total lipid, cholesterol and phospholipid in the liver; and concentration of cholesterol in the serum. A preliminary time study carried out over 6 weeks had indicated that maximal activity of the G-6-Pase enzyme system occurred after feeding the CO and HCO diets for two weeks (fig. 1). Therefore, the time interval chosen for feeding the experimental diets in the current study was 14 to 19 days.

Each animal was stunned by a sharp blow on the head, and decapitated. Two or three milliliters of blood were collected in a centrifuge tube, and allowed to stand at room temperature until enzyme determinations were completed. The liver was removed rapidly, chilled for a few seconds in chipped ice, blotted with filter paper, weighed, and homogenized in two volumes of ice-cold water. One milliliter of the 33% homogenate was diluted immediately to 2.5% with chilled potassium citrate buffer (0.1 M, pH 6.5) for enzyme assays, duplicate 0.5-ml aliquots were pipetted into centrifuge tubes containing 0.5 ml of 60% KOH for glycogen determinations, and the remainder of the homogenate was stored frozen for lipid analyses at a later time.

The G-6-Pase and FDPase assays were based on the rate of release of inorganic phosphate from 0.1 ml of substrate solution (glucose-6-phosphate,[9] 0.06 M; and fructose diphosphate,[10] 0.05 M, respectively) during incubation with 0.1 ml of the diluted homogenate in a Dubnoff water bath at 37.5°C (Freedland and Harper, '57, '59). In addition to substrate and homogenate, the FDPase reaction mixture contained $MgSO_4$ (0.025 M), 0.1 ml; $MnSO_4$ (0.025 M), 0.2 ml; and borate buffer (pH 9.5), 0.5 ml. Endogenous tubes containing all components except substrate were used with each set of determinations. Reactions were stopped at 15 minutes by addition of 1 ml of 10% trichloroacetic acid. Tube contents were filtered, and phosphorous determined by the method of Fiske and Subarrow ('25).

[9] Obtained from Sigma Chemical Company, St. Louis.
[10] See footnote 9.

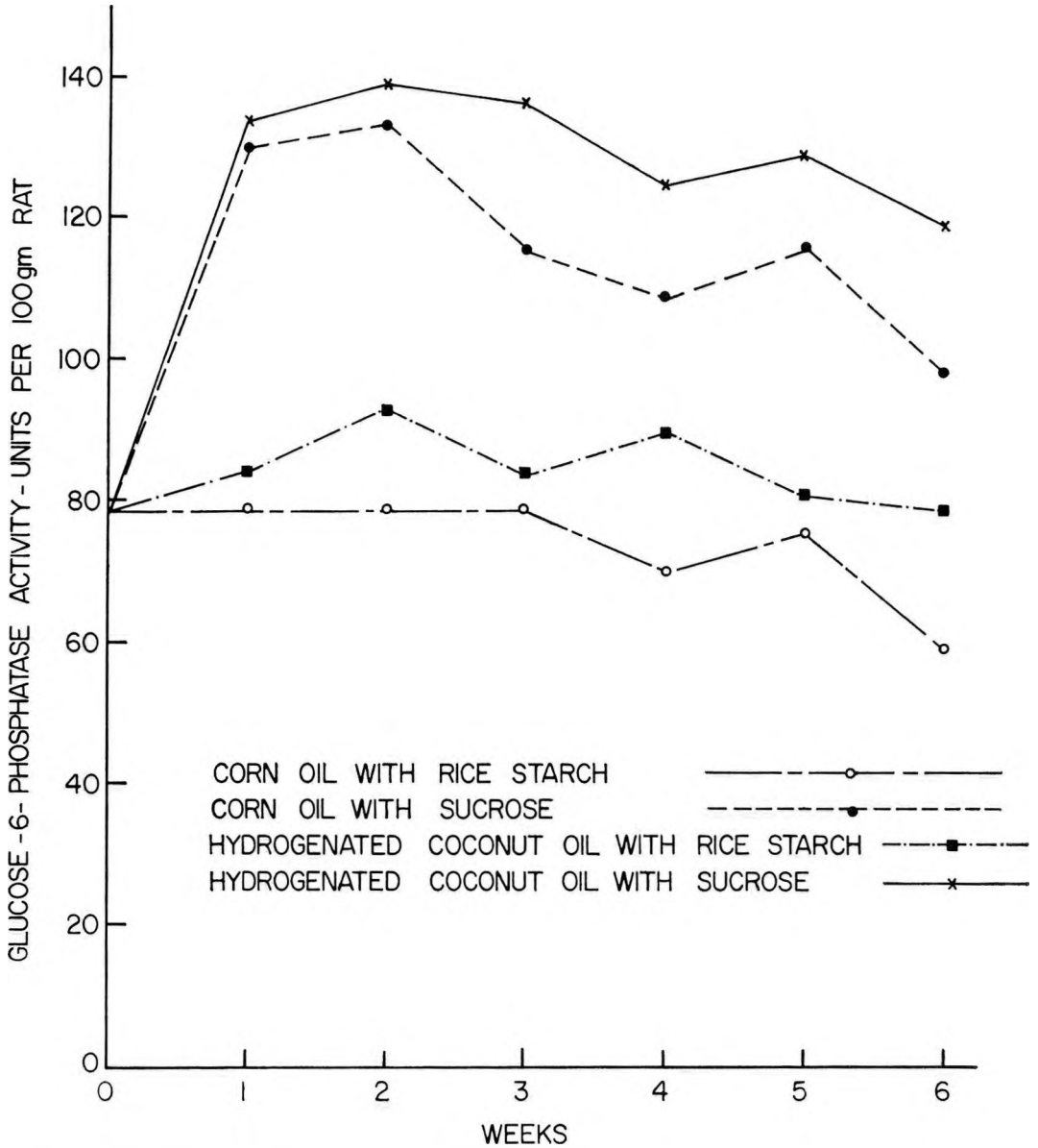

Fig. 1 Changes with time in glucose-6-phosphatase activity in livers from rats fed different combinations of fats and carbohydrates.

Blood samples were centrifuged, serum drawn off, and cholesterol determined directly by the method of Zlatkis et al. ('53), using the color reagent modification of Rosenthal et al. ('57).

Liver glycogen was determined by the nephelometric method of Hansen et al. ('52).

For moisture and lipid analyses, homogenates were thawed at room temperature, and dried at 95°C to a constant weight. The dried livers were ground, and total lipid was extracted with a solvent mixture[11] (Bixby et al., '53; Rosenthal et

[11] Ethyl ether-petroleum ether-95% ethanol mixture, 5:5:2.

al., '57). The fat extract remaining after aliquots had been removed for cholesterol determinations was evaporated to dryness, dried for about 10 minutes more at 95°C, and weighed. The fat was then redissolved in approximately 3 ml of ether, and phospholipids were determined gravimetrically by precipitation with acetone (Yoshida and Harper, '60).

Samples of fat-free residue from dried livers were analyzed for nitrogen by the Kjeldahl procedure.[12]

RESULTS

Body weights and liver nitrogen. Average body weights at the time of sacrifice tended to be about 10% lower in groups fed HCO than in those fed CO. Average weights (gm) with standard errors were as follows: group 1 (CO and RS), 162 ± 4; group 2 (CO and S), 160 ± 3; group 3 (HCO and RS), 150 ± 4; group 4 (HCO and S), 148 ± 3; group 5 (CO + cholesterol and RS), 162 ± 4; group 6 (CO + cholesterol and S), 169 ± 3; group 7 (HCO + cholesterol and RS), 146 ± 3; and group 8 (CO + cholesterol and S), 147±3.

Data from liver assays for nitrogen, enzyme activities, and glycogen are presented in table 1. As the percentage of nitrogen was essentially the same in livers from all groups of rats in this study, differences in values for liver nitrogen per 100 gm of rat are a reflection of differences in liver size in relation to body weight. The relative liver weights from groups fed sucrose were consistently greater than those from corresponding groups fed rice starch.

Enzyme activities and glycogen levels in livers from rats fed no added cholesterol (groups 1 through 4). The activity of the G-6-Pase enzyme system was calculated on the basis of total activity (units per 100 gm of rat) and of specific activity (units per 100 mg of liver nitrogen). On both bases, G-6-Pase activity was significantly greater in livers from sucrose-fed groups than in those of the corresponding rice starch-fed groups ($P < 0.01$). Since the liver G-6-Pase enzyme system catalyzes the release of glucose into the bloodstream for transport to all body tissues, total activity appears to be a more meaningful value than specific activity. Therefore, comparisons will be based chiefly on total activity.

When CO was the dietary fat (groups 1 and 2), the substitution of sucrose for rice starch induced a 61% increase in total G-6-Pase activity (fig. 2a). This degree of stimulation is comparable to that reported by Freedland and Harper ('58) under similar conditions. The results with HCO (groups 3 and 4) were more unexpected, viz., G-6-Pase activity was 34% higher when rice starch was fed with HCO (group 3) than when the same carbohydrate was fed with CO (group 1), indicating that the type of fat, as well as the type of carbohydrate, influenced the activity of this enzyme system. The two effects were only partially additive, since the enzyme activity increased by only 43% when sucrose was substituted for rice starch in the HCO diet, as compared with 61% in the CO diet (fig. 2a).

The FDPase enzyme system responded somewhat differently than did the G-6-Pase. A substantial increase over the control level (CO with RS) occurred only when sucrose was substituted for rice starch in the CO diet (fig. 2a). The saturated fat alone had no effect, and feeding sucrose resulted in only a slightly greater total activity than did feeding rice starch when HCO was the dietary fat. Note that the percentage increase in FDPase activity in group 2 (CO with S) equaled only a little more than one-half the increase in G-6-Pase activity in the same rats (35% as compared with 61%).

Liver glycogen levels, expressed in mg per 100 gm of rat, followed a pattern similar to that of the FDPase enzyme system (fig. 2a). The feeding of sucrose in place of rice starch resulted in the deposition of a significantly greater amount of glycogen ($P < 0.01$) in livers of rats fed CO, but not of those fed HCO.

Effects on enzyme activities and glycogen levels of addition of cholesterol to the dietary fats (groups 5 through 8). Total activities of the G-6-Pase and FDPase enzyme systems, and amounts of glycogen in livers from the 4 groups fed added cholesterol were consistently higher than those

[12] Nitrogen analyses carried out by Agricultural Biochemistry Laboratory, University of Arkansas.

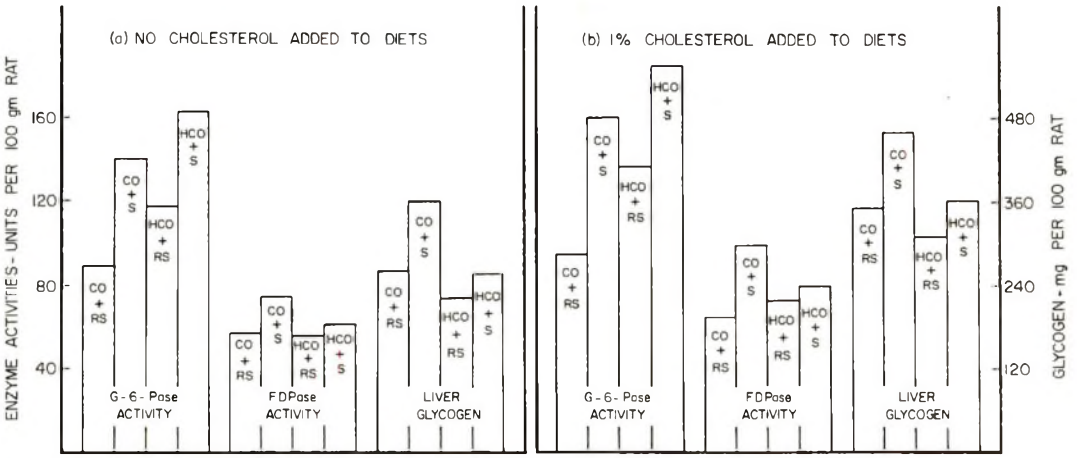

Fig. 2 Activities of the glucose-6-phosphatase and fructose diphosphatase enzyme systems and glycogen levels in livers from rats fed different combinations of fats and carbohydrates for two weeks. CO = corn oil; HCO = hydrogenated coconut oil; RS = rice starch; S = sucrose.

from the corresponding groups not fed added cholesterol (table 1). However, relationships among the groups were practically identical in the two sets (fig. 2b compared with fig. 2a).

Liver lipid and serum cholesterol levels in rats fed no added cholesterol (groups 1 through 4). Concentration of lipid (percentage of dry weight) in livers from rats fed HCO was somewhat greater than that from rats fed CO. Values were as follows: group 1 (CO with RS), 14.98 ± 0.37; group 2 (CO with S), 14.19 ± 0.48; group 3 (HCO with RS), 16.74 ± 0.70; group 4 (HCO with S), 17.47 ± 0.57. Differences between effects of the two fats were significant at the 5% level when rice starch was the dietary carbohydrate (group 1 vs. 3), and at the 1% level when sucrose was the dietary carbohydrate (group 2 vs. 4). It is possible that greater differences may have occurred after a longer period of feeding, since changes in liver lipids tend to develop more slowly than changes in the activities of at least some liver enzyme systems (Arata et al., '56; Carroll et al., '60).

Table 2 summarizes the data from liver lipid analyses and serum cholesterol determinations. When total lipid was expressed as milligrams per 100 gm of rat, the effect of HCO in the diet was less evident than when expressed on a percentage basis. With rice starch the differ-

TABLE 2

Liver lipids and serum cholesterol concentraiton in rats fed different combinations of dietary fats and carbohydrates

Groups[1]	Total liver lipid	Liver cholesterol	Liver phospholipid	Serum cholesterol
	mg/100 gm rat	*mg/100 gm rat*	*mg/100 gm rat*	*mg/100 ml serum*
Group 1, CO and RS	201 ± 7[2]	20 ± 1	61 ± 5	213 ± 6
Group 2, CO and S	227 ± 11	20 ± 1	72 ± 4	193 ± 5
Group 3, HCO and RS	224 ± 11	16 ± 1	84 ± 6	168 ± 5
Group 4, HCO and S	266 ± 11	18 ± 1	85 ± 6	175 ± 7
Group 5, CO + cholesterol and RS	436 ± 17	97 ± 5	77 ± 5	233 ± 13
Group 6, CO + cholesterol and S	527 ± 23	115 ± 9	85 ± 4	229 ± 13
Group 7, HCO + cholesterol and RS	398 ± 16	89 ± 6	97 ± 5	352 ± 19
Group 8, HCO + cholesterol and S	470 ± 16	92 ± 8	103 ± 6	314 ± 23

[1] Abbreviations: CO, corn oil; HCO hydrogenated coconut oil; RS, rice starch; S, sucrose.
[2] Standard error of the mean.

ence was barely significant ($P < 0.10$), whereas with S it was significant only at the 2% level. Total cholesterol tended to decrease and total phospholipid to increase in livers from rats fed HCO as compared with those fed CO. The differences were highly significant ($P < 0.01$) only when the fat was fed with rice starch. Fluctuations in serum cholesterol concentration closely paralleled changes in total liver cholesterol.

Effects on liver lipid and serum cholesterol levels of addition of cholesterol to the dietary fats (groups 5 through 8). Addition of cholesterol to the dietary fat resulted in a profound increase in total liver lipid per 100 gm of rat, a slight increase in total phospholipid, and a fivefold increase in total cholesterol (table 2).

DISCUSSION

The above data support the hypothesis that altering the type of dietary fat can modify responses of glucose-6-phosphatase and fructose diphosphatase enzyme systems to changes in the type of carbohydrate fed. First, the percentage of stimulation of G-6-Pase activity by sucrose was much less with HCO than with CO as the dietary fat (43 vs. 61%). Second, highly significant increases in FDPase activity and in liver glycogen deposition resulted from the substitution of sucrose for rice starch only when CO was fed.

Two further observations made in the course of this study may provide possible clues to mechanisms underlying the above effects. In the first place, HCO had a very significant stimulatory effect on G-6-Pase activity, quite apart from its influence on the response of the enzyme to dietary carbohydrate. It is conceivable that the increased G-6-Pase activity could be a reflection of increased mobilization of glucose for fatty acid synthesis. Evidence that feeding HCO tends to increase fatty acid synthesis via the malonyl CoA route is provided in a recent report by Allman and Gibson.[13] They observed that the addition of HCO to diets of weanling mice that had been deprived of fat induced a tenfold greater increase in fatty acid synthesis than did the addition of CO. Also, the relatively low levels of liver glycogen in rats fed HCO (fig. 2) are consistent with an increased mobilization of glucose.

The other possible clue is the lack of parallelism in the responses of the two enzyme systems. The stimulation of G-6-Pase activity observed on feeding a source of fructose is believed to be due to the conversion of fructose to glucose (Freedland and Harper, '57; Fitch et al., '59). Freedland and Harper ('59) further proposed that a concurrent increase in FDPase activity on feeding fructose indicates that the conversion takes place via fructose-1-phosphate, triose phosphates, and fructose diphosphate. However, two observations in this study suggest that the Embden-Meyerhoff pathway is not the only route used under these conditions for the conversion of fructose to glucose. First, G-6-Pase activity was increased almost twice as much as was FDPase activity by feeding a source of fructose with CO diets; and second, G-6-Pase activity, but not FDPase activity, was increased by substituting sucrose for rice starch in HCO diets. An alternate pathway from fructose-1-phosphate to fructose-6-phosphate, bypassing fructose diphosphate (and thus the increased requirement for FDPase), would involve combination of the triose phosphates with intermediates of the hexose monophosphate shunt (HMS) system with formation of fructose-6-phosphate. Couri and Racker ('59) have suggested that when HMS enzymes are very active, reversal of glycolysis probably proceeds largely by this route. Stimulation of at least one of the HMS enzymes by a 60% fructose diet has been reported by Fitch and Chaikoff ('60). Enhanced HMS activity has also been associated with an increased rate of fatty acid synthesis (Kaplan et al., '56; Siperstein and Fagan, '57 and '58; Tepperman and Tepperman, '58).

On the basis of these considerations, a tentative explanation of enzyme effects observed in this study might be that with the CO diets, fructose conversion to glucose-6-phosphate was accomplished partially via the Embden-Meyerhoff pathway and partially via the HMS; whereas with

[13] Allman, D. W., and D. M. Gibson 1962 Fatty acid synthesis from malonyl CoA in linoleate deficiency. Federation. Proc., 21: 288 (abstract).

the HCO diets, conversion was mainly via the shunt.

Data from lipid analyses indicate that the type of carbohydrate as well as the type of fat in the diet can influence concentrations of lipids in the liver and serum. Responses to the feeding of HCO depended to a considerable extent on the type of carbohydrate fed with the fat. Such interrelationships may play an important role in interpreting results obtained under a variety of experimental conditions.

SUMMARY

The purpose of this study was to determine whether changes in the dietary source of fat would affect the activities of some of the enzymes involved in the metabolism of carbohydrates. Male, weanling rats were fed rations in which levels of all nutrients were identical. Dietary fats were corn oil and hydrogenated coconut oil with and without added cholesterol. Each of the 4 fats was fed with rice starch, as a direct source of glucose, and with sucrose, as partially indirect source.

During the third week of the experiment, livers from all animals were assayed for activities of the glucose-6-phosphatase and fructose diphosphatase enzyme systems, glycogen, total lipid, phospholipid, and cholesterol; and serum was analyzed for total cholesterol.

Responses of the two enzyme systems and glycogen levels in the liver to changes in dietary carbohydrate were modified by the type of fat fed. Similarly, changes in liver lipids and serum cholesterol concentration were influenced by the combination of fat and carbohydrate in the diet.

Possible interrelationships among the above effects were discussed, and the importance of considering dietary constituents other than the specific nutrient under investigation in metabolic studies was stressed.

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Body Iron Levels and Hematologic Findings During Excess Methionine Feeding[1]

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It has been demonstrated that feeding an excess of methionine to rats will produce histologic changes in the pancreas, gastrointestinal tract, and salivary glands similar to those produced by ethionine, its analogue (Kaufman et al., '60, '62; Klavins et al., in press). It has been shown also that the feeding of ethionine causes an increased absorption of iron in rats and mice (Kinney et al., '55a). The effect of methionine upon the absorption of iron has not been studied. For this reason, experiments were devised to study the absorption and deposition of iron in rats fed excess amounts of methionine.

MATERIALS AND METHODS

Sixty-four Sprague-Dawley male albino rats, each weighing approximately 164 gm were divided into 4 groups of 16 animals each. Group 1 received a basal synthetic diet supplemented with 2% of DL-methionine. The animals in group 2 were fed the basal diet and were used as pair-fed controls to those in group 1. Rats in group 3 were fed the basal diet supplemented with 2% of methionine and 2% of iron citrate. The animals in group 4 were the pair-fed controls for group 3 and were fed the basal diet to which 2% of iron citrate was added. The basal diet had the following composition per 100 gm: glucose, 67; casein, 18; corn oil, 11 (containing 0.001 cm^3 of halibut liver oil);[2] and salt mixture 4 (Hegsted et al., '41). Crystalline vitamins were added to supply each 100 gm of diet with the following (in micrograms): pyridoxine hydrochloride, 400; thiamine chloride, 400; riboflavin, 800; calcium pantothenate, 1,500; and nicotinic acid, 2,500. In order that the basal diet would be identical with that used in the earlier experiments with ethionine (Kinney et al., '55a, b) the diets were not supplemented with choline.

When methionine and ferric citrate were added, each replaced equivalent amounts of glucose by weight. The animals were weighed twice a week and killed after 30 days. White and red cell blood counts were made. Hemoglobin was determined by the cyanmethemoglobin method utilizing a commercial standard (Hycel) and read in a Klett colorimeter. Hematocrits were determined by a micromethod. Smears were prepared from the peripheral blood. In one-half of the animals in each group sections were taken from the liver, pancreas, duodenum, and tibia. Liver iron analysis was performed on these animals. The remainder of the animals in each group were used for the iron analysis of the entire animal. In the latter, the entire gastrointestinal tract was removed, washed in saline, analyzed and then the results were included with the analysis of the remainder of the animal. Iron analysis was performed after digestion with sulfuric, nitric, and perchloric acids. Digestion was carried out by placing the entire animal in an Erlenmeyer flask containing 40 ml of reagent grade concentrated sulphuric acid. After the carcass was entirely disintegrated, the flask was placed on a hot plate and the contents heated. As digestion was continued, nitric acid was added periodically until the digest was clear and the fumes were white. The small amount of organic material that remained was digested by adding perchloric acid. This was done in small amounts, not more than 1 cm^3 of perchloric acid being added at a time. A similar procedure was used for the digestion of liver samples and of gut, except that smaller amounts of the acids were used. Iron was determined colorime-

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[2] Haliver Oil.

trically on aliquots of the digest by the method of Kitzes et al. ('44).

RESULTS

A steady gain in weight occurred in all groups. At the end of the experiment there was no statistically significant difference in the weights of the rats fed excess methionine (group 1) and their controls (group 2). The average weight of the animals in group 1 was 224 gm and in group 2 it was 230 gm ($P > 0.1$). On the other hand, when the diets were supplemented with methionine and iron there was a statistically significant decrease ($P < 0.05$) in the weights of the experimental animals (group 3) whose average weight at the termination of the experiment was 214 gm in contrast with 227 gm in the control animals (group 4). When the experimental groups were compared, that is, group 1, fed excess methionine with group 3, fed excess methionine plus 2% of iron citrate, there was no statistically significant difference ($P < 0.1 > 0.05$).

The total food consumption for group 1 was 351 gm/rat and for group 3 it was 363 gm/rat. There was no statistically significant difference in the amounts eaten ($P > 0.1$).

The iron values are recorded in table 1. The average total liver iron value of group 1 receiving the 2% methionine supplement was 1.3 mg, whereas the value in the pair-fed controls was 0.99 mg ($P < 0.05$). There were 15.7 mg of iron/100 gm of liver for group 1 and 13.8 mg iron/100 gm of liver for the group 2, corresponding pair-fed controls ($P > 0.05$). The total body iron for group 1 fed the excess methionine was 8.98 mg and for the pair-fed control (group 2) it was 8.88 mg ($P > 0.1$).

When the diets were supplemented with 2% of iron citrate the average total liver iron was 3.13 mg in the experimental rats fed the excess methionine diet and 2.74 mg in the corresponding pair-fed control groups ($P > 0.1$). In milligrams per 100 gm of liver the experimental values were 45.1 and the control values were 36.1 ($P > 0.1$). The total body iron in the iron supplemented animals was 11.89 mg for group 3 and 10.90 mg for group 4 ($P > 0.1$). There was no statistically significant variation in the liver weights.

The hematologic findings are recorded in table 2. With the methionine excess the hemoglobin values were significantly lower than in the corresponding pair-fed controls ($P < 0.01$). The hematocrit and mean corpuscular hemoglobin values (MCH) were also significantly lower in the animals fed excess methionine ($P < 0.01$ when groups 1 and 2 were compared, $P < 0.05$ when groups 3 and 4 were compared). The mean corpuscular volume (MCV) was significantly lower in the high methionine group (group 1) than in the corresponding pair-fed control group ($P < 0.05$). However, when the diets were supplemented with 2% of iron citrate no statistically significant difference could be demonstrated between group 3 and group 4 ($P > 0.1$). The differences in the mean corpuscular hemoglobin concentration (MCHC) and in the red blood cell counts were not con-

TABLE 1

Iron values

	Experimental groups	Liver iron[1]		Total body iron[2]	Liver wt[3]
		mg/liver	*mg/100 gm liver*	*mg*	*gm*
1	2% Methionine	1.3	15.7	8.98	7.3
2	Basal, pair-fed to group 1	0.99	13.8	8.88	7.2
3	2% Methionine + 2% iron citrate	3.13	45.1	11.89	6.9
4	Basal + 2% iron citrate, pair-fed to group 3	2.74	36.1	10.90	8.1

[1] Average value of eight rats in each group.
[2] Average value of eight rats in each group.
[3] Average weight of 16 rats in each group.

TABLE 2

Hematologic observations

	Experimental groups	No. of animals	Hemoglobin	Hematocrit	MCH[1]	MCV[2]	MCHC[3]	Red blood cells $\times 10^{-6}$	White blood cells $\times 10^{-3}$	Differential W. B. C.		
										Neutrophils	Lymphocytes	Monocytes
			gm/100 ml	%	*gm* $\times 10^{-12}$	μ^3	%			%	%	%
1	2% Methionine	16	13 (11–16)[4]	45 (40–50)	19 (15–23)	63 (54–73)	29 (24–40)	7.10 (6.19–8.40)	20.46 (11.35–31.28)	17.2 (4–37)	80.2 (61–91)	2.5 (0–6)
2	Basal, pair-fed to group 1	16	16 (15–18)	52 (46–58)	21 (17–24)	69 (57–82)	31 (23–36)	7.52 (6.57–9.35)	15.98 (6.58–24.20)	16.4 (6–26)	81.3 (65–92)	2.2 (0–9)
3	2% Methionine + 2% iron citrate	16	15 (12–17)	45 (40–49)	22 (16–26)	65 (58–72)	34 (27–40)	6.94 (6.49–7.77)	23.58 (12.70–29.93)	10.9 (3–31)	87.5 (67–95)	1.6 (0–3)
4	Basal + 2% iron citrate, pair-fed to group 3	16	17 (15–19)	48 (43–51)	24 (20–27)	68 (63–77)	35 (31–42)	7.06 (6.09–7.94)	19.28 (15.33–38.87)	15.6 (4–32)	82.8 (68–94)	1.5 (0–5)

[1] Indicates mean corpuscular hemoglobin.
[2] Indicates mean corpuscular volume.
[3] Indicates mean corpuscular hemoglobin concentration.
[4] Figures in parentheses indicate range.

sidered to be significant in the experimental and corresponding pair-fed control animals. The white blood cell count was greater in the groups fed excess methionine ($P < 0.05$); but there were no statistically significant differences in the differential counts. Only one eosinophil was found in each of two animals in group 4, and one basophil in one animal in group 1. The reticulocytes were increased in the peripheral blood and there was erythroid hyperplasia of the tibial bone marrow in the animals with excess methionine, regardless of iron supplementation.

Pancreatic damage was found in the animals that received excess methionine. The changes were identical to those reported previously (Kaufman et al., '60) and consisted of loss of basophilia, vacuolization of cytoplasm, pyknosis of nuclei and loss of individual acinar cells. In addition, acinar regeneration was similar to that reported after prolonged ethionine feeding (Kinney et al., '55b). Histochemically, the liver iron content was slightly increased in group 1 receiving the 2% methionine when compared with the pair-fed controls. The iron was found predominantly in the Kupffer cells. When the diets were supplemented with 2% of iron citrate, the predominant distribution in both the experimental and control groups was in the parenchymal cells, particularly in the periportal areas. Small amounts of iron were present in the Kupffer cells. There was no difference in the amount of parenchymal iron but there was slightly more iron in the Kupffer cells in group 3 animals, when compared with group 4 animals. The histologic changes in the duodenum consisted of shrinking of the acini of the Brunner's glands. The cytoplasmic basophilia was diffuse, and the nuclei were located in the central portion of the cells (Klavins et al., in press). Histochemically demonstrable iron in the duodenum ranged from zero to 4 + and there was no essential difference between the experimental and the control animals.

DISCUSSION

It has been shown previously that when rats were fed a purified diet containing 0.5% of DL-ethionine there was an in-

crease in the amount of iron absorbed and deposited in the livers (Kinney et al., '55a; DeMonterice et al., '61). Among other factors, pancreatic damage was considered to play some role in iron absorption (Kinney et al., '55a). In the present experiment it appears that the pancreatic damage per se had no direct effect on the absorption of iron since the total body iron was not affected in the experimental animals fed excess methionine, yet pancreatic damage was present although not as marked as in animals fed ethionine.

No increase in iron absorption was noted when rats were fed excess methionine, but a statistically significant increase in liver iron was noted in the rats that received a 2% methionine supplement when compared with the pair-fed controls receiving a basal diet. When 2% of iron citrate was added to both the high methionine diet and to the basal diet, no difference could be observed between these two groups (groups 3 and 4). The increase in liver iron in group 1 can be explained by a shift in body iron since the total body iron was not altered.

The mechanisms by which excess methionine causes the hypochromic microcytic anemia and increased white blood cell counts are not understood. It has been shown that methionine inhibits the absorption of histidine.[3] Since histidine is the most limiting amino acid in hemoglobin synthesis in the rabbit reticulocyte *in vitro* (Borsook et al., '57), it is possible that the anemia observed with methionine excess is produced by the unavailability of histidine for hemoglobin production. This cannot explain the increased erythrocyte turnover (Cohen and Berg, '56) with excess methionine when a more rapid synthesis of hemoglobin was associated with more rapid degradation. On the other hand, the predominant deposition of iron in Kupffer cells and hemosiderosis of the spleen (Van Pilsum and Berg, '50)[4] can be explained by the increased degradation of the hemoglobin. The erythroid hyperplasia could be secondary to the anemia caused by excess methionine.

SUMMARY

Since it is known that ethionine causes an increase in iron absorption and that excessive amounts of methionine in the diet will produce similar histologic changes in the pancreas, gastrointestinal tract, and salivary glands, experiments were devised whereby rats were fed diets containing 2% of DL-methionine for one month. There was no increase in iron absorption under this regimen. The rats receiving excess methionine developed a microcytic hypochromic anemia, erythroid hyperplasia of the bone marrow and an increase in the peripheral white blood cell count.

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Digestibility of the alpha-Cellulose and Pentosan Components of the Cellulosic Micelle of Fescue and Alfalfa[1]

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Many of the criticisms of the proximate analysis as an assay for the nutritional value of feeds are focused on the crude fiber and the nitrogen-free extract fractions in the analytical scheme. Neither the nitrogen-free extract nor the crude fiber bears any definite or regular relationship to any particular one or group of plant constituents (Norman, '35a). Since cellulose constitutes a major portion of the structural framework of plants, alternative analytical schemes that have been proposed (Crampton and Whiting, '42; Ferguson, '42; Ely et al., '53; Sullivan, '55) to account for plant material have generally employed a crude cellulose determination. This determination isolates constituents other than true cellulose, as, for example, in grasses xylan occurs in close association with true cellulose (Astbury et al., '35; Norman, '36b, '37) forming a cellulosic aggregate referred to as a micelle. The Matrone et al. ('46) modifications of the Norman-Jenkins crude cellulose determination isolate this structural micelle. Ferguson ('42) found in a study with wheat straw and wheat-straw pulp that the crude cellulose was digested to the same extent as the associated cellulosans. Later he raised the point that separate determination of cellulose and its associated cellulosans seems unnecessary if both components are digested to the same extent by the animal (Ferguson, '48).

The purpose of this study was to determine the digestibility of the xylan and true cellulose fractions of the micelle directly to ascertain whether this structural unit of the plant is also a uniformly digested unit, namely, a nutritive entity.

EXPERIMENTAL PROCEDURE

Alfalfa (*Medicago satina*) and fescue (*Festuca arundinacea*), representing two forages that vary markedly in relation to the pentosan:α-cellulose ratio, were chosen for the investigation. Five samples of forage were selected from each species in such a manner that a graduated scale of crude cellulose values was represented in each series of samples. The fescue samples were obtained from harvests of two seasons and the alfalfa samples from one. Each sample was fed to two sheep in a conventional digestion trial and the feed and feces subjected to the following analysis. The method of Matrone et al. ('46) was used to isolate the cellulosic micelle. The spectrophotometric method of Adams and Castagne ('47) employing a pentosan to furfuraldehyde conversion was used to determine the pentosan content of the crude cellulose and the total pentosan content of the feed and fecal samples. This method does not determine the hydroxymethyl- and methyl-furfuraldehyde derivatives arising from the substances associated with pentosans, i.e., cellulose, starch, and hexose sugars. True or α-cellulose was determined as that portion of crude cellulose which was insoluble in 17.5% NaOH according to the method of TAPPI ('58).

Sample treatment was as follows: (1) isolation of the crude cellulose from a 1-gm air-dried sample and quantitative transfer of this crude cellulose to the furfural-

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dehyde distilling flask, using 12% HCl to rinse the Erlenmeyer flask, filter stick and rubber policeman for the furfuraldehyde determination, and (2) isolation of crude cellulose from another 1-gm air-dried sample for the α-cellulose determination.

RESULTS AND DISCUSSION

The fractions of the various carbohydrates of fescue and alfalfa feeds are presented in table 1. The cellulosic micelle, as isolated by the crude cellulose method of Matrone et al. ('46), comprises a considerable portion of the plant material in both alfalfa and fescue. Also pertinent is the relatively large amount of total furfuraldehyde-yielding substances in the two forages. When calculated as pentosans these substances accounted for an average of 10.77% of the dry material in alfalfa and an average of 16.68% in fescue. The fescue micelle was 28.12% pentosans, which is in agreement with the values presented for grasses by Ely et al. ('53) and Norman ('36a). The average pentosan content of the alfalfa micelle was 17.20% furfuraldehyde-yielding material calculated to pentosans. The available literature indicates that the furfuraldehyde yield from alfalfa or legume crude cellulose fractions is xylan. Bennett ('40) reported that red clover contains about one-half the amount of furfuraldehyde yield as does bluegrass. Phillips and Davis ('40) observed that furfuraldehyde-yielding constituents account for 77.34% of a hemicellulose fraction of alfalfa hay. Subsequent hydrolysis indicated that the major portion of the hemicellulose fraction was D-xylose. This hemicellulose-β fraction accounted for 4.3% of the moisture-free alfalfa hay. Norman ('35b) reported that the cellulose of bean straw, growing vetch, and mature vetch contained 9.0, 3.0 and 6.3% of xylan, respectively. Nowotonowna ('36) found that the xylan content of some wood gymnosperm celluloses ranged from 5.05 to 11.61%. In view of this information and the specificity of the pentosan method used it is probable that this furfuraldehyde yield from the alfalfa micelle arises from pentosan material.

A considerable portion, 9.08%, of the alfalfa micelle was unaccounted for by either the α-cellulose or micellular pentosans. The exact nature of this undetermined material is not apparent from this study. The average digestibility of the alfalfa micellular pentosans was 40.54%, whereas the average digestibility of the fraction represented by the difference between the crude cellulose and α-cellulose fractions was 52.40%. Since this fraction included both the micellular pentosans and the undetermined material, the unidentified material is not likely to be lignin. Norman and Fuller ('42) stated that mannan in gymnosperms is one cellulosan that accounts for varying portions of crude

TABLE 1

Carbohydrate fractions from fescue and alfalfa

Sample	Cellulose		Pentosans		
	Crude	α-Fraction	Total	Micellular	Non-micellular
		Fescue			
1	23.32	15.69	12.34	5.27	7.07
2	26.89	20.03	16.20	8.73	7.47
3	30.21	18.08	14.72	6.91	7.81
4	32.76	23.68	20.01	10.35	9.66
5	37.08	28.10	20.11	11.53	8.58
Average	30.05	21.12	16.68	8.56	8.12
		Alfalfa			
6	18.55	12.69	8.78	2.94	5.84
7	24.32	17.06	9.68	3.70	5.98
8	27.86	20.73	10.97	5.34	5.63
9	32.43	24.82	11.92	6.09	5.83
10	33.37	24.95	12.50	5.68	6.82
Average	27.31	20.05	10.77	4.75	6.02

TABLE 2

Digestion coefficients of indicated carbohydrate fractions of fescue and alfalfa

Sample	Cellulose		Pentosans		
	Crude	α-Fraction	Total	Micellular	Non-micellular
		Fescue			
1	78.38	80.01	81.78	87.45	77.55
	87.12	85.73	86.30	90.93	82.86
2	92.02	90.60	90.58	94.31	86.23
	90.36	88.66	88.04	91.12	84.45
3	76.99	74.98	74.49	80.50	69.18
	78.52	77.31	76.06	79.06	73.40
4	75.83	75.58	74.54	76.34	72.40
	69.47	69.84	69.28	72.57	65.77
5	52.36	57.69	51.51	59.44	40.85
	62.24	65.33	60.84	66.51	53.23
Average	76.33	76.57	75.34	79.84	70.59
		Alfalfa			
6	69.32	66.06	75.23	56.98	84.40
	70.24	68.34	74.95	54.14	85.40
7	65.19	63.46	66.05	43.98	79.68
	61.84	63.20	62.22	37.82	77.28
8	54.54	58.99	56.81	42.69	70.22
	57.65	60.67	59.56	47.67	70.86
9	52.22	56.44	48.36	35.50	61.77
	51.94	57.52	47.83	40.30	55.69
10	48.95	52.96	42.38	20.64	60.47
	53.72	56.44	45.52	25.63	60.07
Average	58.56	60.41	57.89	40.54	70.78

cellulose fractions and has no association other than with the cellulosic aggregate. Nowotonowna ('36) observed that the mannan content of several woods ranged from 3.84 to 10.14% of the crude cellulose and was associated with it. In view of this information, mannan appears to be the likely constituent of this other fraction of the alfalfa (a gymnosperm) micelle.

Since the fescue samples were selected from studies over a two-year period the stage of maturity pattern is not continuous. However, as the crude cellulose content ranged from 23.32 to 37.08%, the α-cellulose and micellular pentosans ranged from 15.69 to 28.10% and from 5.27 to 11.53%, respectively. The alfalfa samples were selected within one season and showed that the increase in crude cellulose content (18.55 to 33.77%) was directly related to advancing maturity. The α-cellulose content of the plant material increased from 12.69 to 24.95% and pentosan content from 2.94 to 5.68%.

In both fescue and alfalfa the digestibility of the micelle and its component fractions, α-cellulose and micellular pentosans, showed a pronounced decrease with increasing crude cellulose content of the plant material. However, the constituents of the cellulose micelle were digested differently in the two forages under study. The pentosan fraction of the alfalfa micelle was digested to a significantly less ($P < 0.01$) extent than the α-cellulose fraction, whereas with fescue the opposite was true — the pentosan fraction was more digestible than the α-cellulose fraction although not quite significant at the 5% level ($F = 4.79$, F required for $P < 0.05 = 4.96$). These results are shown in table 2.

Thus the cellulosic micelle is not a nutritive entity. Being composed of at least two fractions of different digestibility, the cellulosic micelle of both alfalfa and fescue must be considered heterogeneous with respect to digestibility. The inclusion of the pentosan fraction results in an increase in the digestion coefficient of the fescue crude cellulose and a decrease in the alfalfa crude cellulose digestion coefficient. These opposite effects in the common forages, fescue and alfalfa, negate the increased definition of plant material

by micelle isolation and indicate that a different characterization of this carbohydrate fraction should be used.

The average digestibility of the alfalfa α-cellulose was less than the fescue α-cellulose and the digestibility of the α-cellulose of both forages decreased with the advancing maturity trend. Therefore, even α-cellulose isolation and determination is not a nutritive entity characterization. The non-micellular pentosans of both alfalfa and fescue were digested to about the same extent.

When the micellular pentosans and non-micellular pentosans were treated as a unit, the digestibility was essentially the same as the corresponding α-cellulose fraction. This was true for both the alfalfa and fescue hays. Therefore, an analysis which would isolate the entire furfuraldehyde-yielding fraction plus the α-cellulose fraction would isolate material that would have the same overall digestibility within a given species of forage at a given stage of maturity, thus characterizing a greater portion of the carbohydrate fraction. This aspect warrants further consideration.

SUMMARY

The determination of the digestibility of the components of the cellulosic micelle has shown that the α-cellulose and pentosan constituents are digested unequally, with the micellular pentosans of alfalfa being less digestible than the alfalfa α-cellulose and the reverse situation being true with fescue; hence the micelle can not be considered a nutritive entity in these two forages. Thus the greater characterization of the carbohydrate fraction by micelle isolation may not be warranted. The digestibility of the total pentosans, accounting for 16.68 and 10.77% of the dry matter in fescue and alfalfa, respectively, when compared with the digestibility of the α-cellulose within species, was not different, suggesting that the combination of these two could result in a biologically meaningful partitioning of forages.

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Effect of Diet on Work Metabolism

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It has been generally assumed that carbohydrate is the main and primary energy source during exercise. Fat has been considered only as reserve fuel, used mainly during recovery. However, in recent years, evidence has accumulated which seems to suggest that the plasma free fatty acids are an important fuel for muscular work, and that the carbohydrate metabolism starts to play a major role only in heavy exercise when the oxygen supply of the muscle becomes insufficient (Fritz, '61). It was shown by Fritz and co-workers ('58) that electrical stimulation increased the oxidation of palmitic acid-C^{14} in the rat diaphragm and that the rate of oxidation increased with the concentration of free fatty acids in the medium. According to Andres et al. ('56), the O_2 uptake of the skeletal muscle in man is so high that it cannot be explained by the oxidation of glucose alone. During electrical stimulation of the thigh muscle in anesthetized dogs, a consistent uptake of free fatty acids was found on the stimulated side (Issekutz and Spitzer, '60). Friedberg and Estes ('62) demonstrated the oxidation of palmitic acid-1-C^{14} in the forearm of man. Exercise increased the disappearance rate of injected palmitic-1-C^{14} (Friedberg et al., '60). It therefore seemed of considerable practical importance to study the participation of the two major fuels: fat and carbohydrate, in the energy expenditure during exercise, under different dietary conditions.

In these studies, very light exercise, 300 kpm[1]/minute on the bicycle ergometer, was chosen for two reasons: (a) this work load increases the oxygen uptake to only about 4 times that of the basal metabolic rate (BMR), and simulates more closely the normal activity of man than is the case with heavy exercise; (b) heavier work increases the blood lactic acid concentration as a sign of insufficient oxygen supply to the working muscle. The decrease of tissue P_{O_2} increases the utilization of glucose and the elevated lactic acid level lowers the plasma free fatty acid concentration (Issekutz and Miller, '62). Both these factors tend to shift the metabolism towards carbohydrates.

METHODS

Six healthy young men 19 to 24 years of age served as subjects for this study, living for about 70 days under strictly controlled metabolic ward conditions. The following 4 diets were used: diet 1 was the basal diet, consisting of 3,000 Cal., 73 gm of protein, 107 gm of fat and 463 gm of carbohydrate. Diet 2 was a low protein diet. It contained 33 gm of protein, 114 gm of fat and 462 gm of carbohydrate; 3,000 Cal. Diet 3 was a high fat and low carbohydrate diet with a total caloric value of about 3,500. It consisted of 89 gm of protein, 231 gm of fat and 247 gm of carbohydrate. Diet 4 supplied only 1,600 Cal./day (67 gm of protein, 65 gm of fat and 175 gm of carbohydrate). This offered a possibility to study the combined effect of both low fat and low carbohydrate intake. Two subjects were studied with each of the 4 diets.

The formula diets used in this study were analyzed periodically for protein and fat content. The former was measured by the micro-Kjeldahl methods as described by Niederl and Niederl ('42). The analyses of fat were carried out by the acid hydrolysis method (AOAC, '60).

On the day of the metabolic measurements, starting at 8:00 AM, the urine was

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[1] kpm indicates Kilopond meter; 1 kp is the force acting on the mass of 1 kg at normal acceleration of gravity.

collected for 24 hours. Urinary nitrogen (Niederl and Niederl, '42) and creatinine (Anker, '54) excretions were determined.

The basal metabolic rate was determined after 14 to 15 hours of fasting by using the Noyons BMR apparatus. A plastic hood was placed over the head of the subject, an air flow of 50 liters/min was maintained by a pump and after 30 minutes of complete rest, the O_2 uptake and CO_2 output were read for 5 to 7 minutes. Then the subject started to work on the bicycle ergometer described by von Döbeln ('55) at 300 kpm/min; the pedal frequency was 50 rev/min. This work load induces an O_2 uptake of 0.97 ± 0.07 liters/min and a negligible increase of the blood lactic acid level of about 5.4 ± 1.2 mg/100 ml (Issekutz and Rodahl, '61). After 20 minutes' work, collection of expired air was started. With the aid of a nose clip, a mouthpiece, and a Krogh valve, the expired air was directed through a hose into the plastic hood. An air flow of 50 liters/min diluted and carried the expired air into the Noyons diaferometer. The work was continued for 10 more minutes. Within 5 minutes an equilibrium of 99% was reached in the hood (Issekutz and Rodahl, '61). After this, the O_2 uptake and CO_2 output were recorded continuously for 4 minutes.

The Noyons diaferometer is supplied with a factory-made calibration table which was checked from time to time, as described in a previous paper (Issekutz and Rodahl, '61). As an additional check, immediately after taking the diaferometer readings during the last minute of exercise, expired air was collected in a Douglas bag, the volume of which was then measured by a dry gas meter. Room air and expired air were analyzed according to the micro-method of Scholander ('47). The mean difference in the respiratory quotient (RQ) between the two methods found in 23 experiments was 0.009 with an SD of ± 0.027, and in the O_2 uptake: 0.019 liters/min; SD ± 0.041.

TABLE 1

Diet 1: 3,000 Cal.; protein, 73 gm; fat, 107 gm; CHO, 463 gm

Subject	Days	Urinary N	Respiratory quotient (RQ)			Oxidized during work	
			BMR	Work	Work after sugar	Fat	CHO
		gm/day				%	%
B	4	8.84	0.79	0.83			
	11	9.65	0.82	0.81			
	25	10.66	0.87	0.87			
	32	9.64	0.79	—	0.92	27	73
	39	8.70	0.77	0.85			
	53	10.19	0.86	—	0.93	23	77
	60	9.80	0.84	0.86			
	63	10.34	0.89	0.85			
	71	8.58	0.82	0.86			
		Mean:	0.83	0.85		41	59
		SE:	±0.01	±0.01			
		SD:	±0.04	±0.02			
SH	4	8.62	0.80	0.85			
	11	9.32	0.76	0.80			
	25	9.11	0.89	0.84			
	32	9.01	0.83	—	0.93	23	77
	39	8.11	0.80	0.84			
	53	8.76	0.85	—	0.91	30	70
	60	9.15	0.79	0.83			
	63	9.06	0.77	0.80			
	71	8.80	0.75	0.80			
		Mean:	0.80	0.82		59	41
		SE:	±0.01	±0.01			
		SD:	±0.04	±0.02			

The percentage participation of carbohydrate and fat in the energy metabolism was calculated on the basis of standard tables for nonprotein RQ.

On certain days indicated in the tables 1–4, 100 gm of glucose in 200 ml of water were administered orally immediately after completion of the BMR measurements, and the exercise started 1.5 hours later so that the metabolic measurements were carried out about two hours after the glucose administration.

RESULTS

Table 1 shows the effects of the control diet (diet 1) in two subjects (B and SH) during a 71-day experiment. The average RQ under basal conditions was 0.83 in subject B, and 0.80 in subject SH. During exercise the RQ was slightly higher (by 0.02) and less variable than during rest. This work RQ may mean that fat and carbohydrates were participating in the energy metabolism to about the same extent. When, two hours before exercise, 100 gm of glucose were given orally, the work RQ was increased in every instance to 0.91 to 0.93.

A reduction of the protein intake to 33 gm/day (diet 2), which caused roughly a 50% decrease in the oxidation of proteins, failed to induce any consistent change, either in the basal metabolic RQ or in the work RQ (table 2). The latter was somewhat higher than the resting value. Sugar loads, also here, increased the exercise RQ to 0.90 to 0.95.

Table 3 shows the effect of a high fat and low carbohydrate diet (diet 3). Whereas the basal metabolic RQ fluctuated between 0.75 and 0.83 in one of the subjects (subject A) and between 0.70 and 0.81 in the other (subject B), the exercise RQ showed a gradual decline. After the sub-

TABLE 2

Diet 2: 3,000 Cal., protein, 33 gm; fat, 114 gm; CHO, 462 gm

Subject	Days	Diet	Urinary N	Respiratory quotient (RQ)			Oxidized during work	
				BMR	Work	Work after sugar	Fat	CHO
			gm/day				%	%
P	3	1	10.79	0.75	0.91			
	10	1	9.91	0.76	0.81			
	14	2	—	—	—			
	24	2	4.72	0.85	0.86			
	31	2	4.49	0.81	—	0.95	17	83
	38	2	4.22	0.81	0.83			
	52	2	4.20	0.79	—	0.90	33	67
	59	2	3.80	0.85	0.89			
	65	2	4.41	0.85	0.87			
	70	2	4.29	0.81	0.90			
		Mean RQ with diet 2:		0.82	0.87		43	57
			SE:	±0.01	±0.01			
			SD:	±0.02	±0.03			
K	3	1	11.00	0.84	0.86			
	10	1	10.29	0.83	0.85			
	14	2	—	—	—			
	24	2	5.41	0.82	0.89			
	31	2	5.31	0.87	—	0.91	30	70
	38	2	5.06	0.81	0.86			
	52	2	4.71	0.88	—	0.90	33	67
	59	2	5.09	0.85	0.85			
	65	2	4.40	0.83	0.84			
	70	2	3.86	0.85	0.90			
		Mean RQ with diet 2:		0.84	0.87			
			SE:	±0.01	±0.01			
			SD:	±0.02	±0.02			

TABLE 3

Diet 3: 3,500 Cal.; protein, 89 gm; fat, 231 gm; CHO, 247 gm

Subject	Days	Diet	Urinary N	Respiratory quotient (RQ)			Oxidized during work	
				BMR	Work	Work after sugar	Fat	CHO
			gm/day				%	%
A	4	1	9.37	0.78	0.85			
	11	1	9.79	0.79	0.83			
	16	3	—	—	—			
	25	3	9.79	0.75	0.77			
	32	3	10.29	0.79	—	0.81	63	37
	40	3	10.28	0.83	0.76			
	53	3	11.49	0.76	—	0.80	67	33
	68	3	9.34	0.80	0.74			
		Mean RQ with diet 3:		0.79	0.76		81	19
			SE:	±0.01				
			SD:	±0.03				
B	4	1	8.62	0.76	0.80			
	11	1	9.21	0.78	0.87			
	16	3	—	—	—			
	25	3	11.54	0.70	0.81			
	32	3	10.93	0.74	—	0.87	43	57
	40	3	10.86	0.81	0.78			
	53	3	11.66	0.76	—	0.86	47	53
	68	3	10.33	0.80	0.73			
		Mean RQ with diet 3:		0.76	0.77		75	25
			SE:	±0.02				
			SD:	±0.04				

TABLE 4

Diet 4: 1,600 Cal.; protein, 67 gm; fat, 65 gm; CHO, 175 gm

Subject	Days	Diet	Urinary N	Respiratory quotient (RQ)			Oxidized during work	
				BMR	Work	Work after sugar	Fat	CHO
			gm/day				%	%
D	5	1	8.57	0.78	0.84			
	12	1	8.82	0.85	0.80			
	15	4	—	—	—			
	24	4	10.17	0.70	0.77			
	32	4	9.94	0.79	—	0.79	70	30
	41	4	10.39	0.75	0.74			
	54	4	10.71	0.80	—	0.85	50	50
	68	4	12.22	0.81	0.74			
		Mean RQ with diet 4:		0.77	0.75		84	16
			SE:	±0.02				
			SD:	±0.04				
SH	5	1	9.65	0.74	0.83			
	12	1	9.64	0.74	0.80			
	15	4	—	—	—			
	25	4	11.25	0.70	0.74			
	32	4	11.50	0.83	—	0.84	54	46
	41	4	11.98	0.79	0.74			
	54	4	11.33	0.75	—	0.87	43	57
	68	4	12.20	0.79	0.74			
		Mean RQ with diet 4:		0.77	0.74		87	13
			SE:	±0.02				
			SD:	±0.05				

jects had eaten this diet for 24 days, the work RQ was found to be lower than the resting RQ, and at the end of the study, values of 0.74 and 0.73 were measured, indicating that the exercise energy was supplied mainly by the oxidation of fat. Subject B, who also participated in the control experiment (diet 1) at which time his work RQ was 0.85, had a work RQ of 0.73 after having eaten diet 3 for 52 days. Also in this experiment the glucose load increased the work RQ, although not above 0.87 (table 3).

To determine whether the high fat or the low carbohydrate content of diet 3 was responsible for the low work RQ, two subjects (D and SH) were given a calorically restricted diet which was low in both fat and carbohydrate (diet 4, table 4). The results were similar to those obtained with the high fat diet. After the subjects had eaten this diet for 26 days, the exercise RQ (0.74) was lower than the basal metabolic RQ (0.75 to 0.79). In one subject (SH), we had the opportunity to compare the effects of diet 1 and diet 4. Whereas the average work RQ with the basal diet was 0.82, after the subject had eaten diet 4 for 10 days, his work RQ was 0.74 and remained at this low level for the rest of the study. This may indicate that during work some 87% of the energy was derived from oxidation of fat and only 13% from carbohydrates. Ingestion of 100 gm of glucose two hours before exercise shifted this ratio in favor of carbohydrates.

DISCUSSION

These experiments on 6 subjects consuming 4 diets for long periods showed that it was possible at a light work load (300 kpm/min) to obtain respiratory quotients which indicated a major participation of fat oxidation in the work metabolism. Since it is known that fat cannot be converted to carbohydrate, or only to a very limited extent, and because the approximately fourfold increase of the energy expenditure above the BMR level mainly represents muscle metabolism, the conclusion appears to be justified that under certain circumstances the skeletal muscle utilizes mainly fatty acids for fuel.

Diet is certainly one of the factors that can profoundly influence work metabolism. Christensen and Hansen ('39) reported that high fat and low carbohydrate (5% of total calories) diets decreased the work RQ, whereas high carbohydrate (90% of total calories) and low fat diets had the opposite effect. A few days (3 to 7) of eating these extreme diets was enough to change the metabolic pattern of their trained subjects working at a load of 1,080 kpm/min.

In the present study, diets were used that were within the range of possibility of ordinary dietary habits. The work load used, if maintained for 8 hours, represented a total energy expenditure (including 16 hours' rest) of not more than 3,400 Cal./day. When the fat content of the diet was increased from 107 to 231 gm and the carbohydrate was reduced from 463 to 247 gm (diet 3), the exercise RQ decreased to 0.74. The same effect was achieved, however, when the fat intake was reduced to 67 gm and the carbohydrate was maintained at the level of 175 gm/day (diet 4). This suggests that the actual carbohydrate intake rather than the amount of fat in the diet is the decisive factor, determining whether fatty acids or glucose is the preferred fuel. This assumption was further supported by the observation that ingestion of 100 gm of glucose increased the RQ during exercise. A similar observation was reported also by Wrightington ('42). Such an interpretation is also in accordance with the observations of Lewis et al. ('59) that glucose suppresses the oxidation of palmitic acid but fat cannot depress glucose oxidation.

It is possible that a low carbohydrate diet induces a shifting in the hormonal balance. It is known that in rabbits the glucose tolerance is increased by carbohydrate-rich diets and decreased by low carbohydrate diets; in the latter case also the response to insulin is reduced (Himsworth and Scott, '38). Since insulin not only facilitates the glucose uptake of the muscle but also inhibits the release of free fatty acids in the adipose tissue, and, like the ingestion of glucose, it decreases the blood fatty acid levels (Dole, '56), one may speculate that a diet low in carbohydrate brings about a depressed insulin production and consequently causes a high rate of release of free fatty acids. If so, the

skeletal muscle would be expected to oxidize mainly fatty acids; and when muscle metabolism begins to represent a major part of the overall energy metabolism when changing from rest to exercise, this is revealed by the observed decrease in the RQ.

SUMMARY AND CONCLUSIONS

The participation of fat and of carbohydrate in the energy expenditure during light exercise (300 kpm/min) on the bicycle ergometer was studied in 6 healthy men 19 to 24 years of age, living for periods of about 70 days under strictly controlled metabolic ward conditions while consuming the following diets: (1) 3,000 Cal., 73 gm of protein, 107 gm of fat, 463 gm of carbohydrate (basal diet); (2) 3,000 Cal., 33 gm of protein, 114 gm of fat, 462 gm of carbohydrate; (3) 3,500 Cal., 89 gm of protein, 231 gm of fat, 247 gm of carbohydrate; and (4) 1,600 Cal., 67 gm of protein, 65 gm of fat, 175 gm of carbohydrate. In connection with the metabolic measurements, 24-hour urine samples were collected for nitrogen and creatinine determinations. The basal metabolic rate was determined 14 to 15 hours postprandial using the Noyons BMR apparatus, which was also used for the analysis of expired air collected between 20 and 30 minutes of work at 300 kpm. In separate experiments, metabolic measurements were made during work, two hours following ingestion of 100 gm of glucose.

With the control diet (diet 1), the average respiratory quotient (RQ) during exercise was somewhat higher than during rest. A reduction of the protein intake to 33 gm/day (diet 2), which caused roughly a 50% decrease in the oxidation of proteins, failed to induce any consistent change in the basal metabolic RQ or in the work RQ as compared with diet 1. The high fat and low carbohydrate diet (diet 3) caused a gradual decline in the exercise RQ, reaching values of about 0.74 after 52 days, indicating that the exercise energy was supplied mainly by the oxidation of fat. The 1,600 caloric diet, low in both fat and carbohydrate (diet 4), gave results quite similar to those obtained with the high fat diet. In all cases, ingestion of glucose two hours before exercise caused an increase in the exercise RQ.

These experiments show that it is possible at light work loads (300 kpm/min) to obtain respiratory quotients that indicate a major participation of fat oxidation in the work metabolism. These data also suggest that the actual carbohydrate intake rather than the amount of fat in the diet is the decisive factor, determining whether fatty acids or glucose is the preferred fuel.

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