Statistical Study of the Relationship Between Dietary Linoleate and the Fatty Acids of Heart and Blood Lipids[1]

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One of the practical and increasing problems in biochemical and nutritional work involving tissue lipid analysis arises from the multiplicity of variables encountered. Alkaline isomerization and associated techniques allow the quantitative measurement of some 6 or 7 fatty acid fractions, both in the diet and in each of the tissues. Gas chromatography methods may well increase this number three- or fourfold. Biochemically many of these fatty acids are related to each other and to other dietary components by mechanisms that are still to be established; but not all of the measurable fractions are positively and linearly related with each other. Holman ('60) has used the negative correlation between triene and tetraene fractions and their sharply curvilinear relationships to dietary linoleate as a basis for detecting a deficiency of essential fatty acids (EFA) in experimental animals, and for estimating their minimum requirement.

A systematic study of the statistical relationship between the experimental variables would seem a useful first step toward understanding the nutritional observations and establishing the metabolic interrelations in this system. For the purposes of this statistical study, the data of Holman ('60) were chosen because they provide, for the same animals, alkaline isomerization data on several body components together with information on weight gain and dermatitis score. It is the purpose of the present study to: (a) describe the relationship of each variable to all others with particular attention to their relationship to dietary linoleate; (b) explore those statistical techniques that are particularly suited to handle multivariate problems (with a view toward the added complexities associated with the future treatment of gas chromatography data); and (c) approach the problems of using this wide variety of data as a basis for defining the nutritional state of the individual.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain, weighing an average of 41 gm, were divided into 7 groups of 8 rats each. These groups were maintained with diets of controlled fatty acid composition as described in table 1. The linoleate content, as well as total fat content, of the diets was varied by changing the relative amounts of butter fat and cottonseed oil in the diets. The primary data used in this statistical study as well as the details of the nutritional experiment have been published previously (Holman, '60).

Statistical work was carried out with the aid of the Univac 1103 digital computer. The correlation coefficients and multiple regression equations for estimating dietary linoleate were obtained by using the multiple regression and correlation program of McGowan et al.[4] The factor analysis pro-

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[4] McGowan, D. C., J. Rose and L. Kennedy 1956 A multiple regression and correlation program for the Univac Scientific. 1103 Central Exchange Newsletter, 9: RR-127.

TABLE 1

Body weight, dermal score and tissue lipid composition observed in groups of rats maintained with controlled dietary fatty acid intake

	Group 1		Group 2		Group 3		Group 4		Group 5		Group 6		Group 7	
	Mean	σ	Mean	σ	Mean	σ	Mean	σ	Mean	σ	Mean	σ	Mean	σ
Dietary														
Linoleate[1]	0	—	0.14	—	1.12	—	5.11	—	0.56	—	4.48	—	20.2	—
Total fat[1]	0	—	10.0	—	10.0	—	10.0	—	40.0	—	40.0	—	40.0	—
Weight gain[2]	164	22	224	30	244	30	255	19	231	27	259	25	254	20
Dermatitis score[3]	4.50	1.04	1.21	0.57	0.07	0.19	0	0	0.10	0.22	0.10	0.22	0	0
Cardiac[4]														
Dienes	−3.3	4.0	69	17	212	24	279	32	116	20	228	22	455	51
Trienes	395	35	134	30	41.1	4.9	10.2	6.4	68.1	9.4	34.3	9.8	17.4	4.1
Tetraenes	108	23	135	70	333	29	298	32	236	24	277	50	395	39
Pentaenes	14.6	3.4	28.3	3.6	48.9	4.2	81	12	63.1	5.7	54.7	9.9	174	11
Hexaenes	13.3	2.1	51.1	7.9	52.0	5.8	9.7	1.6	120	15	86.0	17.4	16	12
Plasma[5]														
Dienes	−1.2	1.4	2.0	1.5	18.0	3.2	43	18	16.0	2.6	25	14	55.3	7.3
Trienes	14.1	6.3	7.5	2.5	9.1	1.2	3.3	0.9	5.7	1.2	4.6	1.7	1.7	0.9
Tetraenes	2.9	1.0	6.4	1.6	27.4	5.5	39.1	4.6	12.1	3.8	26.7	2.9	38.8	5.1
Pentaenes	3.8	1.3	1.6	0.3	2.8	0.6	4.5	1.1	3.8	0.7	2.3	0.6	3.8	0.7
Hexaenes	1.5	0.4	1.9	0.5	3.0	0.7	1.1	0.3	4.0	1.1	3.9	0.9	2.0	0.6
Erythrocyte[5]														
Dienes	−0.8	1.6	4.8	1.7	11.3	4.8	13.1	2.3	6.5	3.5	13.7	4.6	22.0	2.7
Trienes	30.6	6.3	16.7	3.6	4.9	1.7	2.0	0.9	4.9	2.0	1.9	1.2	1.8	0.5
Tetraenes	13.7	3.8	27.5	5.6	37.3	9.8	31	11	20.4	9.2	28	14	44.3	7.5
Pentaenes	1.4	0.5	5.5	0.8	4.2	1.0	4.2	1.6	6.2	2.5	4.1	1.7	5.8	1.1
Hexaenes	2.8	1.2	7.9	1.0	5.9	0.6	3.3	1.0	3.8	1.9	2.0	0.9	0.7	0.4

[1] Percentage of total caloric intake.
[2] Grams in 89 days.
[3] Arbitrary units (Holman and Ener, '54).
[4] Mg per 100 gm of fresh tissue weight.
[5] Mg per 100 ml.

cedure is that of Thurstone ('35). In the first step of this procedure the largest correlation in each column is used as an estimate of the communality (Holzinger and Harman, '41). The whole factor analysis is then repeated, using the sum of the squares of the factor loadings as the new communality estimate. The correlation coefficients, regression coefficients and other statistics were computed to at least 5 decimal places in each case, but these values have been rounded for simplicity of presentation.

Correlations between variables

In table 1 are summarized some of the more pertinent experimental data that form the basis for the statistical studies. Other data have been discussed in detail by Holman ('60). Since most of the values in table 1 represent the means from 7 animals (range, 5 to 8) the significance of the difference between any two means can be estimated from the fact that the least significant difference is 1.2 or 1.7 times the standard deviation for P = 0.05 or 0.01, respectively. Interest at this point is directed not so much at the absolute differences between any two of these groups as it is in discovering the systematic and general relationships between dietary linoleate and the variables represented here.

The top lines of tables 2 and 3 show the correlations between dietary linoleate and a group of analytical variables related to the fatty acid composition of the heart, plasma, and red cell lipids, and to the rate of weight gain and the degree of dermatitis observed in the animals. The rest of the correlations in these two tables represent the interrelationship between each one of these experimentally determined variables and all of the others. Each correlation coefficient is based upon 47 pairs of observations. Hence, any correlation greater than r = 0.28 or 0.37 is significant at the 5 or 1% level of probability, respectively.

Among the significant correlations relating to dietary linoleate, there are particularly high positive correlations with heart, plasma and red cell dienes, tetraenes and pentaenes. Significant negative correlations exist between dietary linoleate and the trienes of heart, plasma and red cells. This forms the basis for the suggestion (Holman, '60) that the ratio of triene to tetraene in tissue lipids should be a useful indicator of a dietary deficiency of linoleic acid.

There is a significant negative correlation between dietary linoleate and dermatitis score, namely, the lower the linoleic acid in the diet the greater the tendency for a dermatitis to develop. The dermatitis scores of the feet, tail and coat were all studied, both independently and as related to the total score. The correlation between the total score and its different components ranged between + 0.89 and + 0.97. In view of the high, positive correlations between these different measures, there appeared to be little value in considering the scores for the tail, feet and coat separately. Only the total score is reported in tables 1 and 3.

TABLE 2

Correlations between dietary linoleate intake and the concentration of different fractions of unsaturated fatty acids in cardiac lipid

Variable		Variable number							
No.	Description	2	3	4	5	6	7	8	9
1	Dietary linoleate	0.88	−0.44	0.70	0.93	−0.38	0.88	−0.39	0.56
	Heart fatty acids								
2	Dienes		−0.74	0.90	0.89	−0.25	0.91	−0.69	0.39
3	Trienes			−0.76	−0.56	−0.21	−0.48	0.98	0.12
4	Tetraenes				0.80	−0.06	0.88	−0.74	0.49
5	Pentaenes					−0.24	0.91	−0.52	0.58
6	Hexaenes						0.24	−0.27	−0.12
7	Total PUFA[1]							−0.45	0.74
8	Tri/tetra ratio								0.11
9	Endogenous PUFA[2]								

[1] PUFA indicates polyunsaturated fatty acids.
[2] Endogenous PUFA indicates total PUFA minus linoleate.

TABLE 3

Correlations between a number of chemical and physiological variables related to dietary linoleate intake

Variable No.	Variable Description	Variable number 10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
	Diet																		
1	Linoleate	0.70	−0.60	0.67	0.46	−0.14	0.70	−0.41	0.56	0.80	−0.48	0.56	0.32	−0.64	0.46	−0.40	0.09	0.39	−0.37
	Heart fatty acids:																		
2	Dienes	0.85	−0.71	0.89	0.70	−0.02	0.89	−0.70	0.80	0.89	−0.76	0.67	0.39	−0.50	0.43	−0.70	−0.01	0.62	−0.67
3	Trienes	−0.63	0.73	−0.76	−0.79	−0.24	−0.70	0.98	−0.68	−0.74	0.94	−0.59	−0.60	0.07	−0.26	0.97	0.11	−0.78	0.96
4	Tetraenes	0.75	−0.62	0.88	0.73	0.14	0.82	−0.74	0.84	0.82	−0.81	0.63	0.33	−0.45	0.33	−0.75	−0.08	0.64	−0.73
5	Pentaenes	0.69	−0.67	0.74	0.63	−0.07	0.72	−0.54	0.64	0.81	−0.60	0.59	0.42	−0.58	0.43	−0.53	0.04	0.46	−0.51
6	Hexaenes	−0.37	−0.02	−0.29	0.02	0.77	−0.33	−0.26	−0.25	−0.15	−0.21	−0.20	0.28	0.20	−0.31	−0.27	−0.28	0.12	−0.29
7	Total PUFA[1]	0.72	−0.56	0.77	0.56	0.01	0.77	−0.46	0.71	0.79	−0.58	0.58	0.25	−0.67	0.39	−0.46	0.01	0.43	−0.44
8	Tri/tetra	−0.56	0.67	−0.70	−0.76	−0.28	−0.63	0.97	−0.64	−0.70	0.92	−0.57	−0.62	0.02	−0.25	0.99	0.10	−0.78	0.98
9	Endogenous PUFA	0.22	−0.09	0.26	0.10	0.08	0.27	0.12	0.27	0.30	−0.04	0.18	−0.08	−0.66	0.15	0.10	0.01	−0.05	0.11
	Plasma fatty acids:																		
10	Dienes		−0.61	0.89	0.74	−0.14	0.96	−0.58	0.83	0.77	−0.66	0.53	0.22	−0.48	0.31	−0.57	−0.07	0.57	−0.55
11	Trienes			−0.64	−0.63	−0.00	−0.57	0.76	−0.42	−0.70	0.71	−0.44	−0.46	0.38	−0.21	0.66	0.15	−0.48	0.69
12	Tetraenes				0.82	−0.04	0.96	−0.71	0.96	0.81	−0.78	0.60	0.25	−0.43	0.31	−0.70	−0.09	0.68	−0.68
13	Pentaenes					0.08	0.79	−0.76	0.80	0.63	−0.80	0.42	0.41	−0.26	0.12	−0.75	−0.23	0.64	−0.76
14	Hexaenes						−0.05	−0.29	0.05	0.08	−0.26	0.11	0.34	0.08	−0.00	−0.28	0.05	0.26	−0.33
15	Total PUFA							−0.64	0.94	0.80	−0.73	0.60	0.24	−0.47	0.33	−0.64	−0.06	0.64	−0.62
16	Tri/tetra								−0.62	−0.72	0.92	−0.57	−0.61	0.04	−0.25	0.96	0.11	−0.77	0.97
17	Endogenous PUFA									0.72	−0.72	0.56	0.19	−0.36	0.28	−0.64	−0.08	0.67	−0.62
	Erythrocyte fatty acids:																		
18	Diene										−0.76	0.71	0.53	−0.38	0.55	−0.71	0.08	0.60	−0.69
19	Triene											−0.51	−0.47	0.25	−0.12	0.93	0.30	−0.71	0.90
20	Tetraene												0.67	−0.02	0.88	−0.59	0.65	0.54	−0.56
21	Pentaene													0.23	0.64	−0.62	0.46	0.54	−0.63
22	Hexaene														0.15	0.02	0.40	−0.06	0.01
23	Total PUFA															−0.26	0.88	0.30	−0.26
24	Tri/tetra																0.10	−0.78	0.98
25	Endogenous PUFA																	0.01	0.10
	Response																		
26	Weight gain																		−0.77
27	Score[2]																		

[1] PUFA indicates polyunsaturated fatty acids.

[2] Sum of the dermatitis scores for tail, paws and coat.

In another population of 75 male weanling rats which had been fed a fat-free diet plus 1% of cholesterol for 62 days to induce accelerated EFA deficiency (Holman and Peifer, '60), a seeming relationship was observed between dermatitis and weight gain. The correlation between these variables was found to be $r = 0.38$, for which $P < 0.01$. From this it appears that the more a rat grows when fed a fat-free diet, the more intense will be the dermatitis. The deficient animals which grew more from whatever unknown cause were required to mobilize more EFA for the synthesis of new tissue.

Many of the correlation coefficients shown in tables 2 and 3 agree with biochemical observations. For example, the known conversion of linoleate to arachidonate is reflected in high correlations between dietary linoleate and tetraenoic acids of heart, plasma and erythrocytes. That docosahexaenoic acid is not derived from linoleate is evidenced by the negative correlations between dietary linoleate and the tissue hexaenes in this study. That the triene:tetraene ratios in the three tissues are a measure of the same phenomenon is shown by the extremely high correlations of these ratios to each other (0.96, 0.97, 0.99). This suggests that the triene:tetraene ratio of other tissues would be a valid index of EFA deficiency. This has been found to be true for liver in similar recent unpublished studies in this laboratory. Older but less detailed studies (Rieckehoff et al., '49; Widmer and Holman, '50) suggest that similar effects are to be expected in a wide variety of tissues.

These data also can be used to predict biochemical relationships. For example, the very high correlation between dietary linoleate and heart pentaene suggests that a large proportion of the latter may be synthesized from linoleate. This implies that the terminal bond structure of the two acids is identical requiring that the pentaene be 4, 7, 10, 13, 16–docosapentaenoate.

In the above discussion no mention is made of dietary fatty acids other than linoleate. The cottonseed oil and butter fat were varied systematically so that the observed correlation between linoleic acid and the saturated fatty acids in the diet was $r = +0.01$. The separation of the dietary fatty acid variables was not as clear-cut with respect to the unsaturated components (table 4). This is a fact which is frequently overlooked in nutritional studies, but which is an inevitable consequence of using a simple mixture of natural fats and oils to produce the variation in dietary linoleate level. The observed correlation between dietary linoleate and the dietary intakes of monoenes, trienes, tetraenes, and pentaenes ranged from $r = +0.40$ to -0.35 and the intercorrelations between the trienes, tetraenes and pentaenes in all cases exceeded $r = 0.99$. Hence, the present data cannot in any useful way distinguish between possible effects due to dietary trienes as opposed to dietary tetraenes or dietary pentaenes, and only to a partial and undefined extent distinguish the effect of this entire group of acids from the dietary effects of linoleate. The content of these acids in the dietary fat was so low compared with linoleate that it is assumed to be a minor factor in the present discussion.

Factor analysis representation

Factor analysis techniques provide a direct means of visualizing the relationship between a group of intercorrelated variables. The experimental variables are represented as vectors of approximately unit length extending in various directions from a common origin. When the results can be represented properly in three dimensions, it is convenient to represent the

TABLE 4

Correlation between different fatty acid components in the diet

	Monoenes	Dienes	Trienes	Tetraenes	Pentaenes
Saturated	0.919	0.009	0.941	0.938	0.932
Monoenes		0.402	0.732	0.729	0.717
Dienes			−0.329	−0.330	−0.347
Trienes				0.997	0.998
Tetraenes					0.996

variables as points plotted on the surface of a sphere. Since, in the present data, a number of the variables are negatively related to the rest, it is necessary to show both halves of the sphere, namely, both a positive hemisphere and a negative hemisphere. In figure 1 those variables that have a high correlation with each other are represented by points that lie close together. Those points which lie roughly 90° away from each other on the surface of the sphere have a zero correlation with each other. An example of this may be seen by comparing the point for dietary linoleate with the points representing the other dietary fatty acid components. For two variables which have a very large negative correlation with each other, the two points might be found in different hemispheres, with the second point being roughly the projection of the first point through the origin onto the opposite side of the sphere. A good example of this occurs in the negative correlation between the dermatitis score and levels of tissue triene on one hemisphere as opposed to tissue diene, tetraene and pentaene on the opposite hemisphere. Indeed these two highly, but inversely, related groups can be considered a single distinct complex of experimental variables which constitute a definition of essential fatty acid deficiency. To emphasize this relationship, these two groups have been enclosed with a solid line. The practical value of such a factor analysis representation is that it illustrates, in one simple diagram, the major correlation relationships represented by the hundreds of correlation coefficients in tables 2 and 3.

Encircled in figure 1 is a complex of variables which has a high positive correlation with linoleate intake. This complex includes the dienes, tetraenes and pentaenes of all tissues studied (though some overlapping points were omitted from figure 1 to simplify its appearance). Weight gain is another experimental variable well correlated with this same complex. From its position in the diagram (and the correlations in table 2), weight gain is almost as well correlated with the intakes of other fatty acid components of the diet as it is with linoleate intake. This is in agreement with the observation that linolenic and docosahexaenoic acids will promote weight gain without preventing the dermatitis of EFA deficiency.

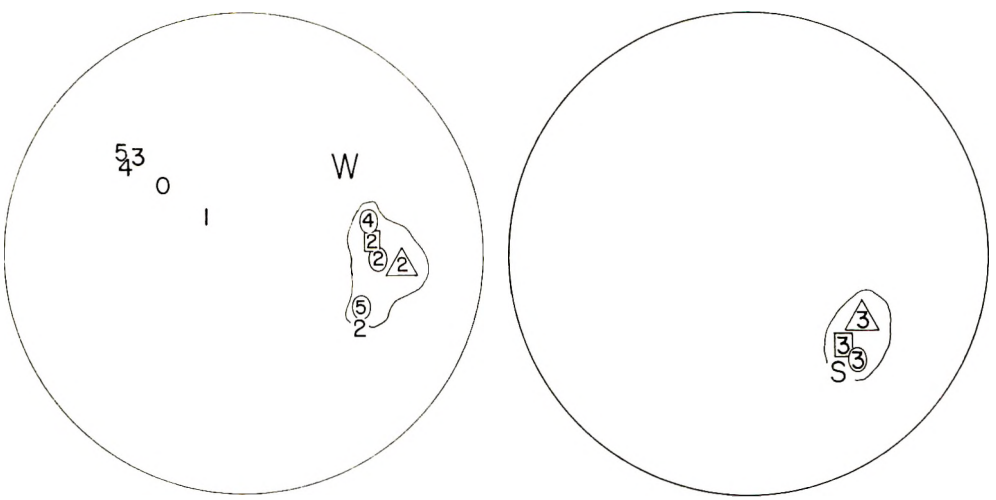

Fig. 1. Factor analysis representation of the relationship between dietary fatty acids and tissue fatty acids, weight gain, and dermal score. All points are represented as being plotted on the surface of a sphere, the two hemispheric views showing the two opposite sides of the sphere. Points designated as zero, 1, 2, 3, 4, and 5 represent the dietary intake of fatty acids containing zero to 5 double bonds, respectively. The numbers enclosed in ovals represent the corresponding unsaturated fatty acids in cardiac lipid; and numbers in rectangles and triangles refer to those from erythrocytes and plasma lipids, respectively. For ease in visualizing the relationship of the two hemispheres the reader should imagine them folded back to back.

The relationship between the different fatty acid components of the diet is also graphically defined in figure 1. The high correlation between triene, tetraene, and pentaene is particularly evident. The clear separation of diene from all of the other dietary lipid variables and the high correlation between dietary diene and the tissue analysis data both suggest that dietary diene is the controlling variable in this experiment.

Estimation of linoleate intake

One of the better ways of demonstrating the degree of correlation between dietary linoleate and the changes occurring in the lipid composition of the heart and blood is to derive a multiple regression equation which will allow the estimation of dietary linoleate intake from a knowledge of tissue analysis data. Such a relationship has practical significance, particularly in relation to discussion of individual variations in linoleate requirement or in studies of the effect of stress conditions upon the dietary requirement for linoleate.

When all of the independent variables of tables 2 and 3 are incorporated into a single multiple regression equation, it is possible to estimate the dietary linoleate within an error of $\sigma = 1.8\%$ of total calories over the entire range from zero to 20% covered by this experiment. The multiple correlation coefficient was $R = 0.98$ (for which $P < 0.01$). Further study of this equation revealed, however, that only 6 of the included variables contributed significantly toward this multiple correlation, and, of these, three were from the cardiac lipid data.

Using only the analytical data for the heart (expressed as milligrams per 100 gm of fresh tissue), the following estimation equation is obtained:

Linoleate as percentage of total calories in the diet $= -5.1 + 0.0426$ diene $+ 0.0165$ triene $- 0.0219$ tetraene $+ 0.0744$ pentaene $+ 0.0060$ hexaene

for which the multiple correlation coefficient is $R = 0.97$ and the error of estimation of dietary linoleate is $\sigma = 1.9\%$ of total calories. This estimate is almost as good as that obtained when the total analytical data of table 2 and 3 were included. The authors make no claim that this or other later specific equations are general in the sense that they will hold true for other species or other experimental conditions. The small error of estimation seen here demonstrates that, under the conditions of this experiment, there is a direct and close relationship between the amount of linoleic acid in the diet and the amounts of certain fatty acids in the cardiac lipid. The polyunsaturated fatty acids occur in the heart in higher concentration and undergo larger changes in concentration and pattern than in other tissues (Rieckehoff et al., '49; Widmer and Holman, '50). Unfortunately this organ is not readily accessible for sampling in living animals.

Similar equations were therefore derived relating either plasma or red cell lipid composition to the concentration of linoleic acid under the diet. In the case of plasma, the estimation equation was:

Linoleate as percentage of total calories $= + 6.6 + 0.162$ diene $- 0.499$ triene $+ 0.188$ tetraene $- 1.96$ pentaene $- 0.191$ hexaene

for which the multiple regression coefficient was $R = 0.77$ and the error of estimating dietary linoleate was $\sigma = 4.8\%$ of calories. Similarly in the case of erythrocyte lipid data, the equation was:

Linoleate as percentage of total calories $= - 0.5 + 0.592$ diene $+ 0.167$ triene $+ 0.094$ tetraene $+ 0.035$ pentaene $- 1.36$ hexaene

for which the multiple correlation coefficient was $R = 0.90$ and the error of estimation of dietary linoleate was $\sigma = 3.3\%$ of calories. From these data it appears that the red cell provides a slightly better analytical sample than the plasma for use in this connection. Neither of the equations derived from blood analysis data, however, are as well related to the dietary intake as that derived from cardiac lipid data. Though this fact may be disheartening from an analytical point of view, it serves to emphasize the close relationship that exists between the dietary intake of adequate amounts of essential fatty acids and the lipid metabolism of heart muscle.

These regression equations have practical as well as theoretical value. Using the difference between the actual dietary linoleate and that estimated with these equations, aberrations in polyunsaturated acid metabolism can be detected. Therefore the influences of physiological state upon

linoleate requirement may be assayed. The equation relating dietary linoleate to heart fatty acids has been applied to several experimental groups in unpublished studies. It clearly distinguishes the apparent dietary linoleate for male and female rats fed the same fat-free diets, confirming a difference in EFA requirement for the two sexes. For male rats, the apparent dietary linoleate has been found to be affected by hypercholesterolemic agents and by dietary saturated or hydrogenated fats. The usefulness of this treatment of nutritional data has been indicated by these studies, and it is hoped that a similar evaluation of human blood samples can be worked out, making possible an estimation of an individual's nutritive status for purposes of nutritional survey and possibly for diagnosis and therapy.

The individual and his group

We have discussed the relationship between the dietary and analytical variables, but have said nothing about the individual animals in this study. In terms of the mathematical procedures involved, it is entirely feasible to look at these same data from a different point of view. More specifically, the animals may be considered as the variables studied and the different analytical measures (chemical, dermal and weight data—but not dietary intake data) as the observations on these variables. The data, considered in this fashion, were correlated and subjected to a factor analysis, and the results are expressed in figure 2. The individual points in figure 2 represent different rats. Since the third and higher dimensions contained little information in this case, the points in this figure are plotted in a two-dimensional representation. Again the axis system and absolute dimensions are not indicated because they are arbitrary and have no meaning in this situation. Those animals that resemble each other in weight gains, dermal condition, and tissue lipid compositions are represented by points lying close together, and those that do not, by points farther apart. To simplify the interpretation, each dietary intake group has been enclosed with a curved line and designated by group number (see table 1).

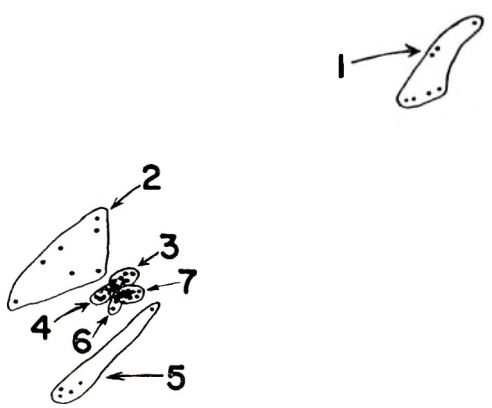

Fig. 2 Factor analysis plot showing the relationship of each experimental animal to all of the others when comparisons are made on the basis of tissue lipid composition, dermal score and total body weight gain. The numerals indicate the group numbers corresponding to those in table 1.

This representation of the data serves to bring out and emphasize three facts.

(a) All groups of animals with linoleate intakes exceeding 1.0% of total calories are highly correlated—almost to the point of being indistinguishable. No animal in any of these groups showed chemical, dermal or weight-gain evidence of linoleate deficiency; that is, no point in groups 3, 4, 6 or 7 was displaced in the direction toward group 1.

(b) The condition of all animals in group 1 (zero linoleate supplement) was distinctly different from all other animals—attesting, for one thing, to the very low level of linoleate activity in the basic test diet.

(c) Groups 2 and 5 (linoleate intake in the range 0.14 to 0.56% of total calories) do not fall in the expected position between groups 1 and 7. They lie very close to (are highly correlated with) the groups with high linoleate intakes. Such a finding would suggest that the biological requirement for linoleate might lie between zero and 0.14% of total calories for these animals. One additional factor is ignored, however, namely, that groups 2 and 5 are displaced from the high intake groups and from each other in a vertical direction. Further examination of the data shows that changes in figure 2 in the vertical di-

rection are related to total fat intake—the upper points (groups 1 to 4) represent animals with total fat intakes of zero to 10% of total calories in the diet, while the lower point (groups 5 to 7) represent animals with 40% of their caloric intake derived from fat. It would therefore appear that in this marginal linoleate intake range, from 0.14 to 0.56% of total calories, the chemical, dermal and weight-gain condition of the animal is dependent upon the total amount of fat in the diet—a factor which is of little importance at high linoleate intakes. The exact nature of the dietary fat components which are effective under these conditions is of interest, but in view of the complexities defined by table 4, it seems desirable to defer any discussion of this matter until work now in progress has been completed. These observations are seen as confirming the statement that, "Linoleate content of 1% of calories represents the level of EFA below which the normal metabolism of PUFA no longer persists" (Holman, '60), as well as pointing a way to the study of the manner in which lipid metabolism is altered in the marginal intake range from 0.1 to 0.5% of the calories.

DISCUSSION

In nutritional work it is frequently desirable to be able to discuss the relative physical and biochemical conditions of different individuals—either man or experimental animals. This need becomes apparent, for example, whenever an attempt is made to "evaluate the nutritional status of an individual" or discuss "individual differences in nutrient requirement." Unfortunately there are few tools available that allow this to be done with certainty in any but the most extreme deficiency conditions. One of the main contributions of the present study, therefore, may be that it describes two general statistical approaches to this problem. The first of these is by use of the multiple regression equations which relate nutrient intake to the chemical composition of the heart and other tissues. Whenever large individual differences in nutrient requirement exist, or whenever some experimental condition markedly changes such a requirement, discrepancies would be expected to exist between the observed nutrient intake and the nutrient intake as computed from tissue analysis data by the aid of a multiple regression equation derived in the above manner. The second of these methods, depicted in figure 2, is by means of a factor analysis. In general, the first of these methods may be more useful when attempting to measure in quantitative terms the extent to which a requirement is altered, whereas the second of these methods is a valuable qualitative tool for use in exploratory work, particularly when it is desired to demonstrate general relationships and interactions.

As an example, one case taken from the present data will be discussed here. In figure 2, group 5 is composed of 5 rats—4 of similar biochemical condition and one quite different. This odd point (rat no. 27) is displaced in the direction of group 1,—that is, rat no. 27 appears more deficient than the others in his intake group. Applying the above multiple regression equation to the cardiac lipid data for the rats in this group, the estimated linoleate intake for the cluster of 4 rats is $1.5 \pm 0.7\%$ of total calories and for rat no. 27 is 0.46% of total calories. Admittedly this information asks more questions than it answers at this point, but at least this procedure defines the problem clearly in terms of specific animals and thus opens the question to experimental attack.

These statistical procedures also present difficulties. One of the more critical ones in the present multiple regression work arises from the fact that some of the more important experimental variables are not linearly related to the dietary intake of linoleate. Other more appropriate statistical approaches to this problem may be possible as soon as further experimental work provides a more precise picture of the curvilinear relationships that exist in this system.

SUMMARY

A number of statistical techniques were applied to the study of tissue analysis, dermal score, and weight gain data obtained from rats maintained with controlled intakes of linoleic acid and total fat. The correlation between each of these variables and dietary linoleate as well as

all other variables was presented. The correlation matrix thus generated was used for multiple regression and factor analysis studies.

By means of multiple regression equations, a close relationship was established between dietary linoleate and heart lipid composition, and a lesser correlation was found to exist in the case of plasma and erythrocyte lipid compositions. The use of such relationships in the study of individual variations in linoleate requirement was discussed.

Factor analysis was used to present a simplified view of the relationships between the different variables discussed above. The results showed, for example, the high positive correlation between dietary linoleate intake and the concentrations of dienes, tetraenes and pentaenes in tissue lipids, and the high negative correlation between dietary linoleate intake and the tissue trienes. Through a variant of this factor analysis procedure, the relationship was depicted between each animal in this study and all of the other animals when their conditions were compared in terms of their tissue lipid composition, dermal score and weight gain. On the basis of this diagram, conclusions were drawn with respect to the nutritional requirement for linoleate and the nature of the change in lipid metabolism that occurs as the linoleate intake was reduced below this minimum figure.

ACKNOWLEDGMENTS

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Metabolic Patterns in Preadolescent Children

IV. FAT INTAKE AND EXCRETION[1]

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Few data are available on fat metabolism in normal preadolescent children. The work of Holt et al. ('19, '22) included a number of studies on fat metabolism of infants and young children. Williams et al. ('43) reported data on fat intake and fecal excretion by normal children receiving mixed diets.

A series of three studies, carried out in 1954, 1956 and 1958, was undertaken to investigate the metabolic patterns of girls, 7 to 9 years of age, receiving a controlled diet, with the level of dietary protein as the chief variable.

This report presents data on fat intake, excretion and apparent digestibility for 35 girls maintained with mixed diets. The effects of fat intake on fat excretion were investigated by regression analyses; correlations were made between fecal fat and excretions of other nutrients.

DESCRIPTION OF STUDIES

The general plan and organization of each of the three studies has been published in the Southern Cooperative Series Bulletin no. 64 ('59). This bulletin also presents a description of each subject and lists the kinds and amounts of foods in the daily diets.

The number of girls participating in each metabolic study and the range in initial body weights for each group were as follows: in 1954, 11 girls, 22.4 to 42.7 kg; in 1956, 12 girls, 22.1 to 33.6 kg; and in 1958, 12 girls, 21.9 to 34.1 kg.

The duration of the studies was 16 4-day periods (64 days) in 1954, 14 4-day periods (56 days) in 1956, and 8 6-day periods (48 days) in 1958.

The controlled diets provided nutrients to meet the recommended allowances of the National Research Council ('53) for children 7 to 9 years of age, except that the amount of protein was varied.

The methods for collecting and preserving the food composites and the fecal composites are described in Southern Cooperative Series Bulletin no. 64 ('59). Analyses for content of fat in wet food composites were carried out by using a modified A.O.A.C. ('55) acid hydrolysis method with a preliminary yeast fermentation treatment.[2] This treatment eliminated possible difficulties in determining the fat content of food composites containing sucrose or fructose. The same modified method was used for slurries of feces except that treatment with yeast was omitted. Determinations for total fat were made on food composites for each diet for alternate periods in 1954 and 1956 and for all periods in 1958. Fecal composites for each subject were analyzed for these same periods. If a girl failed to eat all of the food, a separate food composite was prepared for analyses.

RESULTS AND DISCUSSION

In table 1 are shown the results of fat analyses on food and on fecal composites presented as averages for each group of girls receiving each of the controlled diets. Also presented are the energy and protein

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[1] This study was a phase of the Southern Regional Research Project, "Requirements and Utilization of Selected Nutrients by Preadolescent Children," supported in part by funds appropriated to the U. S. Department of Agriculture under the Research and Marketing Act of 1946, and the Hatch Act, as amended. The Human Nutrition Research Division of the Agricultural Research Service was a cooperator in this project.

[2] Also, unpublished material.

TABLE 1

Average daily dietary energy, protein and fat; average fecal fat, apparent digestibility of fat and source of calories

Year and diet	No. of girls	Energy[1]	Protein[2]		Fat					Caloric distribution[3]		
					Dietary		Fecal		Apparent digestibility	Protein	Fat	Carbohydrate
		Cal.	*gm*	*gm/kg*	*gm*	*S.D.*[4]	*gm*	*S.D.*	%	%	%	%
1954												
1	11	1955	65	2.2	66	7.1	3.6	1.0	94	14	30	56
1956												
2	3(S)[5]	1948	48	2.0	61	—	2.3	—	96	10	28	62
3	3(S)	1966	73	2.7	66	—	2.1	—	97	16	32	52
4	3(L)	2356	59	1.9	74	—	3.5	—	96	10	28	62
5	3(L)	2415	88	2.7	75	—	2.5	—	97	15	28	57
1958												
8[6]	12	2177	22	0.8	78	2.9	3.1	0.7	96	4	32	64
8′		2240	18	0.6	81	4.6	3.5	0.5	96	3	33	64

[1] Energy, determined in an Emerson bomb calorimeter, represents the gross energy or heat of combustion due to the oxidation of the food to its ultimate oxidation products.

[2] Kjeldahl nitrogen × 6.25.

[3] Calculated by applying the general factors of 4 and 9 Cal./gm to values for protein and fat. The percentage of calories from carbohydrate was calculated by difference.

[4] Standard deviation of the observations. Not computed for 1956 because only three subjects participated in each diet group.

[5] Small girls (S), 22.1 to 27.4 kg body weight; large girls (L), 29.9 to 33.6 kg.

[6] Diet 8 was fed for 30 days, diet 8′ for 18 days. Diet 8′ was essentially the same as diet 8 except that milk was omitted to reduce protein.

TABLE 2

Sources of daily dietary fats:[1] calculated total, percentage from animal and vegetable sources and percentage of fatty acids

Year and diet	Total	Fat source		Fatty acids					
				Saturated			Unsaturated		
		Animal	Vegetable	Total	Palmitic	Stearic	Total	Oleic	Linoleic
	gm	%	%	%	%	%	%	%	%
1954									
1	75	59	41	39	21	9	55	42	8
1956									
2–5[2]	77	61	39	40	22	10	54	42	8
1958									
8	80	30	70	33	21	6	61	48	10

[1] Total fat calculated from USDA Handbook no. 8 (Watt and Merrill, '50) and fatty acids from Home Economics Research Report no. 7 (Goddard and Goodall, '59).
[2] Values for diets 2, 3, 4 and 5 were averaged.

(N × 6.25) content of each of the diets as determined in other laboratories (Moschette, '60; James, '60).

Dietary fat. The average daily fat content of the diets in these studies with 35 girls ranged from 61 to 81 gm and provided 28 to 33% of the total calories. Table 2 presents the calculated total fat and fatty acid composition of the diets. Margarine was the main source of visible fat. In addition to the visible fat, the values for total fat include the amounts of fat such as those present in "lean" meat, eggs and vegetables. The average daily amounts of fat in diets 1 to 5 determined by analyses (table 1) are from 87 to 90% of the calculated amounts (table 2). Closest agreement occurred for diet 8 in which the amount of fat as analyzed was 98% of the calculated value. Diets used in 1958 contained little meat, compared with diets 1 to 5; hence the error resulting from estimating the amount of fat in meats was reduced.

Because dietary protein was lower in 1958 than in the two previous studies, the calories were increased by adding fat and carbohydrate foods to diets 8 and 8′ (table 1). Extra calories were provided so that the energy value of these diets would not be a limiting factor in nitrogen storage. For both of these diets 40 gm of margarine, 15 gm of mayonnaise, and 60 gm of fondant (containing 11.4 gm of hydrogenated vegetable oil) were included in each day's menu.

In 1958 fat from vegetable sources (table 2) comprised around 70% of the total fat intake, with 30% from animal sources. In the 1954 and 1956 studies the ratio was reversed. Since vegetable oils, particularly cottonseed oil often used in commercially prepared mayonnaise, are good sources of unsaturated fatty acids, the linoleic acid (10%) and oleic acid (48%) content is somewhat higher in diet 8 as compared with the diets in the other two studies. Diet 8 provided about 8 gm of linoleic acid per day, whereas the 1954 and 1956 diets provided about 6 gm. The total saturated fatty acids decreased from about 40% in the 1954 and 1956 studies to 33% in 1958.

Fat excretion. The range in average daily fecal fat for individual girls was from 2.9 to 4.1 gm in 1954, 1.8 to 3.2 gm in 1956, and 2.5 to 3.9 gm in 1958. The average daily fecal fat for the several diet groups ranged from 2.1 to 3.6 gm (table 1). Certain high fecal values for those who were ill were omitted from the excretion ranges.

The fat excretion of the girls was similar to the excretions reported in the literature. Holt ('19), in a study with 37 normal children one to 10 years of age, found that the average daily amount of fat excreted was 2.48 gm with a range from 0.95 to 5.65 gm for individuals. Anderson ('45) reported excretions ranging from 0.35 to 1.79 gm of fat per day in a group of 11 children, two to 6 years of age. Williams

et al. ('43) reported excretions from 1.6 to 3.8 gm of fat per day for 19 children, 4 to 12 years of age.

Digestibility of fat. In these studies with 35 preadolescent girls the analyzed values for fat intake and fecal output indicate that the average apparent digestibility of the total fat was 94% in the 1954 study and 96% in the 1956 and 1958 studies (table 1). The percentage of apparent digestibility of fat for these children compares favorably with that of those observed by Holt ('19), 93.9%; Williams et al. ('43), 96 to 98%; and Wang et al. ('39), 97.0%.

Effect of fat intake on fat excretion. The relationship between fat intake and fecal fat, on the basis of grams per kilogram of body weight, is shown in figure 1. The effect of the amount of dietary fat ingested on the amount of fat excreted is similar for each study when the data are expressed in terms of per kilogram of body weight. The amount of fat excreted increased about 0.05 gm per kg per day as the fat intake increased 1 gm per kg per day. This increase in fecal fat was statistically significant in 1954 and 1958 as evidenced by the significant regression coefficients of 0.06 and 0.04, but not significant in 1956. The correlation in 1954 was significant at the 1% level ($r = 0.84$) and in 1958 at the 5% level ($r = 0.69$).

In the 1956 study, however, when the fat intake varied among the 4 groups of girls, the regression analysis of the data on *total* fecal fat and *total* fat intake, in grams per day, indicated that the fat in the feces increased significantly, about 0.05 gm, as the fat intake increased 1 gm. The correlation was significant at the 5% level ($r = 0.59$).

Williams et al. ('43) observed that total fat excretion for 19 healthy children, 4 to 12 years of age, tended to increase as fat intake increased from 62 to 113 gm per day.

Evidence that the amount of dietary fat directly influences the amount of fat in the feces does not appear as conclusive for adults as is indicated for children. Wollaeger et al. ('47) concluded that raising the dietary fat resulted in increased fecal fat, whereas other workers (Meyer et al., '55; Krakower, '34) noted little or no relationship between the amount of fat in the diet and that in the feces. Annegers et al. ('48) noted more significant differences in the excretion of fat among individuals

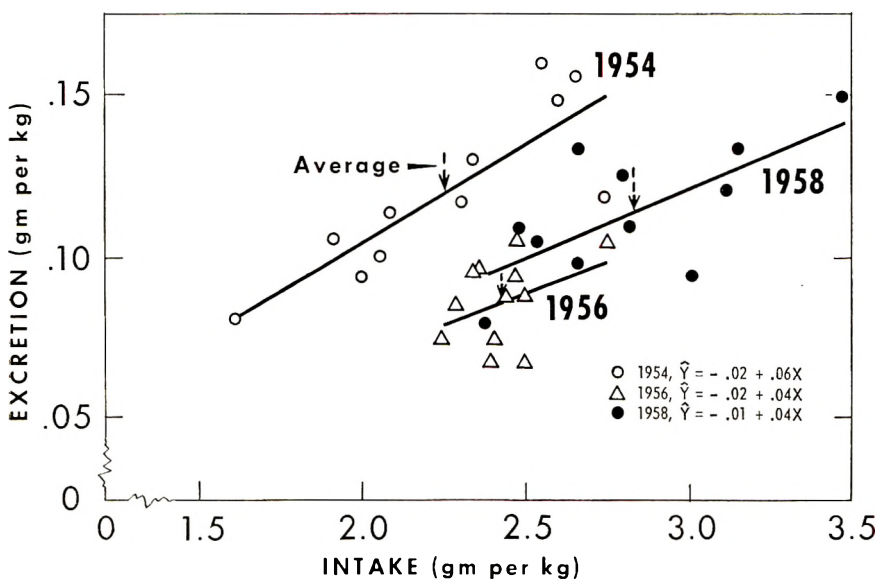

Fig. 1 Relation between fat intake and fecal fat excretion. Each point represents the average for one girl for all metabolic periods in which she was studied. Data for one subject, two periods (1954); two subjects, one period each (1956); and one subject, all periods (1958) were omitted because of illness.

than for the same subjects receiving different amounts or types of fat in the diet.

Effect of protein intake on fat excretion. Nitrogen balances determined for the girls in these studies have been reported (James, '60). Covariance analyses made to determine the effect of differences in nitrogen intake on the amount of fecal fat gave inconclusive results. The effects of many factors, such as kind and amount of carbohydrate and fiber, could not be isolated in the analyses.

Fecal calcium and magnesium excretion as related to fat excretion. Reports on calcium and magnesium for these studies (Schofield, '60) indicate that calcium intake for the 35 girls studied in the three years ranged from about 0.9 to 1 gm, and the magnesium intake from about 120 to 240 mg per day. The relationship between fecal fat and fecal calcium was not clearly defined. The correlation between amounts of fat and calcium excreted in the feces was significant at the 5% level ($r = 0.61$) for the 1954 data but not significant for either the 1956 or the 1958 data. None of the correlations between fecal fat and fecal magnesium were significant.

SUMMARY

The amount of fat in the diets and feces was determined for 35 girls, 7 to 9 years of age, as one segment in a series of metabolic studies. The controlled diets were composed of ordinary foods with the level of protein as the chief variable and with the fat providing from 28 to 33% of the total calories.

For the three series of studies, the average daily intake of fat was 66, 69 and 79 gm and, correspondingly, the average daily fecal fat was 3.6, 2.6 and 3.2 gm. The apparent digestibility of the fat ranged from 94 to 96%.

Under the conditions of these studies the fecal fat of preadolescent girls increased 0.05 gm as the fat intake increased 1 gm, when data were expressed on a per kilogram of body weight basis. This increase in fecal fat was statistically significant in two of the three studies.

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Dietary Adaptation and the Level of Glucose-6-Phosphatase and Phosphohexoseisomerase of Rat Liver

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Adaptation to a high-fat diet has been shown to increase the rate of utilization of fat and to decrease that of carbohydrate in normal and hypophysectomized rats during a subsequent fasting period (Samuels et al., '42a). Blood sugar levels have also been shown to be maintained more efficiently in fat-adapted rats during periods of glycogenolysis (Samuels et al., '42b). The fasting levels of either alkaline or acid liver phosphatase were not altered by prolonged feeding of fat or carbohydrate diets. Fat absorption was shown to produce a transitory increase of alkaline phosphatase activity, which cannot be interpreted as an adaptative response (Nimni, '57). More recently it has been reported that animals fed a high-fat diet maintained a higher liver phosphorylase activity when fasted than those fed a carbohydrate diet (Nimni et al., '58). Further investigation of this pointed toward a difference in the glycogen content of the liver as being partially responsible for these results. Freedland and Harper ('57) have shown that the exclusion of carbohydrate from the diet of the rat leads to an increased glucose-6-phosphatase activity (G-6-Pase) in the liver. In our work we decided to investigate this problem further to establish the enzymatic pattern at different stages of metabolic activity.

G-6-Pase occupies a key position in carbohydrate metabolism. In the liver it can be utilized through different pathways. The enzyme initially responsible for the utilization of glucose-6-phosphate by glycolysis is phosphohexoseisomerase (PHI). Therefore, we investigated the effects of dietary adaptation to high-fat and high-carbohydrate diets on the activity of these enzymes under different metabolic conditions.

EXPERIMENTAL

Male albino rats of the Wistar strain were housed in individual cages and fed ad libitum. Growth was optimal in all instances. On the day prior to the experimental determinations the food was removed from the cages and the animals allowed to fast overnight. On the following morning they were fed a 4-gm ration of their respective diets. Only animals consuming it readily were used in the experiments. The metabolic status of the animals was checked after sacrificing, for evidence of food in the stomach and intestines. The composition of the diets was similar to that of those used previously (Nimni, '57). The high-carbohydrate diet supplied 16% of the total calories as casein and the rest as corn starch. The high-fat diet supplied an isocaloric amount of protein and contained butter as a source of fat. G-6-Pase activity was determined according to the Cori and Cori method ('52) in which inorganic phosphate is released from a glucose-6-phosphate substrate. An aliquot of liver homogenate (0.1 ml) was incubated at 37°C during 15 minutes with 10 μmoles of G-6-P in a 0.075 M maleate buffer at pH 6.5 in a final volume of 0.5 ml. After deproteinizing with 10% trichloroacetic acid, inorganic phosphate was determined in the supernatant by the method of Fiske and SubbaRow ('25). A G-6-Pase unit is the

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amount of enzyme which will liberate 1 μmole of P per minute of incubation. PHI activity was determined in the homogenate by the method of Bruns and Hinsberg ('54). A PHI unit is the amount of enzyme which will produce 1 μmole of fructose-6-phosphate per minute of incubation at 37°C and pH 7.8. Fructose formation was established according to the procedure of Roe ('34), and corrections were made for fructose-6-phosphate formation using the constant of Umbreit et al. ('59).

RESULTS AND DISCUSSION

The effects of feeding a high-fat and a high-carbohydrate diet on the liver G-6-Pase activity of nonadapted animals are shown in figure 1. These animals had been maintained previously with a regular stock diet and were fasted overnight prior to being offered the experimental diet. Determinations were made at zero time and 3, 12 and 30 hours after they were offered the diet. The food was ingested over a 12-hour period. After this time, the remaining ration having been removed from the cage, all animals in the 3- and 12-hour groups were in an active absorption process at the time of sacrifice, as evidenced by the presence of food in the gastrointestinal tract. Initially all animals showed the same level of G-6-Pase activity, corresponding to the fasting stage, with no significant difference in the enzyme activity three hours after feeding. Twelve hours after they began ingesting the diet there was a sharp decrease in the activity of the carbohydrate-fed rats, which returned to the normal basal level following a fasting period. The general patterns observed suggest that during active carbohydrate absorption a change in metabolism occurs which leads to a decrease of the G-6-Pase activity; although fat absorption does not appear to alter the basal metabolic activity at the level of the enzyme under study.

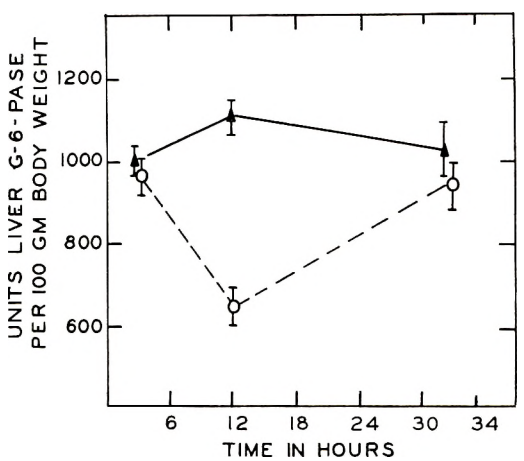

Fig. 1 Effect of a high-fat (▲–▲) and a high-carbohydrate (○–○) diet on the liver G-6-Pase activity of nonadapted rats at different time intervals after feeding. Food was offered at zero time and removed 12 hours later, after which rats were subjected to a 20-hour fast. Each value represents a mean of 8 rats. The standard deviation of the mean is indicated by vertical lines for each point of the curve.

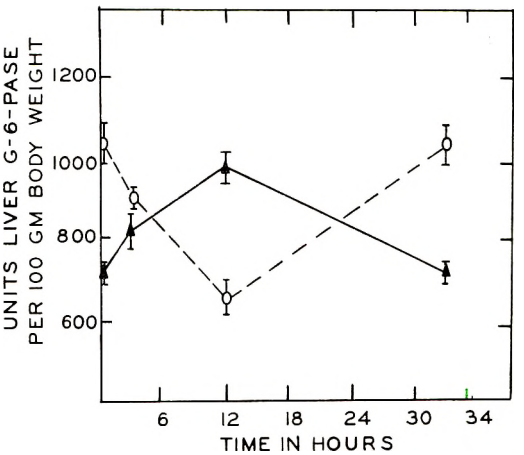

Fig. 2 Effects of a 25-day dietary adaptation to the ingestion of a high-fat (▲–▲) and a high-carbohydrate diet (○–○) on the liver G-6-Pase activity. Food was offered to fasted rats at zero time and removed 12 hours later, after which rats were subjected to a 20-hour fast. Each value represents a mean of 6 to 11 rats.

In figure 2 are shown the results obtained with animals adapted by having been fed fat and carbohydrate diets for 25 days. After overnight fasting the carbohydrate-adapted animals exhibited a significantly higher level of G-6-Pase activity. Liver enzyme activity of the fasted fat-adapted rats increased when they were fed the fat diet; whereas this activity decreased in rats fed the carbohydrate diet, as had occurred with the nonadapted animals fed a similar diet (fig. 1). This similarity in behavior is to be expected, since the stock diet, with a fat content of 4%, is essentially a high-carbohydrate diet. Twenty hours after the food was removed the liver

enzyme activity of the adapted animals returned to that characteristic of normal fasting levels.

That the increased fat metabolism does not produce any signficant change in the activity of this enzyme, when compared with that of the fasting stage, suggests a similarity between the metabolic patterns established at this enzymatic level during fasting and during fat utilization. Excess carbohydrate feeding decreased G-6-Pase activity in the later stages of absorption, this being probably a response to the large amount of glucose being rendered available to the organism and of the increased glycogen stored due to the glycogenic nature of the diet. The possibility that this response may be controlled by a hormonal mechanism is under study. The decreased basal activity of G-6-Pase of the fat-adapted animal would probably explain, if this were a rate limiting step, the mechanism by which these animals maintain higher levels of stored glycogen in the liver during fasting, but not explain the simultaneously increased levels of circulating glucose.

The values obtained when the PHI activity was determined in adapted rats under different metabolic conditions are shown in table 1. The carbohydrate-adapted rats maintained a significantly higher fasting and nonfasting level of PHI activity than the rats adapted to fat over similar periods of time. The differences observed between comparative groups are statistically significant ($P < 0.01$). Experiments performed on nonadapted rats, however, failed to show any difference in PHI activity three and 12 hours after ingestion of fat or carbohydrate.[2] If we consider the activity of PHI as an index of the amount of G-6-phosphate which is metabolized via the glycolitic pathway, it would appear that decreased glucose metabolism via this route occurs in fasted fat-adapted animals when compared with carbohydrate-adapted rats under similar circumstances. This decreased carbohydrate utilization by the fat-adapted animals could also probably account for the higher glycogen stores which are maintained by these animals under fasting conditions.

TABLE 1

Effects of a 30-day adaptation period to high-fat and high-carbohydrate diets on liver phosphohexoseisomerase (PHI) activity measured during absorption and after fasting[1]

Diet	Fasting period	Units liver PHI/100 gm body weight
	hours	
Fat	22	328 ± 6.2[2]
Carbohydrate	22	532 ± 15.0
Fat	—	320 ± 8.5
Carbohydrate	—	495 ± 10.5

[1] Values represent the average of 7 rats.
[2] Standard deviation of the mean.

SUMMARY

Dietary adaptation to high-fat and high-carbohydrate diets was shown to modify the absorptive and post-absorptive levels of liver glucose-6-phosphatase activity in the rat. Fasting levels were higher in the animals previously fed carbohydrate diets.

Fat adaptation, however, decreased the activity of liver phosphohexoseisomerase during absorption and fasting.

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Studies on Phosphorus Metabolism in Dystrophic Chicks[1]

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Numerous studies have shown that abnormal phosphorus metabolism is associated with nutritional muscular dystrophy in a number of laboratory mammals. Most workers have shown increased incorporation of P^{32} into dystrophic muscles, as compared with normal muscles, but no consistent pattern has been shown in the phosphorus composition of dystrophic, as compared with nondystrophic muscles. (Morgulis and Spencer, '36; Goettsch et al., '39; Weissberger and Harris, '43; Cohen and Warringa, '51; Ferdman, '56; Fitch and Dinning, '59).

Since it appeared that a systematic approach was needed to determine the specific phosphorus compounds affected, and since little is known about phosphorus metabolism in dystrophic chicks, studies were undertaken to conduct a systematic assessment of the total phosphorus, trichloroacetic acid-soluble phosphorus, acid-insoluble phosphorus, barium-soluble and barium-insoluble phosphorus, as well as further fractionations of these broad phosphorus groups. Isotope studies were included to determine the specific activities of the phosphorus in the various fractions of dystrophic and normal chicks, as well as the rate of penetration of the tissues of phosphorus as compared with that of potassium and cesium.

EXPERIMENTAL

White Plymouth Rock × Vantress male chicks were used in all experiments. The chicks were obtained from a Cornell flock of hens maintained with a diet low in vitamin E. Previous studies by Nesheim[3] demonstrated that chicks from these hens develop vitamin E-deficiency symptoms, including muscular dystrophy, at an earlier age than chicks from hens fed a normal diet. The chicks were housed at 2 to 3 days of age in electrically heated battery brooders with wire mesh floors. The brooders were kept in rooms with thermostatically controlled temperature. Lighting was maintained 14 hours per day. Selection of the chicks for treatment and pens was completely at random. The date of observation and the severity of muscular dystrophy were recorded periodically, beginning at three weeks of age. Dystrophy was produced in chicks by feeding the basal diet shown in table 1. The controls, or normal chicks, received this diet supplemented with 80 mg of *d*-α-tocopheryl acetate per kg of diet. All diets were fed ad libitum. At the end of the experimental period of 4 to 4½ weeks, most of the chicks receiving the basal diet showed muscular dystrophy, which could be observed in the live chicks as white streaks of degenerated muscle fibers in the breast muscle. In preparing the dystrophic and normal chicks for phosphorus determinations, they were handled and killed at all times in such a manner as to avoid any excess muscular activity. The procedure followed involved injection of 0.5 cm^3 pentobarbital sodium[4] directly into the heart. The skin was slit over the breast and the chick was quickly immersed, breast down, in liquid nitrogen. The entire breast was then removed, wrapped in

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[3] Nesheim, M. C. 1959 Studies on the effect of selenium and other factors on vitamin E deficiency in the chick. Ph.D. Thesis, Cornell University.

[4] Nembutal, Abbott.

TABLE 1

Composition of diet

	%
Casein	15.00
Gelatin	10.00
Glucose	61.725
Stripped lard	4.00
Cellulose	3.00
Mineral mix[1]	5.49
Vitamin mix[2]	0.945

[1] Mineral mixture supplied the following in gm/kg of diet: $CaHPO_4$, 21.51; $CaCO_3$, 14.92; KH_2PO_4, 8.67; NaCl, 6.00; $MgCO_3$, 1.42; $FePO_4 \cdot 4H_2O$, 2.62; $MnCl_2 \cdot 4H_2O$, 0.463; KI, 0.0026; $Cu(C_2H_3O_2)_2$, 0.0119; $ZnCl_2$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.0017; $Na_2MoO_4 \cdot 2H_2O$, 0.0083; KCl, 1.5.

[2] Vitamin mixture supplied the following per kilogram of diet: (in milligrams) thiamine·HCl, 10.0; riboflavin, 10.0; niacin, 50.0; Ca pantothenate, 20; pyridoxine·HCl, 4.5; folic acid, 4; biotin, 0.2; menadione, 0.5; inositol, 250; choline Cl, 1540; diphenyl-p-phenylenediamine, 220; and vitamin A, 5600 I.U.; vitamin D_3, 4410 I.C.U.; vitamin B_{12}, 20 μg.

aluminum foil and placed in a freezer at −28°C until analyses could be conducted. The radioactive compounds were administered either by intravenous or intraperitoneal injection. The specific procedures used and the amounts injected will be described in the discussion of the individual experiments.

The muscle samples were prepared for chemical analysis by the method described by Umbreit et al. ('57). The partition of the phosphorus into its various fractions involved separation of phosphorus compounds according to their solubilities in trichloroacetic acid and subsequently, as barium-soluble and insoluble fractions derived from the acid-soluble phosphorus compounds at pH 8.2. Analyses for phosphorus were conducted by the method of Sumner ('44).

RESULTS AND DISCUSSION

The total phosphorus, percentage of dry matter and percentage of ash of the breast muscle in dystrophic and nondystrophic chicks are presented in table 2. These results show that the percentage of dry matter in dystrophic chicks decreased, whereas the ash and total phosphorus increased in the dystrophic chicks as compared with normal chicks.

The distribution of phosphorus between the acid-soluble and acid-insoluble fractions of breast muscle of dystrophic and nondystrophic chicks is shown in table 3. Apparently, the compound or compounds responsible for the increase in total phos-

TABLE 2

Phosphorus, dry matter and ash composition of breast muscle of dystrophic and nondystrophic chicks

Group	Total P	Dry matter	Ash
	mg/gm dry matter	%	*% dry matter*
Dystrophic	(8)[1]*14.08*[2] ± 1.61[3]	(10)22.62 ± 0.58	(8)7.34 ± 0.82[4]
Nondystrophic	(9) 11.57 ± 0.56	(10)24.73 ± 0.24	(10)5.30 ± 0.03

[1] Figures in parentheses indicate number of indiviudal chicks analyzed.
[2] Figures in italic indicate significant differences from nondystrophic controls ($P < 0.05$). A "t" test for significance was used in all experiments unless otherwise noted.
[3] In all tables ± values represent the standard error of the mean.
[4] In this instance, $P < 0.025$.

TABLE 3

Total muscle phosphorus and total phosphorus of acid-soluble and acid-insoluble fractions of breast muscle of dystrophic and nondystrophic chicks[1]

Group	Total muscle P	Total P acid soluble	Total P acid insoluble
	mg/gm dry matter	*mg/gm dry matter*	*mg/gm dry matter*
Dystrophic	*11.53* ± 0.26[2]	7.91 ± 0.15[2]	3.62 ± 0.16[3]
Nondystrophic	10.59 ± 0.08	6.98 ± 0.25	3.61 ± 0.26

[1] Fifteen chicks individually analyzed for each determination.
[2] Significantly different ($P < 0.005$) from nondystrophic controls.
[3] No significant difference.

phorus in dystrophic muscle is present in the acid-soluble fraction.

In view of this, the next series of experiments was designed to define further the phosphorus compounds present in the acid-soluble fraction in an attempt to elucidate the particular compound or compounds showing the greatest increase in the muscle of dystrophic chicks. After treatment with trichloroacetic acid as previously described, the acid-soluble portion of the muscle extract was further fractionated by the addition of barium acetate at pH 8.2 according to the procedure outlined in detail by Umbreit et al. ('57). Three determinations were conducted on the barium insoluble fraction as follows: (1) a direct phosphorus analysis in which the sample aliquot received no treatment; (2) a 7-minute hydrolysis in which the sample aliquot was heated in a boiling water bath for 7 minutes prior to phosphorus analysis; and (3) a total phosphorus determination in which the sample aliquot was digested by the procedure described by Sumner and Somers ('49) for one hour prior to phosphorus determination. For the barium-soluble fraction, only the direct and total phosphorus analyses were conducted. The phosphorus found by direct analysis of the barium-insoluble fraction constitutes the inorganic fraction; the 7-minute hydrolyzable phosphorus is representative of such compounds as ATP and ADP phosphorus. A residual phosphorus was determined by subtracting the sum of the direct and the 7-minute hydrolyzable phosphorus from the total phosphorus and is representative of such compounds as hexose diphosphate and glyceric acid phosphate. In the barium-soluble fraction, phosphorus found by direct analysis may represent inorganic phosphorus to some extent, but it is probably largely representative of creatine phosphorus (Greenberg, '52). The remaining phosphorus compounds in this fraction are largely intermediate compounds such as hexose phosphates, phosphopyridine nucleotides, and others.

The distribution of phosphorus in the various fractions of dystrophic and nondystrophic breast muscle in the chick are presented in table 4. The total phosphorus and the direct phosphorus in the barium-

TABLE 4

Distribution of phosphorus in various fractions of dystrophic and nondystrophic breast muscle of the chick

Group	Acid-soluble	Barium-insoluble				Barium-soluble	
	Total P	Total P	Direct P	7-Min. hydrolyzable P	Residual P	Total P	Direct P
	mg/gm DM[1]	*mg/gm DM*	*µg/gm DM*	*mg/gm DM*	*µg/gm DM*	*mg/gm DM*	*mg/gm DM*
Dystrophic	(10)[2]7.72 ± 0.18[3]	(9)3.01 ± 22	(9)937[3] ± 144	(9)1.35 ± 0.11 (45.33%)[5]	(9)728 ± 78	(10)4.05 ± 0.41	(10)2.24 ± 0.22[4]
Nondystrophic	(10) 6.72 ± 0.34	(9)2.64 ± 21	(9)523 ± 62	(9)1.46 ± 0.11 (56.48%)	(9)656 ± 93	(10)4.44 ± 0.23	(10)2.82 ± 0.16

[1] DM indicates dry matter.
[2] Figures in parentheses indicate number of individual chicks analyzed.
[3,4] Figures in italic indicate significant difference from nondystrophic controls ($P < 0.025$), except ([3]) where $P < 0.05$.
[5] Seven-minute hydrolyzable phosphorus expressed as % of total P in barium insoluble fraction, ($P < 0.025$ by analysis of variance).

insoluble fraction were increased in dystrophic chicks, but the 7-minute hydrolyzable phosphorus (representing ATP and others) when expressed as a percentage of total phosphorus in this fraction, was significantly decreased in dystrophy as compared with normal chicks. A significant decrease in barium-soluble phosphorus (as creatine phosphate and others) was also observed in the dystrophic as compared with the nondystrophic chicks.

Isotope studies

Specific activity of phosphorus. Since the results presented above showed an *increase* in total phosphorus and inorganic phosphorus but a decrease in certain specific phosphates (probably ATP, creatine phosphate, and others), studies only on the total phosphorus content of the various phosphorus fractions could lead to erroneous results. Because of this, studies were undertaken to determine the specific activities of the phosphorus in the various phosphorus fractions from the muscles of dystrophic and nondystrophic chicks, according to the procedures described by Comar ('55). The specific activities were determined for two different time intervals (4 and 24 hours after administration) in an attempt to estimate the rapidity of incorporation of the isotope in the dystrophic and control muscles. A separate experiment was conducted in which the muscle samples were collected 48 hours after P^{32} injection. Only the specific activity of the total phosphorus in the muscle was determined in the latter investigation.

Single injections of approximately 150 μc per chick of P^{32} in the form of H_3PO_4 in weak HCl were given by intraperitoneal injection to both the dystrophic and nondystrophic chicks. Corrections for body size on the basis of body weight were made in the calculation of the results. The sample collection and fractionation procedure were the same as those described above. A counting standard was prepared and the "percentage of dose" as well as "specific activities" were calculated.

The distribution of P^{32} in the total muscle phosphorus of dystrophic and nondystrophic chicks and the specific activities of the phosphorus are presented in table 5, and indicate that the specific activity of the phosphorus in the deficient chicks was significantly increased at both 4 and 24 hours but no significant difference existed at 48 hours after injection of P^{32}. The increase in specific activity at 4 and 24 hours of the dystrophic muscle as compared with that in normal muscle was approximately 50%, whereas the increase in total phosphorus content of the dystrophic muscle was only about 10% above that of the nondystrophic controls.

In an effort to determine the compound or compounds responsible for this rapid uptake of phosphorus, the specific activity of the phosphorus in the acid-soluble fraction was determined. These results are presented in table 6, and show that the specific activity of the acid-soluble phos-

TABLE 5

Distribution of P^{32} in the breast muscle of dystrophic and nondystrophic chicks

Group	Total phosphorus					
	4 hours		24 hours		48 hours	
	% Dose[1]	Specific activity[2]	% Dose	Specific activity	% Dose	Specific activity
Dystrophic	1.97	*17.65 ± 1.52*[3]	1.93	*16.31 ± 1.47*	0.424	14.72 ± 0.69
Nondystrophic	1.23	11.82 ± 0.72	1.18	11.02 ± 0.50	0.382	14.33 ± 0.83

[1] Per cent dose per gm of dry muscle corrected for body weight $\left(\frac{\%\text{ dose/gm dry matter} \times \text{body weight}}{100}\right)$.

[2] Specific activity of total phosphorus corrected for body weight $\left(\frac{\%\text{ dose/gm dry matter}}{\text{mg P/gm dry matter}} \times \text{body weight}\right)$.

[3] Figures in italic indicate significant difference from nondystrophic chicks ($P < 0.01$).

TABLE 6

Distribution of P^{32} in the phosphorus fractions of the breast muscle of dystrophic and nondystrophic chicks

Group	Hours	Total phosphorus					
		Acid soluble		Barium insoluble		Barium soluble	
		% Dose[1]	Specific activity[2]	% Dose	Specific activity	% Dose	Specific activity
Dystrophic	4	1.35	*17.52 ± 1.47*[3]	0.4591	*16.80 ± 1.46*[4]	1.0543	*21.74 ± 3.54*[4]
	24	1.28	*16.22 ± 1.55*[4]	0.3408	10.23 ± 1.37	0.6891	20.07 ± 3.76
Nondystrophic	4	0.78	11.13 ± 0.72	0.2637	10.43 ± 1.40	0.5719	11.48 ± 0.62
	24	0.74	11.52 ± 0.45	0.2378	9.22 ± 0.28	0.4518	11.49 ± 0.94

[1] Per cent dose per gm of dry muscle corrected for body weight $\left(\frac{\%\ \text{dose/gm dry matter} \times \text{body weight}}{100}\right)$.

[2] Specific activity of total phosphorus corrected for body weight $\left(\frac{\%\ \text{dose/gm dry matter} \times \text{body weight}}{\text{mg P/gm dry matter}}\right)$.

[3] Figures in italic indicate significant difference from nondystrophic controls ($P < 0.005$).

[4] In this instance, $P < 0.025$.

phorus, the barium-insoluble phosphorus and the barium-soluble phosphorus were all significantly increased in dystrophic muscle as compared with the normal controls at 4 hours after administration of the P^{32}. The acid-soluble and barium-soluble phosphorus from the dystrophic muscle also showed increased specific activities at 24 hours after administrations. Since the specific activity of the phosphorus in the barium-insoluble fraction was substantially increased in the dystrophic muscle only at 4 hours after administration of P^{32}, it appears that the P^{32} was incorporated more rapidly into one or more of the labile phosphates in this fraction and did not remain in the fraction as long as 24 hours. The substantial increase in specific activity of the total phosphorus from the barium-soluble phosphates indicates an accelerated incorporation of P^{32} into a phosphorus compound in this fraction. Although creatine phosphate is the likely choice as the compound responsible for this increased incorporation, the fact that several other compounds are present in this fraction makes it impossible to attribute the entire increase in specific activity to creatine phosphate.

Rate of penetration of phosphorus, potassium and cesium. Because of differences found in phosphorus content and the incorporation of P^{32} into various fractions of muscle made dystrophic by vitamin E deficiency, the rate at which phosphorus is transferred from blood to muscle under these conditions was estimated. Moreover, in order to assess whether any such change might be a general permeability phenomenon, and because of the reported decrease in muscle potassium in muscular dystrophy (Nesheim et al., '59), a similar determination was undertaken for the penetration of potassium. The latter study was made as a double tracer experiment, using cesium as the second tracer, because it was felt that additional information might be forthcoming from learning something of the behavior of this element which has physiologic properties similar to sodium as well as to potassium (Hood and Comar, '53).

The concentrating capacity of a tissue for an ion may be estimated by injecting

the radioisotope of that ion into an animal and comparing the activity in 1 gm of tissue with that in 1 gm of plasma after a period short enough to obviate appreciable loss from the overall system or to allow return of the isotopic ion to plasma, and yet long enough to permit appreciable accumulation by the tissue. Actually the average activity concentration of plasma over the experimental period should be used rather than the final value. Because of the experimental difficulties in obtaining an average, however, the final value can provide a satisfactory approximation and the error be minimized by using a slow injection route. By this technique, one can determine what fraction of the element in the plasma enters the tissue. By knowing the ion concentration in plasma, the actual amount of ion which penetrates the tissue per unit of time can also be calculated (Hevesy, '48).

Two groups of 5 chicks each, showing muscular dystrophy of similar degree, were chosen for these isotope studies. Two groups of 5 normal chicks were randomly selected for controls.

All isotope injections were subcutaneous. Those injected with P^{32} (150 μc, as H_3PO_4 in weak acid) were sacrificed two hours after injection; those with the K^{42}-Cs^{137} mixture (250 μc K^{42}; 30 μc Cs^{137} as chlorides in weak acid), one hour after injection.

The P^{32} samples were counted with a thin end-window Geiger-Mueller detector. The K^{42}-Cs^{137} samples were counted with a well-type scintillation detector, using a thallium-activated sodium iodide crystal. Corrections for background and, where necessary, for coincidence were made; no self-absorption corrections were necessary. The K^{42} was counted as soon as possible after collection of the samples, care being taken to record the exact time of day. The Cs^{137} content was determined by recounting 5 days later when the K^{42} had decayed to an undetectable level; the K^{42} content was then determined by difference and the percentage of dose calculated by reference to a standard curve plotted from data on the K^{42} content of a dose aliquot against time.

The penetration of phosphorus into muscle is shown in table 7. The penetration rate of 1 μg/min. for the controls agrees exactly with the figure reported by Kalckar et al. ('44) for perfused rabbit muscle. Phosphorus penetration rate was significantly greater in the dystrophic than in the normal muscle ($P < 0.05$).

Similar calculations were made for the penetration of potassium into the muscle, liver and kidney of the dystrophic chick. The results are summarized in table 8, along with data on the relative behavior of K^{42} and Cs^{137}. In all three tissues, 1.5 times as much potassium entered the tissues of the dystrophic chicks as compared with the controls. In the muscle, this means an increased turnover of K, since flame spectrophotometric analyses revealed no difference in K content of muscle between groups (mean values of 478 μg/gm in dystrophic and 465 in controls). Presumably a similar steady state obtains in the other tissues also, although the physiologic reasons for increased K turnover in liver and kidney are not apparent. Perhaps this is related to potassium migration and amino acid transport noted by Riggs et al. ('58).

The fact that the muscle, unlike liver and kidney, in the dystrophic chicks

TABLE 7

Penetration of P in muscle of dystrophic chicks[1]

Group	Plasma inorganic P	Plasma P^{32}	Muscle P^{32}	P penetration of muscle
	μg/ml	*% of dose/ gm × 10⁴*	*% of dose/ gm × 10⁴*	*μg P/min./gm*
	A	B	C	[(C/B) ×A] ÷ min.
Dystrophic	58.3 ± 2.2	3.93 ± 0.97	*19.2 ± 12.9*	*2.21 ± 1.05*[2]
Nondystrophic	58.3 ± 2.7	3.19 ± 0.32	6.18 ± 1.36	0.946 ± 0.262

[1] Experimental time was two hours.

[2] Figures in italic indicate significant difference from nondystrophic controls ($P < 0.05$).

TABLE 8

Penetration of K, K^{42} and Cs^{137} in muscle, liver and kidney of dystrophic chicks

Group	Tissue	Relative activities % of dose/gm tissue / % of dose/gm plasma		Relative penetration	K penetration rate in tissue
		Cs^{137}	K^{42}	Cs^{137}/K^{42}	$\mu g\ K/min./gm$
Dystrophy	muscle	*4.09*[1]	20.8	0.20	*51.2*
Control	muscle	2.86	16.7	0.17	34.2
Dystrophy	liver	12.4	28.2	*0.46*	*69.1*
Control	liver	14.9	22.6	0.66	46.7
Dystrophy	kidney	36.9	22.1	1.70	*54.2*
Control	kidney	32.0	16.4	1.96	33.8

[1] Figures in italic indicate significant difference from nondystrophic controls ($P < 0.05$).

picked up more Cs^{137} than in the controls appears to indicate a change in the permeability characteristics of the muscle cell membrane, as compared with the cells of liver and kidney in the dystrophic chicks.

SUMMARY

Results are presented which show: (1) that the total muscle phosphorus content is greater in dystrophic chicks than in normal controls; (2) that the increase in total phosphorus is due largely to a specific increase in the inorganic phosphorus fraction in dystrophic muscles; (3) that the 7-minute hydrolyzable phosphorus (representing ATP and others) was significantly decreased, as was the barium-soluble phosphorus (representing creatine phosphate and others) in the dystrophic as compared with nondystrophic muscles.

Isotope studies showed increased penetration rates of phosphorus, potassium and cesium in the muscles of dystrophic versus nondystrophic chicks. An increased turnover of potassium also was shown to occur in the dystrophic muscles. These results indicate that the increased incorporation of phosphorus into dystrophic muscles may be due to a general increase in permeability of these muscles rather than a specific increase in phosphorus turnover. The results showing a decrease in such compounds as ATP and creatine phosphate in the dystrophic muscles which showed, at the same time, an increase in uptake of total phosphorus, indicate that phosphorus metabolism may be specifically affected during dystrophy in the chick.

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Comparative Vitamin B_6 Activity of Frozen, Irradiated and Heat-Processed Foods[1]

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Recently, investigators in several laboratories have demonstrated that vitamin B_6 is more important in human nutrition than was generally believed from earlier observations. Coursin ('54) and Molony and Parmalee ('54) reported that convulsive seizures which developed in infants receiving a proprietary liquid canned milk formula were dramatically cured by administering pyridoxine.

Greenberg et al. ('49) and Vilter et al. ('53) reported that humans receiving diets deficient in vitamin B_6 or the antagonist desoxypyridoxine excreted large amounts of xanthurenic acid in the urine following a test dose of tryptophan. Rafe and Plonko ('56) carried out tryptophan load tests on 17 children under 4 years old. Twelve of the 17 children excreted large amounts of urinary xanthurenic acid following administration of the tryptophan. These authors believed that the appearance of xanthurenic acid in the urine of the children indicated a need for pyridoxine supplementation. Wachstein ('56) reported that patients with typical symptoms of toxemia of pregnancy excreted significantly larger amounts of xanthurenic acid following a tryptophan load test than occurs in women with uncomplicated pregnancies. These data were interpreted as evidence that the intake of vitamin B_6 was below that required for normal tryptophan metabolism.

Day et al. ('57) reported that there was approximately 25% loss in vitamin B_6 activity when beef was sterilized with gamma radiations, and Richardson et al. ('58) reported that the loss of pyridoxine was greater than that of riboflavin when an aqueous solution containing a mixture of B vitamins was treated with 2.79 megarads of gamma radiation. Harris ('59) has pointed out that various methods of processing human foods have tended to decrease their vitamin B_6 content and suggested that further studies of the vitamin B_6 content of the diet used in the United States are warranted. Extensive studies have been carried out recently on the effect of processing and storage on the vitamin B_6 activity of milk products. These studies, as well as some of the problems involved in assays for vitamin B_6, have been reviewed recently by Woodring and Storvick ('60).

Since vitamin B_6 may be more labile than had been assumed, it seemed desirable to compare the loss of vitamin B_6 activity in foods that had been preserved by gamma radiation with that of the same foods which had been preserved by conventional methods. The results are described in this paper.

EXPERIMENTAL

Beef liver, boned chicken, cabbage, green beans, lima beans and sweet potatoes were frozen, irradiated with 2.79 and 5.58 megarads[2] of gamma rays and canned by conventional heat treatment. Assays were run on each food initially and after storage for an average of 9 and 15 months. The initial values were obtained within one to 4 months after the foods were received at this laboratory.

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[1] These studies were supported by the Office of the Surgeon General Department of the Army under contract DA-49-007-MD-582. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.
[2] Rad indicates a unit of ionizing radiation corresponding to an absorption of energy in any medium of 100 ergs per gm (1 rad in tissue = 100/93 rep).

All foods used in this test, except frozen lima beans and canned (heat-processed) lima beans, green beans and sweet potatoes were supplied and processed by the Quartermaster Food and Container Institute of the Armed Forces (QMFCI).[3] The other foods were purchased from a local market in quantities sufficient for the entire experiment. All foods were packed in no. 2 or no. 10 tin cans. Frozen foods were kept at − 20°C, and irradiated and canned foods were stored at ambient temperatures which ranged from about 24° to 27°C. It was assumed that all treatments of a food supplied by the QMFCI were processed from the same original lot. In most cases, two to 6 no. 2 cans or one no. 10 can of food was sufficient for a complete assay. The cans used in individual tests were taken at random from the original supply. Precautions were taken to insure a uniform sample for each individual assay. In this procedure the contents of two to 6 no. 2 cans or one no. 10 can were run through a meat grinder, thoroughly mixed and divided so that each sublot was sufficient for two days. Each sublot was sealed in a no. 2 can and stored at − 20°C until immediately before it was fed.

Assay procedure. The rat growth procedure was used for the assay of the vitamin B_6 activity of the various foods. An excess number of 25- to 28-day-old rats of the Texas A. and M. strain were placed in individual cages and given the vitamin B_6-deficient diet. The composition of this diet is shown in table 1. At the end of a two-week depletion period, males weighing 60 to 75 gm and females weighing 50 to 65 gm were selected for the assays. They were arranged in individual cages in groups of 5 males and 5 females each (10 rats per group) so that the average weight of each group was essentially the same for a given assay. The average weight of groups in different assays ranged from 60 to 65 gm.

Pyridoxine hydrochloride was used for the standard and was fed at levels of zero, 1, 3, 5, 10 and 15 μg per rat per day 6 days weekly for 4 weeks. A standard was run with each assay. The pyridoxine was dissolved in water and the solution was pipetted into individual casters. Each treatment of food was fed at three levels.

TABLE 1

Composition of the vitamin B_6-deficient diet

	gm
Casein[1]	25.0
Sucrose[2]	65.5
Woodpulp[3]	3.0
Mineral mixture[4]	5.0
Lard	0.4
Choline chloride	0.1
	mg/100 gm[5]
Menadione	0.75
α-Tocopherol	2.0
Thiamine·HCl	1.0
Riboflavin	1.0
Ca pantothenate	3.0
Niacin	2.0
Folic acid	0.2
Biotin	0.02
Vitamin B_{12}	0.002
	I.U./100 gm
Vitamin A	3000
Vitamin D	400

[1] Labco, The Borden Company, New York.
[2] Cerelose, Corn Products Company, New York.
[3] Solka Floc BW-40, Brown Company, Berlin, New Hampshire.
[4] Richardson and Hogan ('46).
[5] The authors are indebted to Merck Sharp and Dohme, Rahway, New Jersey for generous supplies of vitamins and to Lederle Laboratories, Pearl River, New York for folic acid.

The wet food was weighed on a torsion balance and administered to individual rats in glass casters. The basal vitamin B_6-deficient diet was removed from the cages at approximately 8:00 A.M. and the casters containing the supplements were put into the cages immediately. All supplements were usually consumed within two to three hours and the diet was returned to the cage within 4 hours.

Rats in the group receiving no pyridoxine failed to gain weight and many developed typical acrodynia dermatitis. Those receiving 1 μg of pyridoxine gained an average of 1.5 to 3.5 gm per week, and a few developed acrodynia. Those receiving 15 μg per day gained from 20 to 25 gm per week and all animals were normal.

Preliminary tests were run to determine the weight of each treatment of food required to give an average gain ranging from 4 to 15 gm per week. The levels of food were selected so that the average gain of one group of rats would be 4 to 6 gm; the second would be 8 to 10 gm and the

[3] Chicago, Illinois.

third, 12 to 15 gm. As often the case in biological assays, ideal gains were not always obtained for every food. For example, the weights of frozen lima beans fed in one assay were 1.0, 2.5 and 4.0 gm of wet food per day and the average gains were 7.2, 13.8 and 16.3 gm per week, respectively. Lima beans irradiated with 5.58 megarads were fed at levels of 1.0, 2.0 and 3.5 gm of wet food per day and the corresponding average gains were 3.8, 7.3 and 10.8 gm per week. The average weekly gain in weight was plotted against the dose of pyridoxine or dose of food in grams per day on log-log paper. The standard curve and the curves for frozen and irradiated lima beans in this assay are shown in figure 1.

RESULTS AND DISCUSSION

This investigation was carried out primarily to compare the vitamin B_6 activity of foods sterilized by the irradiation process with that of the same foods preserved by conventional methods. The rat growth procedure was used because it measured the available vitamin B_6 in the foods and no attempt was made to determine the proportion of pyridoxine, pyridoxal or pyridoxamine or bound vitamin B_6.

Even though the primary purpose was to compare the vitamin B_6 activity of irradiated foods with that of foods preserved by conventional methods, the data show that there was an effect of storage as well as of treatment. Both of these effects are discussed.

Effect of storage on vitamin B_6. The vitamin B_6 activity of foods obtained in the initial assay (one to 4 months) and after they had been stored an average of 9 (8 to 10) and an average of 15 (12 to 18) months are summarized in table 2. The frozen, canned and irradiated beef liver, boned chicken and cabbage presumably were prepared from the same original lot of food in each case. Since frozen lima beans and canned lima beans, green beans and sweet potatoes were purchased locally, the values represent the vitamin B_6 activity that may be present in similarly processed foods. They do not necessarily represent the relative vitamin B_6 activity in relation to that of the same food preserved by another process. The vitamin B_6 activity of every treatment of beef liver,

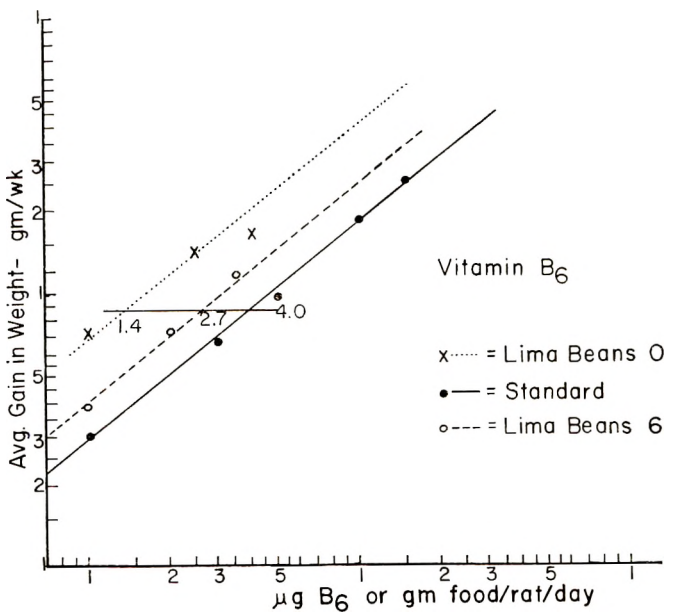

Fig. 1 Curves typical of those obtained in vitamin B_6 assays. Gain in weight was plotted on log-log paper against μg of the standard or grams of food per rat per day. Lima beans 0 indicates frozen, and lima beans 6, 5.58 megarads of gamma radiation. Numbers on the curves represent weight of food containing 4 μg of vitamin B_6 activity.

TABLE 2

Vitamin B_6 activity of frozen, irradiated and canned foods

Food	Av. storage period	Vitamin B_6			
		Frozen	Heat-processed	Irradiated	
				2.79 megarads	5.58 megarads
	months		*µg/100 gm wet food*		
Beef liver	initial	1375	400	1375	1133
	9	1550	—	1400	1200
	15	1125	143	600	640
Activity retained, %		82	36	44	56
Boned chicken	initial	600	340	410	375
	9	950	250	390	310
	15	460	280	390	250
Activity retained, %		77	82	95	67
Cabbage	initial	120	113	75	63
	9	140	53	87	48
	15	93	65	58	48
Activity retained, %		93	58	77	76
Green beans	initial	90	—	60	40
	9	90	78	53	47
	15	50	50	33	31
Activity retained, %		55	64	55	77
Lima beans	initial	175	78	133	117
	9	272	110	153	130
	15	220	130	130	160
Activity retained, %		126	166	98	137
Sweet potatoes	initial	125	53	65	30
	9	140	90	88	76
	15	179	73	95	57
Activity retained, %		143	138	128	190

[1] Percentage of the original activity retained after storage $= \frac{\text{final}}{\text{initial}} \times 100$.

boned chicken, cabbage and green beans was less after these foods had been stored for 15 months than it was initially. In order to express the effect of storage on the vitamin B_6 activity of the food on a quantitative basis the value in micrograms of vitamin B_6 in the final assay (15 months) was divided by the intial value and multiplied by 100 to convert to percentage of vitamin B_6 activity retained. These values are shown in table 2. In general, the activity retained after storage was higher in boned chicken and cabbage than in beef liver and green beans. Heat-treated and irradiated beef liver retained the least activity of any of these foods. Even though the vitamin B_6 activity of these 4 foods varied widely, there was consistently less activity in all treatments after they had been stored 15 months. This overall decrease of vitamin B_6 activity was similar to the decrease observed in milk on storage. Gregory ('59) observed a 60% loss of vitamin B_6 in milk and milk products on storage.

This decrease in vitamin B_6 activity on storage was not observed in lima beans and sweet potatoes. Every treatment of these foods either remained the same or increased in vitamin B_6 activity with storage. This apparent increase in vitamin B_6 activity of lima beans and sweet potatoes may be explained in two ways. First, it is possible that initial values were too low and thus caused an apparent increase in activity after these foods had been stored. Second, if some vitamin B_6 had been liberated from the bound form and had become available during storage, the activity as measured by the rat-growth procedure

could have actually increased. Further studies will be required to establish whether this possibility is plausible.

Effect of treatment on vitamin B_6 activity. There was less vitamin B_6 activity in heat-treated and irradiated foods than in the frozen foods at each corresponding storage period. In order to express this difference on a quantitative basis, the percentage of the activity in the treated food to that in the frozen food was calculated for each corresponding storage period. These data are summarized in table 3.

The initial activity of heat-treated beef liver was only 29% of that in the frozen liver and at 15 months the activity of the heat-treated beef liver was only 13% of that in the frozen food. The loss in vitamin B_6 in this particular food ranged from 70 to 87% depending upon the length of storage time before consumption. The vitamin B_6 activity of boned chicken, cabbage and green beans which had been irradiated or heat-treated was about 45 to 60% of that in the frozen food. In general, the vitamin B_6 activity retained in the treated food was variable, but with one exception the amount retained in the irradiated foods was essentially the same or greater than that retained in the heat-treated foods. Since varying loss of activity occurred in 4 of the frozen foods during storage, the total relative loss of activity from that in the original food would even be larger for these 4 foods.

The relative loss of activity for different treatments of lima beans could not be calculated because the frozen, heat-treated and irradiated lima beans were not from the same original lot. Similiarly the relative activity for canned green beans and sweet potatoes could not be calculated but the relative activity of irradiated sweet potatoes was about 40 to 50% of that in frozen food even though the activity increased in each case during storage.

SUMMARY

The vitamin B_6 content of 6 foods which had been frozen, canned by conventional heat treatment and irradiated with 2.79 and 5.58 megarads of gamma rays was obtained using the growth of rats as the assay procedure. The vitamin B_6 activity of beef liver, cabbage, boned chicken, green beans, lima beans and sweet potatoes was

TABLE 3

The relation of vitamin B_6 activity of treated food to that of the frozen food

Food	Av. storage period	Activity retained[1]		
		Heat-processed	Irradiated	
			2.79 megarads	5.58 megarads
	months	%	%	%
Beef liver	initial	29	100	82
	9	—	90	77
	15	13	53	57
Boned chicken	initial	57	68	63
	9	26	41	33
	15	61	85	54
Cabbage	initial	94	63	53
	9	38	62	34
	15	70	62	52
Green beans	initial	—[2]	67	44
	9	—	59	52
	15	—	66	62
Sweet potatoes	initial	—[2]	52	24
	9	—	63	54
	15	—	53	32

[1] Activity retained $= \frac{\text{treated}}{\text{frozen}} \times 100$ at corresponding storage periods.

[2] Activity was not calculated because the treated foods were not from the same lot as frozen foods.

obtained initially and after 9 and 15 months' storage. The vitamin B_6 activity in every treatment of beef liver, boned chicken, cabbage and green beans was less after the food had been stored 15 months than initially. Every treatment of lima beans and sweet potatoes resulted in the same or a higher activity after storage for the same period. The vitamin B_6 activity of the heat-treated and irradiated foods was approximately 40 to 60% of the activity of the frozen foods at each corresponding storage period.

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Comparative Vitamin K Activity of Frozen, Irradiated and Heat-Processed Foods[1]

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Synthesis of vitamin K by microorganisms in the intestinal tract is usually considered sufficient to meet the needs of most animals and because of this synthesis, dietary vitamin K deficiency in man and many other animals under normal conditions is practically unknown. A high incidence of hypoprothrombinemia has been reported in rats receiving diets that contained sulfonamides to prevent intestinal synthesis of vitamin K (Black et al., '42; Day et al., '43; Kornberg et al., '44). A high incidence of brain hemorrhage was reported also in infant rats born to mothers receiving a synthetic diet that contained no added source of vitamin K (Brown et al., '47). Richardson et al. ('56) reported that the vitamin K activity of diets containing relatively small amounts of menadione (vitamin K_3) or of vitamin K_1 or K_5 was markedly decreased by irradiating with sterilizing doses of gamma rays. When large amounts of the pure compounds were added to the diet, sufficient vitamin K activity remained after irradiation to prevent hemorrhages. Practically no destruction of vitamin K occurred when a diet containing dehydrated alfalfa leaf meal was irradiated or when fresh spinach was irradiated. Metta et al. ('59) reported that a large number of male rats receiving a diet containing 35% dry weight each of irradiated beef and flour died from internal hemorrhages in less than 8 weeks. The hemorrhagic syndrome did not develop in females and was prevented in the males by the addition of vitamin K to the diet.

These observations indicated that intestinal synthesis or absorption of vitamin K may not be sufficient to prevent a deficiency when the diet is low in vitamin K or composed chiefly of irradiated foods. In view of the possibility that a diet for humans which contained mostly irradiated foods might be deficient in vitamin K, it was decided to compare the relative vitamin K activity of foods which had been preserved by irradiation and by other conventional processes. The results are described in this report.

EXPERIMENTAL

Asparagus, broccoli, cabbage, cauliflower, green beans and spinach were frozen, irradiated with 2.79 and 5.58 megarads[2] of gamma rays and canned by conventional heat treatment. The food preserved by each process was assayed for vitamin K activity initially within two to 4 months after it had been received at this laboratory. The assays were repeated twice and the food had been stored an average of approximately 9 and 15 months when the determinations were completed. The frozen foods were stored at $-20°C$. The irradiated and heat-processed foods were stored at ambient temperature which ranged from 24° to 27°C. The Quartermaster Food and Container Institute of the Armed Forces supplied and processed each food used for the vitamin K assay except the heat-processed green beans. The canned green beans were obtained from a local grocery and stored in the laboratory in the same manner as the other canned and irradiated foods.

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[2] Rad indicates a unit of ionizing radiation corresponding to an absorption of energy in any medium of 100 ergs per gm (1 rad in tissue = 100/93 rep).

Assay procedure. Prothrombin times of chick plasma were used to evaluate the vitamin K activity of the foods. The procedure was essentially the same as that described by Bliss and György ('51). After a two-week depletion period, menadione, which was used as a reference standard, and the lyophilized foods were administered in gelatin capsules to the chicks each day for a 7-day period. Prothrombin times were then determined 24 hours after the last supplement was given.

Day-old White Leghorn cockerels obtained from a local hatchery were separated at random into groups of 10 chicks each so that the average weight of each group was essentially the same. All chicks received the same vitamin K-deficient diet throughout the experimental period. The composition of this diet was the same as that described by Richardson et al. ('58) except that menadione was omitted. Menadione was used as the standard and was run with each assay. Quick and Stefanini ('48) suggested that either menadione or natural vitamin K_1 could be used as a reference standard. Menadione has been adopted by the U. S. Pharmacopeia ('55) as the reference standard in vitamin K assays.

Supplements of menadione and of foods were started at the end of a two-week depletion period. The amounts of menadione administered to 9 groups of chicks were 0.7, 1.4, 2.1, 2.8, 3.5, 5.25, 7.0, 14.0 and 35.0 μg per chick per week, respectively. Each food was fed at three levels. The average weight of the chicks in the various groups was 115 to 130 gm when the supplements were started and the chicks gained 45 to 60 gm per group during the week they received supplements. The gains were the same regardless of the amount of menadione or amount of food administered or the final plasma prothrombin times. Chicks receiving 0.7 μg of menadione per week usually had average plasma prothrombin times of 65 to 70 seconds, whereas those receiving 35.0 μg per week had normal prothrombin times which, under our conditions, ranged from 15 to 20 seconds.

Preparation of foods. Preliminary trials showed that the wet foods could not be given quantitatively in gelatin capsules and therefore all foods were lyophilized before they were fed. A sufficient amount of wet food for one assay was mixed in a mechanical blender and about 10 gm of the blended food was poured into a 90-mm diameter petri dish. The contents were frozen rapidly in a deep freeze and 4 to 6 dishes containing the frozen food were then placed in the chamber of a VirTis Freeze Mobile for 14 hours. The pressure in the chamber was approximately 0.5 mm Hg and temperature of the condensing trap was approximately − 50°C.

The entire lot of a dry food for one chick was weighed accurately, divided into 7 approximately equal portions and packed into 7 gelatin capsules. This procedure insured that each chick within a group received the same total weight of food during the assay. The menadione was administered in ethanol in quantities so that the maximal amount of the ethanol solution administered per chick per day was 0.3 ml. Preliminary tests were run to obtain an estimate of the weight of food required to give plasma prothrombin times between 20 and 70 seconds. An attempt was then made to select three levels of each food to give three different average prothrombin times within this range. One example illustrates the weight of foods administered and the average prothrombin times obtained. In one assay 0.6, 1.0 and 1.4 gm dry weight of frozen broccoli was fed per chick per week. The corresponding average plasma prothrombin times were 48, 34 and 36 seconds.

Thromboplastin. A thromboplastin solution was prepared from lyophilized chick brain tissue by mixing 1 gm of dry tissue with 20 ml of physiological saline in a 50-ml centrifuge tube. The tube containing the brain suspension was placed in a water bath at 56°C and stirred frequently for 10 minutes. The suspension was cooled rapidly, centrifuged and the supernatant liquid siphoned off. This thromboplastin solution was mixed with an equal volume of 0.2 M calcium chloride solution immediately before use.

Blood samples. On the 8th day after the supplements were started, 1 ml of blood was taken by heart puncture from each chick using a 1.0-ml syringe and a one-inch no. 21 needle. The blood was emptied into a 12-ml conical centrifuge tube containing 0.1 ml of 0.1M sodium oxalate.

After centrifuging, 0.1 ml of the oxalated plasma was pipetted into a second 12-ml centrifuge tube. Two-tenths milliliters of the thromboplastin-calcium chloride solution were added and the time, in seconds, required for the plasma to clot was obtained with a stop watch. Prothrombin times were determined in a constant temperature bath at 37°C.

Calculation of vitamin K activity. The vitamin K activity was calculated by plotting prothrombin time against dose of standard and dose of food on log-log paper. This method is similar to that suggested by Jacques ('41) who showed a similar relationship between thrombin concentration and plasma clotting times. A standard curve and three points obtained for each of two foods are shown in figure 1. Since a line parallel to the standard could not always be drawn through the points for prothrombin times, the vitamin K activity in micrograms was read from the prothrombin times for each level of food. This value together with the dry weight of food administered and the weight of solids in 100 gm of the wet food administered were used to obtain the vitamin K activity per 100 gm of wet food.

RESULTS AND DISCUSSION

The average and the low and high values obtained for the vitamin K activity of each of 6 foods preserved by the different procedures and stored for periods ranging from an average of 3 to 15 months are summarized in table 1.

This study was carried out primarily to determine whether sterilization by ionizing radiation was more destructive of vitamin K activity than conventional methods commonly used for the preservation of foods. In some instances, higher values were obtained in heat-treated and irradiated foods than in the frozen foods. There also appeared to be an increase in vitamin K activity in a few instances after the food had been stored. These changes may be real, but they have not been interpreted in this manner because of the large differences between the low and high values obtained for the three levels of food. Furthermore, values obtained in different assays frequently did not agree. One explanation for this variation may be that the food from the same cans was not used in different assays and the vitamin K activity of the food from various cans actually was different. In our opinion, a great deal of the variation was the result of difficulty in carrying out the vitamin K assay procedure. Regardless of the variability in results, it was concluded that there was no appreciable loss of vitamin K activity in the foods preserved by any process or when stored for 15 months.

Green beans and cauliflower had very little vitamin K activity and large amounts of dried foods had to be administered. Attempts to obtain values for cauliflower were discontinued after preliminary tests showed that amounts of food required to

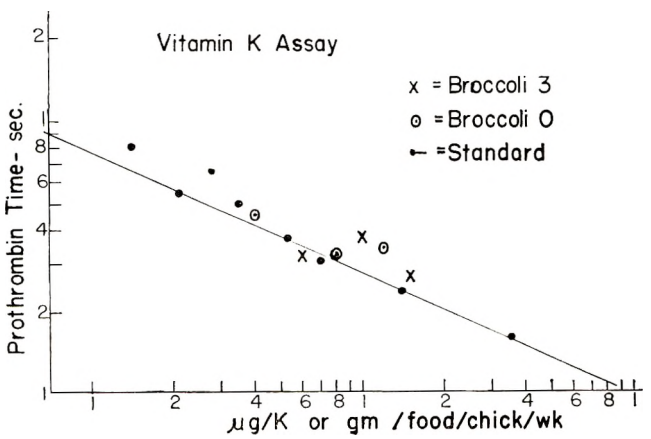

Fig. 1 A standard curve and prothrombin times for three levels of two foods each plotted on log-log paper. Broccoli 3 indicates food irradiated with 2.79 megarads, and broccoli 0, frozen food.

TABLE 1

Vitamin K activity of frozen, heat-processed and irradiated foods

Food	Av. storage period	μg Vitamin K activity/100 gm wet food			
		Frozen	Heat-processed	Irradiated	
				2.79 megarads	5.58 megarads
	months	*Av.*	*Av.*	*Av.*	*Av.*
Spinach	initial[1]	130(107–153)[2]	146(112–180)	230(288–173)	182(203–162)
	9	74 (43–110)	79 (67–92)	183(172–194)	
	15	75 (74–76)	149(111–255)	267(200–333)	213(181–278)
	Av.[3]	93	125	227	198
Broccoli	initial	65 (55–74)	70 (61–80)		35 (20–49)
	9	62 (43–74)	71 (48–93)	72 (44–110)	62 (50–77)
	15	61 (35–77)	62 (50–76)	76 (40–123)	34 (15–59)
	Av.	63	68	74	44
Cabbage	initial	37 (34–40)	44 (41–46)	38 (35–40)	42 (37–46)
	9	59 (49–61)	56 (31–80)	102 (77–138)	59 (49–61)
	15	73 (49–87)	61 (43–86)	93 (46–159)	66 (34–120)
	Av.	56	54	78	56
Asparagus	initial	21 (15–26)	33 (31–35)	37 (26–48)	34 (27–42)
	9	51 (42–62)	66 (59–78)	36 (27–44)	68 (52–84)
	15	28 (23–35)	24 (19–30)	40 (35–44)	37 (33–42)
	Av.	33	41	37	46
Green beans	initial	22 (17–32)	41 (33–55)	17 (14–18)	
	9	24 (24–)[4]	18 (18–)[4]	28 (17–49)	44 (22–77)
	15	37 (29–46)	26 (9–50)	17 (17–)[4]	71 (25–111)
	Av.	28	28	20	58
Cauliflower		<10	<10	<10	<10

[1] Initial values were obtained within 2 to 4 months after the foods were received at the laboratory.
[2] Numbers in parentheses represent the high and low values obtained for the three levels of food.
[3] Average value for three storage periods.
[4] Only one value available.

give a valid assay would be impractical to administer in gelatin capsules.

SUMMARY

The vitamin K content of 6 foods which had been frozen, canned by conventional heat treatment, and irradiated with 2.79 and 5.58 megarads of gamma rays was obtained using the chick assay procedure. Foods assayed for vitamin K were asparagus, broccoli, cabbage, cauliflower, green beans and spinach. The values for vitamin K activity were higher in some instances and lower in a few cases in the treated foods than in the frozen foods. Similar variations in values were obtained after the foods had been stored 15 months. These differences were attributed chiefly to variations in values obtained for different levels of a food and it was concluded that there was no appreciable difference in vitamin K activity caused by the method of preservation or by storage.

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The authors gratefully acknowledge the technical assistance of Mrs. Jacqueline Godwin in carrying out the assay.

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The Effect of Niacin-Deficient, Tryptophan-Low and Protein-Deficient Diets on the DPN/DPNH Ratio in Mouse Liver[1]

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The tissue pyridine nucleotide level and the ratio of oxidized to reduced forms are influenced by the availability and the capacity of the tissue to convert nicotinic acid to the various pyridine nucleotide forms. Kaplan et al. ('56) thus demonstrated that massive injections of nicotinamide into rats *increased* total pyridine nucleotide levels in the liver for 16 to 24 hours following the injections. The effects of niacin deficiency or protein deficiency on liver diphosphopyridine nucleotide (DPN) levels and on the ratio of the oxidized to reduced forms will be presented later.

Burch et al. ('55) indicated that rats fed small amounts of DPN precursors developed low levels of oxidized pyridine nucleotides (diphosphopyridine nucleotide plus triphosphopyridine nucleotide [TPN]) in blood cells and liver; analyses were not performed for the reduced forms (DPNH + TPNH). In addition, the extraction method and the fluorometric determination used by these investigators usually yielded lower nucleotide levels than those obtained by the method of Spirtes and Eichel ('54).

Recently, Tulpule ('58) reported changes in total pyridine nucleotide levels and in the ratios of the oxidized to the reduced components in livers of rats fed a protein-deficient diet. Tulpule's method of extraction and determination resulted in values for DPN + TPN and DPNH + TPNH and not for the diphospho- and triphosphopyridine nucleotide entities separately. Since Glock and McLean ('55) have reported liver DPN/DPNH ratios differing widely from TPN/TPNH ratios, changes in the overall ratios of DPN + TPN/DPNH + TPNH would be difficult to interpret from the point of view of possible alterations in individual nucleotides. The method used by Tulpule also yielded lower *reduced* pyridine nucleotide levels (and therefore high DPN + TPN/DPNH + TPNH ratios) when compared with that used by Spirtes and Eichel ('54). Consequently, it was decided to repeat this work using the latter method, which has the added advantage of yielding separate values for the reduced and oxidized DPN forms as well as higher total values for the reduced moieties.

EXPERIMENTAL

Diet and animal preparation. White, Swiss, male mice weighing 23 to 26 gm were used. These animals had been supplied with a diet of commercial dog chow[2] and water fed ad libitum from the time they were weaned. In table 1 are listed the ingredients of the basal experimental diets, diet A for the niacin-deficient group and diet B for the protein-deficient group. The diets were isocaloric but did not contain the same amount of protein nitrogen. Sulfathalidine was included in diet A to inhibit the bacterial synthesis of niacin (Levy et al., '53). The control group of mice in the niacin-deficiency study received a supplement of 5 mg of niacin per 100 gm of the basal diet. For the production of protein deficiency with diet B, the vitamin-free casein was replaced by an equicaloric amount of sucrose. The control group of animals for each deficiency study (8 mice) was pair-fed with a group of equal size fed the deficient diet. All of

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[1] This investigation was supported by Grants A-2165 and B-730 from the National Institutes of Health, Bethesda, Maryland.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 1
Composition of experimental diets

Ingredient	Diet A[1]	Diet B[2]
	%	%
Vitamin-free casein[3]	4.00	25.00
Vitamin-free casein-hydrolyzate[4]	3.00	0.00
Gelatin[5]	10.00	0.00
DL-Methionine[6]	0.20	0.00
Dextrose	0.00	63.00
White corn meal[7]	10.00	0.00
Corn oil[8]	5.00	5.00
Salts (Wesson, '32)[4]	5.00	5.00
Agar[5]	2.00	2.00
Sulfathalidine	3.00	0.00
	ml/day	*ml/day*
Vitamin B mixture[9]	1	1
Vitamins A, D, E, K[10]	1	1
Calories/gm diet (calculated)	3.86	3.97
Nitrogen/gm diet (Kjeldahl)	0.012	0.018

[1] Control diet for niacin-deficiency experiments. This diet is low in tryptophan (determined by calculation according to Block and Bolling, '45). Niacin was omitted for the deficiency diet.

[2] Control diet for protein-deficiency experiments; vitamin-free casein was omitted from the deficiency diet and replaced isocalorically by sucrose.

[3] Labco, The Borden Company, New York.

[4] General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Difco Laboratories, Detroit.

[6] Courtesy of Merck and Company, Rahway, New Jersey.

[7] Purchased from a feed dealer; exhaustively extracted with absolute ethanol; air-dried.

[8] Mazola, Corn Products Refining Co., New York.

[9] Vitamin B mixture: (percentage composition) pyridoxine·HCl, 0.001 (diet A), 0.002 diet B); Ca pantothenate, 0.010 (diet A), 0.010 (diet B); for the remainder the same amounts are present in diets A and B; riboflavin, 0.002; thiamine·HCl, 0.002; nicotinic acid, 0.005; choline chloride, 0.150; inositol, 0.020; folic acid, 0.001; biotin, 0.002; courtesy of Merck and Company.

[10] Composition of mixture: vitamin A, 850 I.U./ml; vitamin D, 85 I.U./ml; tocopherol, 0.0010%; naphthoquinone, 0.001%; courtesy of Endo Laboratories, New York.

the animals were housed in metabolism cages. A known amount of diet was placed in a weighed, white ointment jar and put into the metabolism cage during the mid-afternoon. The next morning the food jars were removed and weighed. The amount of food consumed by each group was recorded daily. No records were kept of the consumption of water, which was offered ad libitum. The mice were weighed twice weekly in groups. When the experimental mice had lost approximately 25% of their weight as recorded at the start of the experiment, they were selected for pyridine nucleotide assay studies.

Method for the extraction and determination of DPN and DPNH. The method of Spirtes and Eichel ('54) was used. The necessary alcohol dehydrogenase was made by the method of Racker ('50) from air-dried bakers' yeast[3] and stored at −15°C in 3 ml of a saturated solution of $(NH_4)_2SO_4$. DPN[4] was found to be 85 to 90% pure by the alcohol dehydrogenase assay (Racker, '50). DPNH was prepared chemically from the DPN by reduction with hydrosulfite (Gutcho and Stewart, '48) or enzymatically with alcohol dehydrogenase (Racker, '50).

All the inorganic chemicals used were either of reagent[5] or analytical reagent[6] grade.

Total nitrogen was determined by a modification of the microKjeldahl method. Caloric value of the diet was calculated.

RESULTS

For 99 days a group of 8 white Swiss mice, originally weighing 23 to 26 gm each, was fed diet A from which niacin had been omitted. A similar group started to receive the complete diet A a day later and was pair-fed with the first group. Both consumed 6.75 gm per mouse per day at the start of the experimental period; consumption decreased to 3.73 gm per mouse per day near the end of the experimental period. The group consuming the niacin-deficient diet showed an average decrease in individual mouse weight from 23.8 gm to 14.7 gm, whereas consumption of the controls dropped from 24.2 to 19.7 gm (table 2). The recorded difference in weight between these two groups at the time of death was statistically significant ($P < 0.05$). This P value, as well as those to be mentioned later, was calculated from Student's t test values for small samples (Fisher, R. A., '54). A value of $P = 0.05$ or less was assumed to indicate a significant difference between the two groups being compared. Although the total liver size of the deficient animals was smaller

[3] Fleischmann Company, New York.

[4] Sigma Chemical Company, St. Louis.

[5] Baker Chemical Company, Phillipsburg, New Jersey.

[6] Mallinckrodt Chemical Works, St. Louis.

than that of the control group at the time of death, statistical analysis of these figures revealed no significant difference. Two additional groups, each consisting of 8 mice, were supplied with diet B, one fed the complete diet and one, the same diet but with protein replaced isocalorically by sucrose. The average weight per mouse in each group was 25.8 gm at the beginning of the experimental period (table 2). Although the pair-fed control group maintained its weight after receiving the diet 24 days, the weight of the protein-deficient group decreased by approximately 50%. The food intake decreased from 4.6 gm per mouse per day at the start to 4.2 gm per mouse per day near the end of the experimental period of 25 days. Concomitantly with the sharp, significant weight drop ($P < 0.001$) occurring in the protein-deficient group, a decrease in *total* liver weight was noted. Further analysis of the latter finding revealed no significant difference between the two groups, when calculations were based on milligrams of mouse liver per gram of mouse. When total nitrogen per gram of liver was calculated, however, a significant decrease ($P < 0.02$) occurred for the protein-deficient group. This would be expected for organs deficient in protein.

The DPN and DPNH values observed for the livers of mice consuming diet A are shown in table 3. Both the DPN and DPNH values, and consequently the total diphosphopyridine nucleotides, decrease significantly. The percentage decrease of

TABLE 2

Whole animal liver weights and liver nitrogen of niacin-[1] and protein-[2]deficient mice[3]

	Days fed diet	Av. weight of animals		Wet weight of liver at death	Mg liver/ gm animal	N in 518 mg liver at death
		At start of experiment	At death			
		gm	*gm*	*mg*		*mg*
Controls[1]	98	24.2 ± 1.8[4]	19.7 ± 1.2	750 ± 109	38.2 ± 6.4	
Niacin-deficient[1]	99	23.8 ± 1.9	14.7 ± 1.6	589 ± 152	40.3 ± 7.4	
P			$P < 0.05$	$P = 0.4$	$P > 0.3$	
Controls[2]	24	25.8 ± 2.2	26.7 ± 2.1	945 ± 193	35.3 ± 4.6	18.8 ± 4.2
Protein-deficient[2]	25	25.8 ± 2.0	13.7 ± 1.2	518 ± 75	38.5 ± 7.9	14.2 ± 2.2
P			$P < 0.001$	$P < 0.001$	$P > 0.1$	$P < 0.02$

[1] Experimental animals were fed diet A (table 1) with or without niacin.
[2] Control experimental animals were fed diet B (table 1). The protein-deficient group was fed diet B with casein replaced isocalorically by sucrose.
[3] Eight animals/group.
[4] Standard error of the mean.

TABLE 3

DPN and DPNH levels in livers of niacin- and protein-deficient mice[1]

	DPN	DPNH	DPN + DPNH	DPN/DPNH
	μg/gm wet weight			
Controls[2]	471 ± 72[3]	236 ± 53	707 ± 95	2.10 ± 0.62
Niacin-deficient[2]	308 ± 61	163 ± 36	472 ± 85	1.99 ± 0.34
P	$P < 0.001$	$P = 0.01$	$P < 0.01$	$P = 0.9$
Controls[4]	602 ± 71	205 ± 35	807 ± 74	3.01 ± 0.65
Protein-deficient[4]	240 ± 62	187 ± 43	427 ± 85	1.31 ± 0.36
P	$P < 0.001$	$P > 0.4$	$P < 0.001$	$P < 0.001$

[1] Eight animals/group.
[2] Animals fed diet A (table 1) with or without niacin.
[3] Standard error of the mean.
[4] Controls were fed diet B (table 1). The protein-deficient group was fed diet B with casein replaced isocalorically by sucrose.

both forms is the same and therefore the calculated DPN/DPNH ratio remains the same for both groups of mice.

Results for the study involving animals ingesting diet B are also tabulated in table 3. In this case the liver DPN values decreased from 602 μg/gm wet weight to 240. DPNH levels, however, were not lowered significantly. As a result, the total diphosphopyridine nucleotide values are significantly lowered ($P < 0.001$), as are the DPN/DPNH ratios. The latter dropped from an initial level of 3.01 to 1.31 ($P < 0.001$).

DISCUSSION

Using the method of Feigelson et al. ('50) for the extraction and determination of tissue pyridine nucleotides, Tulpule ('58) reported that rats fed a protein-deficient diet showed a lowering of the ratio of oxidized to reduced components. Both pyridine nucleotide components were lowered, the oxidized forms more so than the reduced. The Feigelson method has two main disadvantages: (1) a portion of the reduced pyridine nucleotides may be destroyed because they are extracted at a pH below 7.0 (Fisher et al., '48); and (2) this method determines DPN + TPN and DPNH + TPNH. The ratios of the diphospho- and triphosphopyridine nucleotides are not determined separately. Since Glock and McLean ('55) noted these ratios to be different in liver (DPN/DPNH = 1.8, TPN/TPNH = 0.05), a decrease in the overall ratio determined by the Feigelson method (namely, DPN + TPN/DPNH + TPNH), would not give information as to whether the DPN or the TPN components, or both, have changed. The Spirtes-Eichel method ('54) for the extraction and determination of diphosphopyridine nucleotides results in higher DPNH values than the combined DPNH + TPNH observed when using the former method. In addition, the total DPN + DPNH is usually higher than the total di- and triphosphopyridine nucleotides observed by Feigelson et al. The present experiments, carried out using the Spirtes-Eichel method, differed from Tulpule's results in that the DPN component, alone, decreased (table 3) in the livers of protein-deficient mice. The decrease in the DPN/DPNH ratio recorded in the same table was therefore much greater than that noted by Tulpule ('58) for DPN + TPN/DPNH + TPNH. The reason that DPN alone was altered in the present protein-deficiency experiments is not known. Figures from the protein deficiency study involving liver size (table 2) indicate that the liver weight per gram of total mouse weight is *not* significantly lower for the deficient animals, even though these same protein-deficient mice possess significantly smaller *total* liver weights. Total body and liver weight thus decreased by approximately the same percentage, namely, 55 and 51%, respectively (table 2). The observation of Tulpule ('58) that the livers from protein-deficient animals are more fatty than those from normal controls is one indication that these organs may actually be *more* deficient in protein than the animal considered as a whole.

Although the complete picture of dietary protein deficiency in the experimental group of mice developed 25 days after they started to receive the diet, 99 days were required for the development of a marked niacin deficiency as detected by the nutritional and pharmacological criteria applied by Levy et al. ('53). Part of the loss in weight shown by the deficient mice is no doubt attributable to the reduced food intake of the deficient group and the rest to the restriction of niacin intake.

Previously Williams et al. ('50) had shown that rats fed a tryptophan-free diet developed lower levels of total pyridine nucleotides than normal controls. The authors were unable to increase these totals even after addition to the food ration of 1.5 mg of niacin per 100 gm or several times this amount. Similar results were noted by Williams et al. ('51) for rats fed a nonprotein basal diet. Tryptophan added to the experimental diets, however, raised the pyridine nucleotides to a high level. Also in the present experiments, mice consuming a control diet containing 88 mg % of tryptophan[7] developed less liver DPN than those fed a control diet containing tryptophan at 450 mg per 100 gm. This is further evidence that tryptophan plays an important role in the establishment of the pyridine nucleotide level in mouse liver. A similar role for niacin can be deduced from table 3, in which it is shown

[7] See table 1, footnote 1.

that mice fed a tryptophan-low, niacin-deficient diet formed less liver DPN and DPNH than those receiving the same diet to which 5 mg of niacin per 100 gm had been added. The liver DPN/DPNH ratios of the former group remained unchanged indicating that both the DPN and DPNH were lowered to the same degree. Whether tryptophan and niacin increase the pyridine nucleotide levels in mouse and rat livers directly by increasing the amounts of DPN precursors available or indirectly by stimulating the formation of DPN-synthesizing enzymes cannot be decided from the present experiments or from any in the literature.

SUMMARY

1. When compared with normal control animals, niacin-deficient mice developed the same percentage decrease of diphosphopyridine nucleotide (DPN) and DPNH liver values. This resulted in an unchanged DPN/DPNH ratio.

2. Protein-deficient mice developed a sharply lowered liver DPN content and an unchanged DPNH level; the DPN/DPNH ratio of this group was therefore markedly lowered compared with its control.

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Distribution and Excretion of F^{18} Fluoride in Beef Cattle[1]

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Fluoride occurs in almost all foods and water for animals and is of concern in livestock production primarily because of its excessive ingestion (Mitchell and Edman, '52; Hobbs et al., '54; Suttie et al., '58).

Of the radioactive isotopes of fluorine, F^{18} is the only one which is useful for large animal research since all of the others have shorter half-lives. Tracer studies using this isotope offer additional means for obtaining information on the metabolism of fluoride, since quantitative research with this element has been hampered due to small concentrations in soft tissues (Singer and Armstrong, '59). Radioactivity determinations of F^{18} can be conducted without elaborate processing, but the short half-life of 109.7 min. for F^{18} (Carlson et al., '59) necessitates the use of this radioactive tracer near the source of supply.

This study was undertaken to establish F^{18} tracer techniques in beef cattle and to determine whether long-term ingestion of fluoride would influence F^{18} metabolism and excretion.

EXPERIMENTAL

Approximately 400 mc of F^{18} obtained from 20 min. of cyclotron bombardment of 500 mg of NaF were used in each of 5 trials. The material was dissolved in a minimum of HCl and made to 20-ml volume so that approximately 80 mc of F^{18} were administered to each animal intravenously using a shielded syringe as described by Hansard ('51).

Two 18-month-old beef heifers averaging 660 pounds were used in the first trial, and 12 beef cows, 9 years old, averaging 1020 pounds, were divided into 4 groups of three each for the other 4 trials. The two heifers were used to further develop techniques for using this short lived radioisotope tracer as described by Perkinson et al. ('55) and Chamberlain.[2] The cows had been fed three levels of fluoride for 8 years as described by Hobbs et al. ('54). The basal ration contained 7 ppm of fluoride by analysis, and 40 and 50 ppm of fluoride were added as sodium fluoride in the other two treatments. Approximately 18 hours before dosing, the animals, in groups of three, including one from each level of fluoride feeding, were placed into metabolism stalls as described by Hobbs et al. ('50).

The second and third cows in each group were dosed approximately 50 and 112 min. respectively, after the first cow. Since they were slaughtered 4 hours after dosing, these spaced intervals provided sufficient time to obtain samples for immediate counting. Each dose was accurately measured and a standard reference solution was drawn, diluted and counted with the tissue samples. The dosing solutions were essentially free of radioactive contaminants as measured by a multi-

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[2] Chamberlain, C. C. 1959 Some effects of chronic and acute fluoride levels on metabolism and distribution of F^{18} in selected tissue of cattle. Ph.D. Thesis, Iowa State University, Ames, Iowa.

channel analyzer and by plotting the decay curve against the normal decay of F^{18}. Three milliliters each of whole blood, plasma, plasma protein-free filtrate and urine were counted directly using well-type scintillation counters. Other tissue samples not exceeding 4 gm in weight were placed into tared scintillation tubes, counted, then weighed. Except for liquids, most tissue samples were taken in duplicate, counted for 2 min. and the average count recorded.

Samples were counted in 16 × 150-mm glass tubes in a Nuclear-Chicago DS-5 scintillation well counter fitted with a 2 × 1¾ inch NaI(T1) crystal, at a standard counting volume of 3 ml. The minimum detectable limit of counting was taken as a net sample count equal to background, or approximately 400 cpm. Net counts ranged from this figure to approximately 5×10^4 cpm.

Speed was important due to the rapid decay of the radioisotope. The usual schedule required the cyclotron use for 20 min. at 8:00 A.M. which permitted dosing the first animal by 9:30 A.M. Normally this permitted counting of all samples by 9:30 P.M. A few urine samples with very high activity were allowed to decay overnight before counting. All counts minus background were converted to percentage of original dose using the decay of the standard with appropriate dilutions. All of the data are presented as percentage of dose per milliliter for liquids and per gram for other tissues, with the exception of the one line shown in figure 1, which was based on total dose in the blood.

Blood samples were taken from the jugular vein on the opposite side from which the dose was given. The blood samples were heparinized, plasma removed by centrifugation and plasma protein-free filtrate obtained using trichloroacetic acid. Saliva samples were taken by swabbing the mouth using a slightly moist sponge. The sponge was then squeezed to obtain a 3-ml saliva sample for counting. Urine samples were obtained directly from the bladder using an indwelling catheter which was installed a few minutes prior to dosing of the animals. By this procedure, most of the urine was removed from the bladder before dosing and a continuous flow of urine was maintained during the sampling period

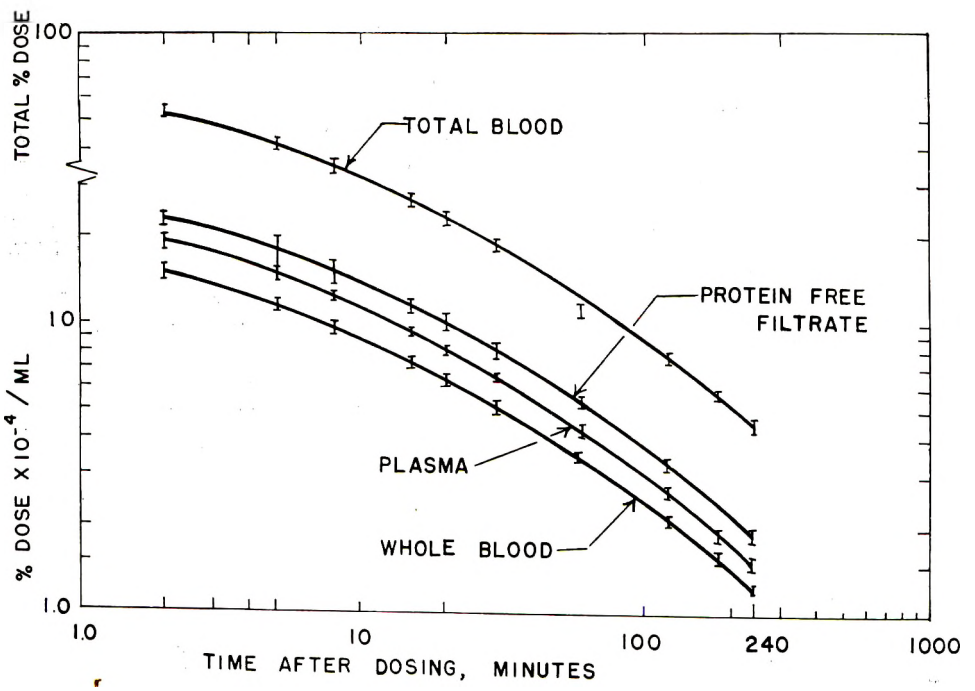

Fig. 1 F^{18} levels in blood from mature cows after intravenous dosing.

Fecal samples were obtained directly from the rectum using rubber gloves and a plastic sleeve. Four hours after dosing, each animal was slaughtered by conventional methods of stunning and bleeding. Samples of bone, teeth, viscera, and visceral contents were collected for subsequent chemical and radiochemical analyses. Fetal membrane samples were also obtained from the 10 pregnant cows. In determining stable fluoride, samples were treated with low-fluoride calcium oxide in platinum dishes, dried and gradually ashed in a muffle furnace until a temperature of 550°C was reached, and maintained at this temperature for one to two hours. Samples were ashed to constant weight. Fluorides in ashed samples were separated by the distillation method of Willard and Winter ('33), and determined by the procedure of Nielsen ('58).

RESULTS AND DISCUSSION

No statistically significant differences were noted in F^{18} tissue levels which could be associated with the previous feeding of stable fluoride; therefore, all of the F^{18} data were pooled for the mature cows, and values for the yearling heifers were averaged separately. Data presented in figure 1 show that F^{18} disappeared from the blood rapidly. Two minutes after dosing only 53.5% of the dose was found in the blood assuming the blood volume as 7.7% of body weight as reported by Dukes ('55). Two-hundred-forty minutes after dosing, the fraction of the dose remaining in the blood had dropped to 4.5%. These values approached a straight line when plotted on log-log paper. Recycling and excretion of the F^{18} probably altered the slope of the line. The F^{18} level in the protein-free filtrate remained higher than in the plasma or cells. The differences were real as the standard error of the means did not overlap as shown in figure 1. Data reported by Seppilli et al. ('57) showed that more fluoride in blood serum was in the free form than in the bound form when the concentration was low.

Data presented in figure 2 show that the major pathway for F^{18} excretion was in the urine. The average urinary concentration of F^{18} reached a peak of 40.4 ×

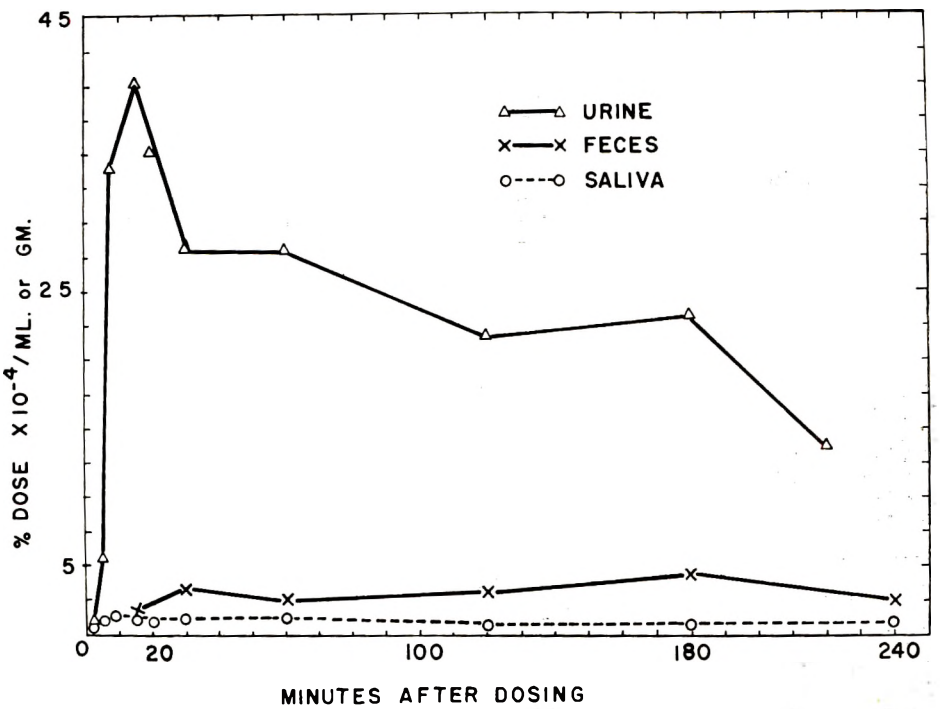

Fig. 2 Average concentration of F^{18} in excreta and saliva from mature cows.

10^{-4}% of dose per ml compared with 7.5 $\times$ 10^{-4} per ml for blood at 15 min. after dosing with F^{18}. A similar trend was noted in humans orally dosed with F^{18} (Carlson et al., '60). Saliva levels of F^{18} reached a peak of 1.3 $\times$ 10^{-4} per ml at 5 min. after dosing compared with a blood level of 11.4 $\times$ 10^{-4} at this same time. The concentration of F^{18} in urine, saliva and feces varied and showed no relationship to the previous dietary treatment of the animals. The highest level of F^{18} in the feces was noted at 180 min. after dosing when the average concentration was 4.1 $\times$ 10^{-4} per gm compared with a whole blood level of 1.6 $\times$ 10^{-4} per ml at 180 min. A possibility exists, however, that the F^{18} concentration in this portion of the gastrointes-

TABLE 1

F^{18} and stable fluoride in selected tissues

	Mature cows		Yearling heifers	
	% Dose $\times 10^{-4}$/gm	Stable fluoride	% Dose $\times 10^{-4}$/gm	Stable fluoride
		ppm		*ppm*
Gastrointestinal tract				
Reticular wall	2.10 ± 0.58[1]	4.06 ± 0.88		
Reticular contents	0.96 ± 0.13			
Rumen wall	2.70 ± 0.44	4.48 ± 1.04	7.26	0.40
Rumen contents	0.61 ± 0.11		1.23	0.06
Omasal septum	1.49 ± 0.29		1.83	0.63
Omasal contents	1.11 ± 0.15		1.55	1.20
Abomasal wall	0.75 ± 0.18	0.52 ± 0.04	0.30	0.27
Abosamal contents	0.71 ± 0.20		0.32	0.47
Small intestine	0.56 ± 0.04		0.26	0.31
Small intestine contents	1.20 ± 0.29		0.65	0.22
Large intestine	0.55 ± 0.06	7.00 ± 1.71	0.48	0.26
Large intestine contents	2.30 ± 0.48	15.56 ± 3.77	4.96	0.60
Vital organs				
Lungs	5.37 ± 0.77	0.59 ± 0.08	9.23	0.42
Kidneys	3.14 ± 0.27	0.80 ± 0.14	2.16	0.37
Liver	0.85 ± 0.12	0.23 ± 0.03	0.54	0.23
Heart	0.43 ± 0.04	0.20 ± 0.02	0.20	0.48
Pancreas	0.55 ± 0.08	0.15 ± 0.02		
Glands				
Mandibular salivary	0.67 ± 0.15			
Parotid salivary	0.74 ± 0.04			
Thyroid	0.97 ± 0.14	0.62 ± 0.08	1.38	0.98
Adrenal	0.48 ± 0.02		0.46	0.29
Pituitary	0.80 ± 0.12			
Mammary tissue	1.27 ± 0.13			
Bones				
Metacarpal	1.66 ± 0.26			
Metatarsal	1.66 ± 0.23			
Rib 9	6.10 ± 0.50			
Rib 10	7.83 ± 0.83			
Mandible	5.42 ± 1.00			
Fetal jaw tooth	0.75 ± 0.56			
Fetal metatarsal	2.14 ± 0.30			
Fetal mandible	3.69 ± 0.40			
Fetal rib	1.28 ± 0.26			
Other tissue				
Skin	1.34 ± 0.28			
Maternal placenta	0.60 ± 0.04	0.49 ± 0.05		
Fetal placenta	0.25 ± 0.04	0.26 ± 0.03		
Aorta	0.77 ± 0.05	3.37 ± 1.38	0.50	0.56
Gastrocnemius muscle	1.10 ± 0.16			
Blood	1.25 ± 0.08	0.10 ± 0.03		

[1] Standard error of mean.

tinal tract may have accumulated over most of the 180 min. after dosing.

The average F^{18} and stable fluoride levels in selected tissues for the cows and heifers are presented in table 1. Since no significant differences were noted in the levels of F^{18} which could be associated with the three levels of dietary fluoride, the data for each tissue were averaged for the 12 cows. The average percentage of dose $\times 10^{-4}$ per gm for the gastrointestinal tract varied from 2.70 for the rumen to 0.55 for the large intestine, whereas that for the contents showed respective values of 0.61 and 2.30 (fig. 3). These data indicate that fluoride administered intravenously, and which is recycled by the saliva and perhaps through the respiratory tract, is taken into the walls of the lining of the upper end of the intestinal tract and excreted into the contents of the lower part of the tract. In general the results obtained for the heifers followed the same trend as those for the cows. In contrast, Perkinson et al. ('55) reported total F^{18} instead of concentration and noted 5.2% of the dose in rumen of sheep two hours after intravenous dosing which they interpreted as F^{18} excretion through the rumen wall into the contents.

In the vital organs high concentrations of F^{18} were observed in lung and kidney tissue. Since the urine is a major pathway of F^{18} elimination, it is not unexpected that the kidney showed a higher level of F^{18} than blood at the time of sacrifice (3.14 vs. 1.25×10^{-4} of dose/gm).

These observations confirm the work of Wallace-Durbin ('54) who demonstrated in rats that the kidney concentration of F^{18} was greater than that in the blood at 4 hours after dosing; however, it is in contrast with the observation that the kidney was the only soft tissue that consistently showed a higher F^{18} concentration than the blood. Data in table 1 show that the F^{18} in blood was lower than in the following soft tissues: reticular wall, rumen wall, lungs, and kidneys. The vascularity of the kidney has been suggested as an explanation of the high concentration of F^{18} in the kidney (Wallace-Durbin, '54) and this appears to be a logical explanation for the high content in the lungs. The liver, a highly vascular tissue, had only two thirds of the level of the blood, however (table 1). Since data in figure 2 show that F^{18} fluoride is excreted in the urine, feces and saliva, it appears that F^{18} may be concentrated in soft tissues which are associated with elimination of body wastes. The high average concentration of 5.37×10^{-4}% of dose for lungs of the cows poses the possibility of respiratory elimination of F^{18}; however, F^{18} would not be expected to volatilize for elimination through the lungs. Using $CaCl_2$, traces of F^{18} were found in the expelled air, and the

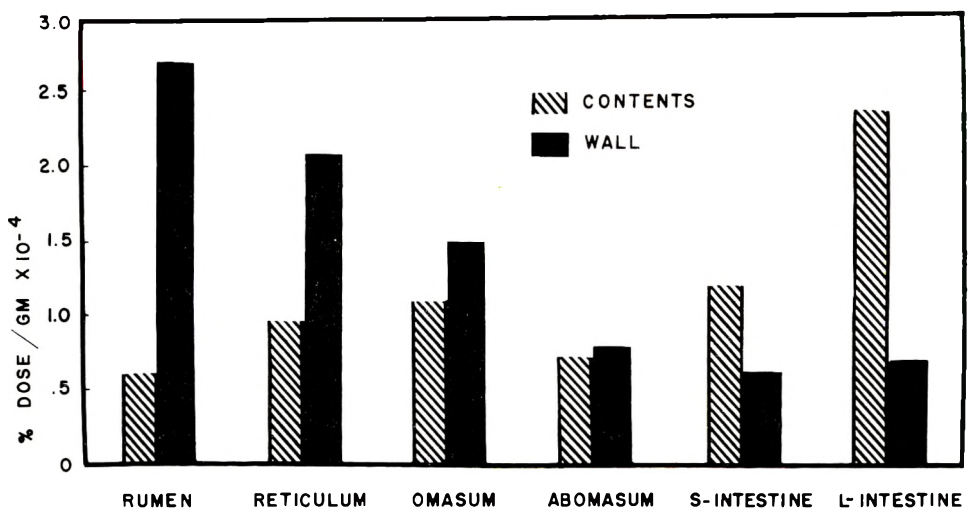

Fig. 3 Concentration of F^{18} in gastrointestinal tract at 240 min. after dosing.

mucus obtained from the nostrils of the cows had a high concentration of F^{18}. Mucus from the respiratory tract may account for elimination of F^{18}. This mucus is readily passed into the digestive tract which would permit the recycling of F^{18} into the blood stream. The ciliated lining of the trachea and bronchial tubes contribute to the removal of liquids from the respiratory system. The beating action of the cilia propel mucus and waste material toward the mouth. Under normal conditions these cilia are efficient and their actions are influenced by chemical changes in the blood (Best and Taylor, '55). The possibility of F^{18} being held or stored in lung cells which have an affinity for F^{18} must also be considered. The stable fluoride levels in the lung and kidney were lower than in other vital organs; hence, the specific activity of lung tissue and kidney tissue was high. Further research is needed to explore the significance of the affinity of the lungs for F^{18}.

Glandular tissues were low in F^{18} except for the mammary tissue which had a concentration almost identical with the blood level at the time of slaughter. Obtaining representative samples of bone for F^{18} analyses was difficult; however, even in the mature cows the rib and mandible showed a relatively high concentration (table 1). The oldest fetus was about 6 months, but the fetal metatarsal contained more F^{18} than the corresponding mature tissues, whereas the mandibles and rib presented the opposite results. These data indicate that there is more exchange of fluoride with the rib and mandibles than with the long bones of mature animals.

The exchange of blood constituents from the dam to the fetus and the existence of a fetal barrier had been demonstrated for many substances. In this study the F^{18} level was 0.60% of dose $\times 10^{-4}$ in the maternal placenta compared with 0.25 for the fetal placenta. From these results it appears that part of the F^{18} passes to the fetus but there appears to be a barrier to the free passage of F^{18}, which is in agreement with the results of Maplesden et al. ('60) on rats and rabbits fed stable fluorides.

In contrast with the data on radioactive fluoride, the stable fluoride concentration in selected tissues varied in most tissue with the level of fluoride in the ration of the cows as shown in table 2. These data

TABLE 2

Stable fluoride analyses and F^{18} specific activity of selected tissues from mature cows

	Ration fluoride in ppm					
	7		47		57	
	Stable fluoride[1]	Specific activity[2]	Stable fluoride	Specific activity	Stable fluoride	Specific activity
	ppm		*ppm*		*ppm*	
Reticular wall	2.03 ± 0.48[3]	1.57	5.74 ± 3.69	0.52	4.76 ± 0.51	0.09
Rumen wall	1.29 ± 0.86	1.53	7.34 ± 3.20	0.49	5.44 ± 0.45	0.45
Abomasal wall	0.44 ± 0.04	1.32	0.53 ± 0.08	2.45	0.58 ± 0.07	0.97
Large intestine, contents	4.0 ± 1.52	0.40	24.00 ± 6.88	0.06	20.00 ± 4.92	0.17
Large intestine, wall	5.33 ± 1.66	0.11	5.50 ± 0.49	0.11	9.00 ± 2.00	0.05
Aorta	0.74 ± 0.08	0.99	4.09 ± 3.22	0.20	4.99 ± 2.63	0.15
Heart	0.14 ± 0.01	2.64	0.23 ± 0.04	1.78	0.23 ± 0.03	2.17
Lung	0.38 ± 0.05	13.95	0.83 ± 0.17	8.69	0.62 ± 0.11	6.55
Liver	0.18 ± 0.04	4.11	0.34 ± 0.07	2.62	0.25 ± 0.02	3.72
Kidney	0.38 ± 0.06	7.11	1.10 ± 0.42	2.66	0.96 ± 0.16	3.76
Pancreas	0.11 ± 0.04	4.64	0.16 ± 0.09	2.69	0.17 ± 0.03	3.53
Thyroid	0.45 ± 0.03	1.62	0.86 ± 0.31	0.96	0.62 ± 0.06	2.02
Placenta	0.41 ± 0.12	1.51	0.59 ± 0.11	1.10	0.50 ± 0.06	1.12
Fetal placenta	0.32 ± 0.07	0.81	0.24[4]	0.92	0.23 ± 0.04	1.13
Blood	0.05 ± 0.01	24.00	0.09 ± 0.04	15.67	0.14 ± 0.06	8.28

[1] Wet basis.

[2] Specific activity/gm $= \frac{\%\text{ of dose} \times 10^{-4}}{\text{ppm stable fluoride}}$.

[3] Standard error of mean.

[4] Only one sample.

are in contrast with the observations reported by Wagner et al. ('58) who found no increase in fluoride concentration of soft tissues of rats fed additional fluoride for 90 days. Data in table 2 also show that, in general, as the dietary fluoride increased, the specific activity of the tissue decreased.

SUMMARY

Blood, saliva, urine and fecal samples from two beef heifers and 12 mature beef cows given tracer levels of F^{18} showed that F^{18} was distributed and excreted rapidly. After 2 min., 53% of the dose remained in the blood, but after 240 min., only 4% of the dose remained in the blood. The F^{18} in the protein-free filtrate was higher than in the plasma or cells. Tissue samples taken at 240 min. after dosing showed high concentrations of F^{18} in lung, kidney, rumen wall, reticular wall and in bone. Continued feeding of fluoride did not influence metabolism or excretion of F^{18}. Stable fluoride varied with the levels of fluoride fed.

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A program utilizing radioisotopes of very short half-life (109.7 min. for F^{18}) necessitates co-ordinated participation of many individuals so as to obtain reliable data within time limitations. A partial listing of the group involved includes R. C. McIlhenny, R. A. Teekell, R. H. Mayo and R. G. Buescher from the UT-AEC Agricultural Research Laboratory; R. G. White and Caroline S. Boyd from the Animal Husbandry-Veterinary Science Department of the University of Tennessee; and J. L. Shupe, R. Lamborn and H. M. Nielsen from the Utah State University.

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The Regulation of Depot Fat by Linoleic Acid

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Many biological properties of triglycerides differ with the chain lengths of the fatty acids involved. For instance, it has been known for some time that rats fed triglycerides consisting mainly of long-chain saturated acids have higher linoleic acid requirements than those fed a fat-free diet (Evans and Lepkovsky, '32; Barnes et al., '59). In contrast, it has been observed that the linoleic acid requirements of rats fed triglycerides made up of shorter-chain acids are about the same as those of animals fed a fat-free diet (Kaunitz et al., '60a).

The two types of triglycerides have also been shown to differ in their effects on neutral fat deposition in the rat, with medium-chain triglycerides being less conducive to deposition of fat than long-chain triglycerides (Kaunitz et al., '58). This quantitative difference in neutral fat deposition suggested that it might be worthwhile to study the composition of the neutral fat of rats fed these two types of triglycerides in conjunction with various levels of linoleic acid. The results of these studies are reported in this paper.

MATERIALS AND METHODS

The medium-chain (MCT) and long-chain (LCT) saturated triglycerides were prepared from coconut oil and other palm kernel oils by fractionation of the split fatty acids and reconstitution of the desired fractions into triglycerides after their composition had been checked by gas-liquid chromatography. The conversion was accomplished by reacting the fatty acid fractions with molar ratios of glycerol until the amounts of free fatty acid and glycerol were below 5%. The crude triglyceride fractions were alkali-refined, deodorized, and filtered and then subjected to a vigorous stream of hydrogen under pressure in the presence of 0.3% nickel catalyst (0.1% nickel) at a temperature of 180 to 200°C for several hours. The resulting saturated material was cooled, filtered to remove the catalyst, decolorized by steam, and refiltered to give an edible grade oil.

MCT represented about 15% of the original coconut oil and was a clear, thin, odorless liquid having a melting point below zero degrees and an iodine number of zero. The LCT represented about 60% of the original coconut oil and was a solid fat with melting point of about 40°C and an iodine number of zero.

The weanling male albino rats used in these studies were born of mothers which had been transferred to a fat-free diet (table 1) without added linoleic acid about 4 days before birth of their litters. In order to deplete them of linoleic acid, the young were reared with this diet until they were transferred to the experimental diets. A few days after birth, all young were pooled, sorted as to sex and size, and redistributed to the mothers so that each mother received 6 small, 7 or 8 medium, or 9 large male young. This was done to obtain more uniform rats. At 27 days of age, the young were weaned, earmarked and weighed. At 31 days, they were reweighed, and matching males were distributed into experimental groups of 8 each so that the average weights of the groups were equal at 27 days and again at 31 days. At this point, they were transferred to the experimental diets. At 103 days of age, the animals were killed. The bodies of fat surrounding the testes were weighed, and pooled samples were frozen for subsequent gas chromatographic analyses.

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TABLE 1

Composition of fat-free diet[1]

	%
Casein, alcohol-washed[2]	30
Dextrose[3]	64
Salt mixture, U.S.P. 13	3.5
Ca carbonate	0.5
Cellulose[4]	2
	mg/kg
Choline dihydrogen citrate	1000
Inositol	1000
p-Aminobenzoic acid	300
Nicotinamide	100
Vitamin K[5]	10
Thiamine·HCl	2
Pyridoxine·HCl	4
Riboflavin	4
Ca pantothenate	10
Folic acid	2.5
Biotin	0.025
Vitamin B_{12} (0.1% trituration)	5
Ascorbic acid	25
α-Tocopheryl acetate	100
Free α-tocopherol	20
β-Carotene	10
Vitamin D_2	0.5

[1] We wish to thank Dr. Leo Pirk, Hoffman-La Roche, Inc., Nutley, New Jersey, for most of the vitamins used in these experiments, and Dr. M. L. Tainter, Sterling-Winthrop Research Institute, Rensselaer, New York, for the vitamin D_2.

[2] Nutritional Biochemicals Corporation, Cleveland.

[3] Cerelose, Corn Products Refining Company, New York.

[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[5] Synkayvite, Hoffman-La Roche, Inc., Nutley, New Jersey.

The experimental diets included the fat-free diet given in table 1, similar ones supplemented with 0.08 and 2% of linoleic acid, and corresponding series of diets containing 20% of MCT or 20% of LCT, added at the expense of the carbohydrate. The low linoleic acid supplement of the MCT and LCT diets was 0.1% instead of 0.08% to compensate for the lower intake by the rats of these calorically higher diets.

For each gas chromatographic analysis, a pooled sample of testicular fat bodies was ground with sand, boiled with water under N_2, cooled, and treated with petroleum ether. The liquid fraction was washed with water, and the ether was evaporated at 100°C under reduced pressure. Five grams of the resulting oil were reacted with 3 ml of methanol containing 0.3% $NaOCH_3$ at 85°C for 10 minutes. The esters were extracted with petroleum ether and washed until neutral to phenolphthalein. The esters were analyzed on a Barber-Colman Gas Liquid Chromatograph using a radium cell. A 0.1-μl sample of ester was injected into a 6-foot column of Chromosorb W (80-100 mesh) coated with 20% Glutarate LAC 38[1] at 180°C. The cell temperature was 230°C and the applied cell potential was 750 volts; the flash heater temperature was 290°C; the sensitivity was 1×10^{-6}. Argon was used as the carrier gas at a flow rate of 150 ml per minute.

RESULTS AND DISCUSSION

In figure 1 are given average body weights at the time of death. As pointed out previously, there was little difference between weights of corresponding groups fed the MCT and fat-free diets. The groups fed LCT with little or no linoleic acid gained significantly less than the corresponding groups fed the other diets—an indication of increased linoleic acid requirements with these triglycerides. Similarly, Pfeifer and Holman ('59) found that inclusion of hydrogenated coconut oil depressed growth more than the basic fat-free diet.

Testicular fat bodies were studied because they are a distinct organ, the weight of which reflects the total neutral fat content of the rat (Hausberger, '37; Stoerk and Porter, '50). This suggested the use of this fat as representative of the total neutral fat, although it is known that depot fat varies in composition in different parts of the body.

In addition to considering the absolute weights of the fat bodies, we have often found it useful to relate them to body weight, a relationship which is not linear. For this purpose, the fat body weights were compared with those of control rats of the same body weight which had been fed a similar diet containing 10% of lard, and the differences between the two were expressed as percentages of the control organ weights. These percentages of "deviation from normal" of the experimental groups could be used for comparison of

[1] Wilkens Instrument and Research, Inc., Walnut Creek, California.

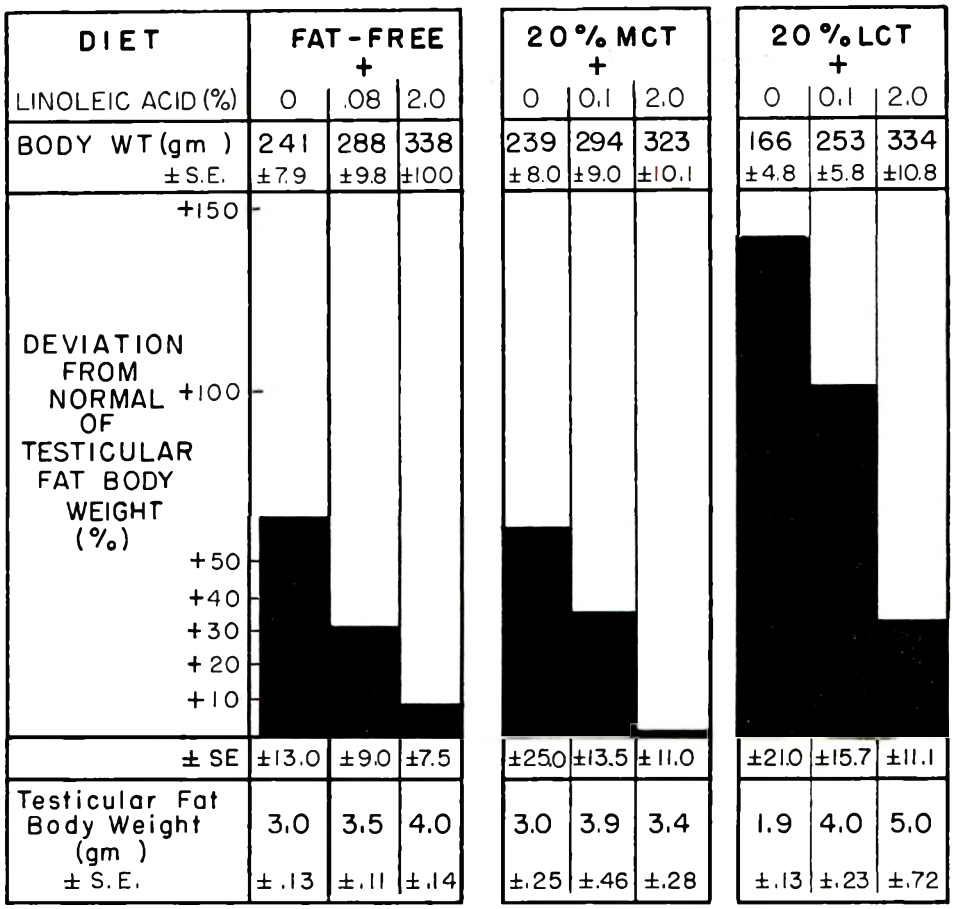

Fig. 1 Body weight, testicular fat body weight, and percentage of deviation from normal of the fat body weight of animals fed diets without fat or with 20% of medium-chain (MCT) or long-chain (LCT) saturated triglycerides and various levels of linoleic acid.

TABLE 2

Fatty acid composition of the special fats, medium-chain (MCT) and long-chain (LCT) saturated triglycerides, and of the testicular fat bodies of rats fed various levels of linoleic acid (LA) in diets containing these fats or no fat

Fatty acid	No. of carbons	Composition of dietary		Fatty acid composition of testicular fat bodies of rats fed diets containing							
				No fat +			20% MCT +		20% LCT +		
		MCT	LCT	No LA	0.08% LA	2% LA	No LA	2% LA	No LA	0.1% LA	2% LA
Caproate	6	7.5					trace	trace			trace
Caprylate	8	78.4	1.0			0.1	0.9	5.8			trace
Caporate	10	14.3	5.1				0.8	5.7	0.4	trace	0.6
Laurate	12	trace	44.8	trace	trace		0.7	2.0	16.2	22.9	26.3
Lauroleate									trace	0.5	1.2
Myristate	14	trace	25.1	1.7	1.4	1.5	2.7	2.6	14.2	18.6	23.5
Myristoleate				trace	0.2	0.5	0.3	0.9	trace	0.8	0.9
Palmitate	16		8.2	29.1	30.4	28.4	31.4	31.5	25.1	25.9	21.3
Palmitoleate				20.7	21.5	17.5	16.1	11.7	9.4	6.7	5.3
Stearate	18		15.6	1.0	1.7	0.5	1.2	1.7	2.2	1.6	4.0
Oleate				47.4	44.8	30.4	45.7	26.0	32.5	22.6	10.9
Linoleate						21.1		11.1	trace		6.0

groups having different average body weights. The actual procedure and the standard organ weight-body weight curves have been described in detail elsewhere (Kaunitz et al., '60b). The percentages of deviation given in the histogram in figure 1 show that the testicular fat bodies of all groups fed LCT were heavier in relation to body weight than those of the corresponding groups fed the fat-free and MCT diets.

In table 2 are summarized the results of the chromatographic analyses of the MCT and LCT which were used in these experiments and of the pooled samples of fat bodies.

The composition of the fat bodies of the group fed the fat-free diet with no linoleic acid (and more or less also those of the group fed the fat-free diet + 0.08% of linoleic acid) represented the endogenous fat of the animals. It contained about 30% of palmitate, 21% of palmitoleate, and 45 to 47% of oleate, values which are similar to those given for rats fed a low-fat diet (Longenecker, '39). The absence of linoleate from the depot fat of animals fed little or no linoleate has been observed repeatedly (Gregory and Drummond, '32; Mead, '57). A dietary supplement of 2% of linoleic acid led to a marked deposition of linoleate, as described by Spadola and Ellis ('36). If it was assumed that the linoleate deposited was derived from the diet and if the remaining components were calculated only in relation to each other, little change in the relative amounts of these acids occurred, with the possible exception of oleate, which constituted 39% of the non-linoleate part. Therefore, it would seem that feeding of linoleic acid led to a mild decrease in the oleate. The amount of fatty acids having chain lengths shorter than C_{16} was small, which is in agreement with previous reports that short-chain acids are not easily deposited in the neutral fat (Eckstein, '29; Powell, '30).

When MCT was fed without added linoleic acid, the composition of the fat bodies was similar to those observed in the corresponding groups fed fat-free diets. Only 1.7% of C_8 and C_{10} acids and no caproate were deposited although MCT consisted almost entirely of these acids. When 2% of linoleic acid was added to the diet, substantial amounts of caprylate and caporate were noted; linoleate was deposited, but not as much as with the fat-free diet. The oleate content of the fat bodies was roughly half that observed when MCT was fed without linoleic acid.

The influence of LCT on the composition of the depot fat was much more pronounced than that of MCT. The animals deposited large amounts of C_{12} and C_{14} acids, and addition of linoleic acid increased this markedly. Even 0.1% of linoleic acid had a definite effect. The fact that so small a quantity had this rather specific effect on fat deposition invites speculation as to a vitamin-like action for small amounts of linoleic acid.

The increased deposition of shorter-chain acids under the influence of linoleic acid when LCT was fed took place at the expense of oleate. When 2% of linoleic acid was fed, less linoleate was deposited than with MCT and the fat-free diet. Since the total linoleic acid intakes of the three groups were about the same for the experimental period, the LCT group must have metabolized more linoleic acid, which is more direct evidence of the higher linoleic acid requirements of animals fed LCT.

It is known that "the nature of the fat deposited in the adipose tissue is determined by the balance between the fat in the diet, the fat synthesized in the body and that broken down in metabolism" (Wertheimer and Shapiro, '48). The results of the present studies permit some amplification of this statement. Dietary linoleate leads to a decrease of neutral fat in relation to body weight and, at the same time, to the deposition of a fat more nearly resembling the dietary fat. This is true at least for the shorter-chain acids of the fats fed. It may also be true for the longer-chain acids inasmuch as the stearate content of the fat bodies of the rats fed LCT and 2% of linoleic acid was higher than with any other diet.

Since the depot fat is a dynamically active tissue which is available for cell structure, this function of linoleic acid may have some significance.

SUMMARY

1. Matching groups of weanling male rats were fed purified diets containing either no fat, 20% of a mixture of saturated triglycerides (MCT) containing 7% of C_8, 78% of C_8, and 14% of C_{10} acids, or 20% of a mixture of saturated triglycerides (LCT) containing 5% of C_{10}, 45% of C_{12}, 25% of C_{14}, 8% of C_{16}, and 16% of C_{18} acids. The diets were supplemented with zero, 0.1, or 2% of linoleic acid. After the rats had received the diet for 71 days, they were killed and the testicular fat bodies pooled and subjected to gas-liquid chromatography.

2. When the fat-free diets were fed, the depot fat consisted of not more than 2% of acids having chains shorter than C_{16}. The main constituent was oleate, the level of which declined when 2% of linoleic acid was added to the diet. With the latter diet, 21% of linoleate was noted in the depot fat.

3. With MCT and no linoleic acid, the depot fat was similar to that of the animals fed the corresponding fat-free diet. With 2% of linoleic acid, substantial amounts of C_8, C_{10}, and C_{12} acids were deposited; the oleate content declined from 45 to 26%, and the linoleate level was 11%.

4. With LCT, the depot fat contained a large amount of laurate and myristate. Linoleic acid supplementation increased their levels at the expense of oleate, with 0.1% of linoleic acid having a considerable effect. With 2% of linoleic acid in the diet, the depot fat contained 6% of linoleate.

5. It is concluded that linoleate regulates the type of fat deposited; it leads to a decrease in neutral fat in relation to body weight and facilitates the laying down of a depot fat more representative of that in the diet.

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Comparative Effects of Estradiol and Coumestrol Diacetate, a Nonsteroid Estrogenic Substance, on Lipid Metabolism in the Male Rat

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Recently, coumestrol, a nonsteroid, estrogenic substance characterized as 6′, 7-dihydroxybenzofuro (3′, 2′-3, 4) coumarin (fig. 1) was isolated from ladino clover (*Trifolium repens*) (Bickoff et al., '58a,b). The material has weak estrogenic activity when compared with diethylstilbestrol or estradiol, but is about 30 times more active than another plant estrogen, genistein, when tested by the mouse uterine assay (Bickoff et al., '59; Bickoff et al., '57).

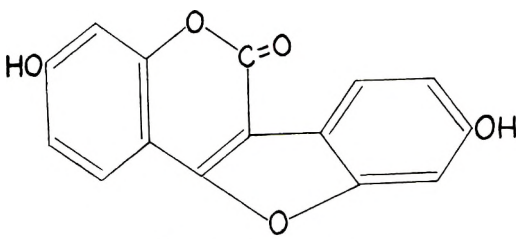

Fig. 1 Coumestrol.

Until recently, the limited availability of coumestrol had prevented extensive study of other physiological effects produced by prolonged feeding. It is well known that estrogens influence lipid metabolism in a number of species (Loeb, '42; György et al., '49; Okey et al., '56; Stamler et al., '56; Eilert, '53) and that in cockerels estrogens have been beneficial in improving carcass quality (Lauffer, '57). Therefore, when a supply of synthetic coumestrol diacetate became available,[1] its effect on liver and plasma lipids, food intake, growth and adrenal and testicle weights were compared with changes produced by relatively small doses of estradiol benzoate.

EXPERIMENTAL

Long-Evans weanling male rats were fed a 1% cholesterol-supplemented diet until their weights reached 150 gm. At that time, half of the animals were castrated under light ether anesthesia and were started on the experimental diets two days after the operation. An equivalent number of unoperated animals were started on similar diets at the same time. Weekly, cumulative food consumption records were kept for each rat.

The basal ration was essentially the 15% egg albumin-casein diet of Okey and Lyman ('57) in which the choline had been raised to 0.09%, and corn oil had been substituted for the lard. All diets except that for the control group contained 1% of cholesterol, which was dissolved in the oil prior to mixing. Coumestrol diacetate was mixed in the diet in such a way that 15 gm of diet contained either 1, 3, 5, 10, or 15 mg of the compound. In most cases, the animals ate slightly less than 15 gm of food daily; hence, actual intake of coumestrol by the rats was less than indicated.

Estradiol benzoate[2] was diluted with sesame oil, and 0.1 ml was injected subcutaneously three times a week after the animals had started to receive the diet. One group of controls, fed the basal diet, was injected with an equivalent amount of oil at the same time. The animals were fed the diets and supplements for 21 days and, following an overnight fast, they were anesthetized with pentobarbital sodium, and heparinized blood plasma was ob-

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[1] Synthetic coumestrol diacetate was generously supplied by E. M. Bickoff of the Western Regional Research Laboratory, Albany, California. Estrogenicity was determined by mouse uterine assay.

[2] Progynon-B, Schering Corporation, Bloomfield, New Jersey.

tained by heart puncture. Adrenal glands, liver and testicles (from normal rats) were removed and weighed immediately. Liver and plasma were extracted as previously described (Okey and Lyman, '57). Total liver lipid was determined by a modification of the method of Bloor ('28). Cholesterol was analyzed by the method of Sperry and Webb ('50). Phospholipids were approximated from lipid phosphorus (Sumner, '44) by multiplying values obtained by 25. In the first experiment, lipid-soluble phosphorus from the plasma was determined directly on the original alcohol: ether (3:1) extract. In the second experiment, however, we used a petroleum ether extract of the lipids (Okey et al., '59); hence, the primary extractant was evaporated and re-extracted into petroleum ether.

RESULTS

In table 1 are presented the results obtained from rats administered estradiol benzoate or fed 1, 3, and 5 mg of coumestrol diacetate daily. At 30 and 100 μg per week, estradiol benzoate reduced food consumption and growth, depressed testicle weights, and caused enlargement of the adrenal glands. Coumestrol diacetate had no inhibitory effects on food intake or growth in either the normal or castrate animals. Testicle and adrenal weights remained in the normal range. Liver cholesterol in the estradiol-treated rats appeared reduced, with no change in liver phospholipids. A definite lipotropic action by the hormone was noted when glyceride lipid was calculated. Plasma cholesterol and phospholipid were increased considerably above nontreated controls. At the three levels of coumestrol that were fed, however, no changes such as those produced by estradiol were apparent, although a suggestion of activity at the 5-mg dose was noted in the small rise in plasma cholesterol. From the results, it seemed possible that the amount of coumestrol diacetate fed was insufficient to produce an estrogenic response. Consequently, a second experiment was performed in which 10 and 15 mg of coumestrol were fed daily. The results were compared with those of rats started at the same time and which received injections of 15 and 30 μg of estradiol benzoate weekly. As shown in table 2, a level of 15 mg of coumestrol diacetate daily had no obvious effect on the indices measured, when compared with the changes produced by the low level of estradiol benzoate.

DISCUSSION

The lack of any estrogenic effect by coumestrol diacetate was unexpected. Since castrate animals showed no more response than normal rats, antagonism by endogenous androgen of testicular origin could not account for the inactivity. Previous work had shown that 0.5 mg of coumestrol, when fed to mice over a 7-day period, produced a strong uterine response (Bickoff, '57). Three milligrams of coumestrol diacetate fed similarly to weanling female rats gave a similar response.[3] If the results from the uterine assay may be extrapolated to the adult animal, it seems unlikely that a dose of 15 mg of coumestrol daily should be below an effective level. In the experiments presented in this study, the highest dosage used was at least 35 times the quantity effective in the young rat and 200 times that shown to affect the immature mouse. The diacetate of coumestrol is apparently well utilized, and exerts its physiological effects to the same degree as the nonacetylated material (Bickoff et al., '60). Therefore, the absence of any physiological activity that might be associated with estrogenicity of the compound in the male rat suggests that coumestrol may be either rapidly degraded by the liver in the adult rat, as certain other estrogens are (Shipley et al., '50), or, perhaps that the material has only a specific uterine effect in the rat without the usual influence on other organs and metabolic systems.

In contrast with the estrogen genistein (4′, 5, 7-trihydroxyisoflavone), which has been shown to be quite toxic in the mouse (Matrone, '56), coumestrol diacetate apparently exerts no deleterious effects on food consumption or growth and does not adversely affect testicular or adrenal weights.

It has been suggested that plant estrogens may be responsible for certain favor-

[3] Personal communication, A. L. Booth, Western Regional Research Laboratory, Albany, California.

TABLE 1

Growth, organ weights and liver and plasma lipids of rats given estradiol benzoate or fed 1, 3, or 5 mg of coumestrol diacetate daily

Diet[2]	Condition[3]	Food eaten per day	Final weight	Testicle weight	Adrenal weight	Liver[1]				Plasma	
						Cholesterol	Total lipid	Phospholipid	Glyceride[4]	Cholesterol	Phospholipid
		gm	*gm*	*gm/100 gm body weight*	*mg/100 gm body weight*	%	%	%	%	*mg/100 ml*	*mg/100 ml*
Control basal (no cholesterol)	C	14.6 ± 0.4[5]	248 ± 6	—	19.0 ± 0.1	0.29 ± 0.02	3.6 ± 0.5	2.9 ± 0.1	0.5 ± 0.2	43.7 ± 3.1	97.8 ± 3.2
Basal	N	15.8 ± 0.5	256 ± 6	0.94 ± 0.08	16.2 ± 0.8	2.41 ± 0.30	19.8 ± 2.9	2.4 ± 0.2	15.0 ± 2.4	45.6 ± 1.7	99.6 ± 3.3
Basal	C	14.7 ± 0.5	253 ± 4	—	18.1 ± 0.8	2.79 ± 0.25	15.6 ± 2.6	2.6 ± 0.4	10.2 ± 2.8	39.7 ± 3.7	92.2 ± 3.9
Basal + 30 μg estradiol/week[6]	N	14.2 ± 0.8	222 ± 1	0.76 ± 0.12	29.4 ± 1.5	1.76 ± 0.22	11.2 ± 1.6	3.0 ± 0.1	6.4 ± 1.4	57.9 ± 3.2	120.6 ± 6.9
Basal + 30 μg estradiol/week	C	14.5 ± 0.5	221 ± 1	—	27.6 ± 0.7	1.70 ± 0.37	9.0 ± 0.6	3.0 ± 0.1	4.3 ± 0.5	56.8 ± 7.8	124.4 ± 14.1
Basal + 100 μg estradiol/week	N	13.4 ± 0.6	209 ± 1	0.64 ± 0.12	26.5 ± 1.8	1.84 ± 0.49	10.7 ± 2.2	2.5 ± 0.3	6.4 ± 2.6	69.7 ± 10.9	154.0 ± 14.1
Basal + 100 μg estradiol/week	C	12.7 ± 1.0	211 ± 6	—	30.0 ± 2.6	2.22 ± 0.50	9.9 ± 1.6	2.9 ± 0.1	4.8 ± 1.1	93.0 ± 7.6	115.9 ± 12.7
Basal + 1 mg coumestrol/day	N	14.4 ± 0.3	246 ± 10	1.00 ± 0.03	15.9 ± 1.6	2.72 ± 0.14	14.4 ± 1.0	2.8 ± 0.3	8.9 ± 3.0	33.0 ± 0.7	104.0 ± 10.4
Basal + 1 mg coumestrol/day	C	14.1 ± 0.3	246 ± 2	—	19.8 ± 0.8	2.68 ± 0.27	14.7 ± 2.0	2.9 ± 0.1	9.1 ± 1.5	42.9 ± 2.3	98.0 ± 12.3
Basal + 3 mg coumestrol/day	N	15.6 ± 0.1	258 ± 4	0.96 ± 0.03	15.6 ± 0.3	2.85 ± 0.18	16.8 ± 1.8	2.8 ± 0.3	11.1 ± 1.8	32.6 ± 4.1	81.5 ± 7.1
Basal + 3 mg coumestrol/day	C	14.2 ± 1.0	239 ± 13	—	19.2 ± 0.8	2.35 ± 0.43	11.9 ± 2.5	2.8 ± 0.1	6.7 ± 2.1	36.4 ± 8.8	97.6 ± 4.0
Basal + 5 mg coumestrol/day	N	15.6 ± 0.3	259 ± 15	0.91 ± 0.04	17.8 ± 1.2	2.19 ± 0.37	13.1 ± 1.4	2.9 ± 0.2	8.0 ± 1.0	53.4 ± 7.1	107.0 ± 8.5
Basal + 5 mg coumestrol/day	C	15.4 ± 0.2	259 ± 6	—	18.4 ± 0.9	2.47 ± 0.33	13.4 ± 1.4	2.9 ± 0.1	8.0 ± 1.0	53.1 ± 4.6	102.0 ± 4.6

[1] Values expressed on wet weight basis.
[2] Basal diet indicates diet containing 1% of cholesterol; 5 or more rats in all groups.
[3] N indicates normal; C indicates castrate.
[4] Glyceride fraction obtained by subtracting cholesterol and phospholipid from total lipid.
[5] Standard error of the mean.
[6] Estradiol benzoate in sesame oil injected three times weekly; coumestrol diacetate was fed mixed in the diet.

TABLE 2

Growth, organ weights and liver and plasma lipids of rats given estradiol benzoate or fed 10 and 15 mg of coumestrol diacetate daily

Diet[1]	Condition[2]	Food eaten per day	Final weight	Testicle weight	Adrenal	Liver[3]				Plasma	
						Cholesterol	Total lipid	Phospholipid	Glyceride[4]	Cholesterol	Phospholipid
		gm	*gm*	*gm/100 gm body weight*	*mg/100 gm body weight*	%	%	%	%	*mg/100 ml*	*mg/100 ml*
Control basal (no cholesterol)	N	14.7 ± 1.1[5]	272 ± 4	0.72 ± 0.01	15.1 ± 0.9	0.51 ± 0.11	6.5 ± 0.6	3.1 ± 1.5	2.9 ± 0.6	52.9 ± 4.6	46.6 ± 9.1
Basal	N	14.5 ± 0.7	264 ± 6	0.83 ± 0.04	14.8 ± 0.5	2.60 ± 0.20	16.2 ± 1.3	2.7 ± 0.1	10.9 ± 0.2	45.6 ± 4.0	47.5 ± 8.3
Basal	C	12.9 ± 1.0	253 ± 5	—	14.9 ± 0.6	3.34 ± 0.50	17.5 ± 3.0	2.7 ± 0.1	11.5 ± 2.9	55.3 ± 1.5	45.7 ± 7.9
Basal + 15 μg estradiol/week[6]	N	11.2 ± 0.5	221 ± 4	0.49 ± 0.01	25.2 ± 0.3	3.94 ± 0.68	12.9 ± 2.3	2.7 ± 0.1	7.3 ± 1.2	102.2 ± 21.6	56.2 ± 8.8
Basal + 15 μg estradiol/week	C	12.3 ± 0.5	224 ± 4	—	25.7 ± 0.9	3.31 ± 0.50	11.6 ± 1.4	2.8 ± 0.0	5.5 ± 0.7	138.6 ± 3.7	66.1 ± 8.8
Basal + 30 μg estradiol/week	N	10.7 ± 0.3	205 ± 7	0.63 ± 0.09	26.8 ± 0.6	3.86 ± 0.62	10.5 ± 1.2	3.0 ± 0.1	3.6 ± 1.4	103.4 ± 22.9	62.6 ± 16.4
Basal + 30 μg estradiol/week	C	11.3 ± 0.3	212 ± 3	—	28.0 ± 1.1	3.15 ± 0.59	11.0 ± 1.2	3.0 ± 0.1	4.8 ± 0.5	100.0 ± 10.9	51.5 ± 7.5
Basal + 10 mg coumestrol/day	N	13.5 ± 0.1	259 ± 10	0.93 ± 0.03	16.4 ± 1.0	3.27 ± 0.20	16.2 ± 4.1	2.7 ± 0.1	10.2 ± 1.4	47.5 ± 7.4	41.5 ± 7.7
Basal + 10 mg coumestrol/day	C	13.2 ± 0.6	267 ± 10	—	18.5 ± 0.8	3.34 ± 0.24	15.5 ± 1.2	2.7 ± 0.1	9.5 ± 1.2	53.9 ± 4.6	20.2 ± 3.4
Basal + 15 mg coumestrol/day	N	13.6 ± 0.2	265 ± 4	0.96 ± 0.04	16.5 ± 0.5	2.80 ± 0.18	17.0 ± 1.2	2.8 ± 0.1	11.4 ± 1.4	53.4 ± 8.6	—

[1] Basal diet indicates diet containing 1% of cholesterol; 5 or more rats in all groups.

[2] N indicates normal; C indicates castrate.

[3] Values expressed on wet weight basis.

[4] Glyceride fraction obtained by subtracting cholesterol and phospholipid from total lipid.

[5] Standard error of the mean.

[6] Estradiol benzoate in sesame oil injected three times weekly; coumestrol diacetate was fed mixed in the diet.

able effects on milk production seen in grazing animals.[4] Although the present results indicate that coumestrol exerts no obvious estrogenicity in the male rat, there is the possibility that polygastric animals, such as sheep and cows, could derive some benefit from ingestion of the material.

The lipotropic action of estrogens on fatty livers induced by diet, has been observed by others (György et al., '47; Plagge et al., '58). Miller and Ellis ('60) have shown that, with choline-deficient fatty livers, diethylstilbestrol reduced only the nonphosphorus-containing lipid fraction of the liver. A similar response to estradiol appears to occur in the cholesterol-induced fatty liver. Although liver cholesterol appeared to be reduced in the first experiment, no such change was observed in the second experiment. The liver glyceride fraction, however, was definitely reduced and, regardless of the dose of estradiol benzoate injected, the effect on this liver lipid fraction and plasma cholesterol was quite similar. The degree of response did not seem to be related to the amount of estrogen administered. Plasma cholesterol response to the estrogen was much less than that observed by Okey and Lyman ('56) who administered 50 and 150 μg of estradiol benzoate per week. The difference in response obtained in the two experiments seems to be associated more with the type of fat fed than with the hormone dose. In the present experiment, corn oil was substituted for the lard[5] used by Okey and Lyman. Others have shown that corn oil effectively reduces plasma cholesterol in cholesterol-fed rats and chicks (Avigan and Steinberg, '58; Jones et al., '56) and in some unknown way prevents the rise in cholesterol esters observed in cholesterol-supplemented female rats fed certain fats or oils (Okey et al., '59).

The results presented in table 1 for plasma phospholipids are comparable with those reported by others, and show an increased response as does the cholesterol, to estrogen treatment. The phospholipid values in table 2, however, were obtained from a petroleum ether extract of the primary alcohol:ether extract after evaporation to dryness *in vacuo*. This treatment reduced the apparent phospholipid content of the plasma by nearly half, but had no noticeable influence on cholesterol. A similar re-extraction of liver lipids gave almost complete recovery of lipid-soluble phosphorus; hence, the effect appears to be characteristic of the plasma lipoid phosphorus compounds. Ellis and Maynard ('37) reported that the primary alcohol: ether extract from bovine plasma contained phosphorus other than phospholipid, and suggested extraction into petroleum ether in order to purify it. Kirk et al. ('34) recommended a similar procedure. On the other hand, Egsgaard ('48) and Zilversmit and Davis ('50) considered the primary alcohol:ether extract of dog or human plasma to contain only phospholipid phosphorus. Since the petroleum ether insoluble phosphorus fraction comprises such a large proportion of what generally is accepted as "phospholipid," it seems advisable to investigate it further. Investigations on the major constituents of this material are being made.

SUMMARY

Increasing levels of the diacetate of coumestrol, an estrogenic substance isolated from clover, were compared with estradiol benzoate, in the normal and castrate male rat, for their effect on food intake, growth, testicle and adrenal size, and liver and plasma lipid distribution. In both normal and castrate groups, injected estradiol benzoate (15, 30, or 100 μg per week) produced depressed growth, testicular and adrenal changes, a rise in plasma cholesterol, and a lipotropic effect on the glyceride fraction of the cholesterol-induced fatty liver. No comparable changes were produced with the oral administration of 1, 3, 5, 10, and 15 mg of coumestrol diacetate daily. It appears, therefore, that although coumestrol has a definite estrogenic effect on the uterus of the young female rat, the compound is inactive and apparently nontoxic for the adult male animal.

ACKNOWLEDGMENTS

The authors wish to thank Makiko Shinoda and Elaine Ranker Monson for

[4] Pope, G. S. 1954 The importance of pasture plant oestrogens in the reproduction and lactation of grazing animals. Dairy Sci. Abstr., *16*: 334.

[5] Primex, Procter and Gamble, Cincinnati.

technical assistance in certain aspects of the work. Appreciation is also expressed to Dr. Ruth Okey for her advice on some of the analytical methods.

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The Effects of Chain Length on the Metabolism of Saturated Fatty Acids by the Rat[1,2]

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Previous investigators have shown that the chain length (Rittenberg et al., '37; Geyer et al., '48, '51; Weinman et al., '50; Lossow and Chaikoff, '55), the form of fatty acid and route of its administration (McCalla et al., '57; Lossow and Chaikoff, '55) and the nutritional state of the animal (Bragdon, '58) will affect the rates of oxidation of fatty acids to CO_2. In the experiments reported here, the effects of chain length upon the rates of metabolism of four C^{14}-carboxyl-labeled saturated fatty acids (butyric, caprylic, lauric, palmitic) were studied. In each case the tagged fatty acid was administered by stomach tube in the form of randomly mixed, synthetic triglycerides of identical chemical composition.[4]

MATERIALS AND METHODS

1. Preparation of C^{14}-labeled fats. The four C^{14}-labeled triglycerides (C_4, C_8, C_{12}, and C_{16}) were prepared by reacting the appropriate acyl chloride with the proper amount of glycerol in the presence of pyridine. The resulting C^{14}-tagged triglycerides were purified by alkaline-wash removal of unreacted acyl chloride. Chemical analyses were not obtained because of limited amounts of sample available.

Larger amounts of C_4, C_8, C_{12}, and C_{16} untagged triglycerides, for use in diluting the tagged triglycerides, were prepared by standard interesterification procedures. The interesterification procedure involves reacting methyl esters with glycerol in vacuo at 100°C using benzyl trimethyl ammonium hydroxide as a catalyst. When necessary, residual hydroxyl values were reduced by reaction of the product with acid chloride in the presence of pyridine. The trilaurin and tripalmitin were purified by crystallization, tricaprylin by deodorization, and tributyrin by distillation. Analyses for the untagged glycerides are given in table 1.

In order to obtain a triglyceride identical in all respects except that the C_4 acids were tagged in one sample, the C_8 in another, et cetera, the tagged and untagged C_4, C_8, C_{12} and C_{16} triglycerides were randomized with safflower oil. The safflower oil contained 11.2% of saturated, 7.3% of oleic, 75.3% of linoleic and 0.5% of linolenic acids, and gave the following constants: acetyl value, 0.15; iodine value, 144.7; saponification value, 192.4. The random rearrangement was carried out by agitation of the fat mix with 0.5% sodium methoxide catalyst in xylene suspension at 70 to 80°C for 45 minutes. The catalyst was neutralized with 20 ml of 10% phosphoric acid. The randomized mix was dissolved in ethyl ether, water-washed, dried over sodium sulfate, and the ether removed with N_2 agitation on a steam bath. The triglyceride was then steam-deodorized at 130 to 140°C for one hour at 1 mm pressure. Compositions before randomization, yields, and specific activities are shown in table 2.

The success of the randomization was evidenced by the fact that the unrandomized mixes solidified above 30°C, whereas

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[2] The Nutrition Foundation contributed to the partial support of this investigation.

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[4] Kindly prepared by the Procter and Gamble Company, Cincinnati.

TABLE 1

Analysis of untagged triglycerides

	Acetyl value	Hydroxyl value	Saponification value	Saponification value (theory)
Tributyrin	0.27	0.5	537	556
Tricaprylin	6.50	26.4	351	357
Trilaurin	0.47	13.6	262	263
Tripalmitin	0.50	5.8	210	209

TABLE 2

Composition of triglycerides prior to randomization[1]

	Sample BUT	Sample CAP	Sample LAU	Sample PAL	Sample CONTROL
	gm	*gm*	*gm*	*gm*	*gm*
Safflower oil	60.0	60.0	60.0	60.0	240.0
C^{14}-labeled tributyrin	12.3				
C^{14}-labeled tricaprylin		6.5			
C^{14}-labeled trilaurin			5.8		
C^{14}-labeled tripalmitin				6.2	
Tributyrin	2.7	15.0	15.0	15.0	60.0
Tricaprylin	15.0	8.5	15.0	15.0	60.0
Trilaurin	15.0	15.0	9.2	15.0	60.0
Tripalmitin	15.0	15.0	15.0	8.8	60.0
Totals, gm	120.0	120.0	120.0	120.0	480.0
Yield, gm	119.5	116.8	118.1	116.1	454.9
Yield, %	99.8	96.3	98.4	96.8	94.8
Specific activities mc/gm	0.082	0.073	0.065	0.063	0.000

[1] α-Tocopherol, 0.1%, was added to each sample.

all of the randomized samples solidified at 9°C and were liquid at 21°C. The four C^{14}-tagged randomized blends were made to 120 gm by addition of untagged mix (control sample). α-Tocopherol (0.1%) was added to retard oxidation and the samples were kept frozen until used.

2. *Experimental procedures.* Albino male rats (Sprague-Dawley strain), weighing approximately 160 gm, were fasted overnight, and then were administered 0.8 ml (0.75 gm) of a tagged triglyceride by stomach tube while under very mild ether anesthesia. Each animal was immediately placed in a Delmar metabolism cage ventilated with CO_2-free air maintained at 25° to 28°C, and the expired CO_2 was collected in 4N sodium hydroxide (CO_2-free). The alkali solution was replaced at intervals and the total CO_2 was precipitated as $BaCO_3$. The amount of radioactivity in each sample of $BaCO_3$ was determined by counting at infinite thickness using a Tracerlab TGC-14, thin window, flow counter. The amount of radioactivity exhaled in the breath during the period represented by the sample was then calculated.

During the 48-hour collection period the animal was maintained with a purified diet fed ad libitum (table 3). The feces and

TABLE 3

Composition of synthetic-type diet

	Content
	gm/100 gm
Casein	30.0
Cystine	0.3
Cornstarch	49.8
Salt mixture[1]	4.0
Vitamin mixture[2]	0.9[3]
Control fat (untagged)	15.0

[1] USP XIV, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Vitamin mixture: (mg/100 gm diet) thiamine·HCl, 0.4; riboflavin, 0.5; pyridoxine·HCl, 0.4; niacin, 2.0; Ca pantothenate, 2.0; inositol, 200.0; biotin, 0.03; folic acid, 0.25; choline Cl, 300.0; ascorbic acid, 10.0; cobalamin (B_{12}), 0.005; menadione, 0.3; *p*-aminobenzoic acid, 10.0; α-tocopherol, 10.0; also vitamin A, 1250 I.U. and vitamin D, 125 I.U.

[3] The vitamin mixture was brought to 0.9 gm with cornstarch.

urine voided during this time were collected. At the termination of the 48-hour test period, the contents of the colon were removed.

The radioactivity in the urine, combined feces and colon contents was determined by the combustion technique described by Kesten et al. ('37), the CO_2 evolved being collected in alkali, precipitated as $BaCO_3$ and counted in the same manner as the breath samples. Four rats were fed palmitic-1-C^{14} acid glyceride and groups of three rats were fed the other tagged glycerides. The animal weights ranged between 152 and 168 gm.

RESULTS AND DISCUSSION

The average percentage of radioactivity recovered from the colon and the feces of rats 48 hours after the stomach-tube feeding of each of the C^{14}-tagged saturated fatty acid glycerides is shown in table 4. These data represent unabsorbed activity. The absorption of the shorter-chain fatty acids approached 100%, whereas that of the palmitic acid was definitely lower (86.5%). This latter value is significantly different from the values obtained for the other three acids at the 95% confidence level (Snedecor, '56).

Bloom et al. ('50, '51) found that the percentage of C^{14} absorbed from the intestine of rats after decanoic-1-C^{14}, lauric-1-C^{14}, and palmitic-1-C^{14} acids which had been given enterally was 95 to 99%, 95 to 98% and 86 to 93%, respectively. The results in table 4 are consistent with these findings.

The percentage of radioactivity recovered, on a per hour basis, in the breath of rats during each collection period is shown in figure 1. The values have been corrected for the radioactivity recovered in the colon and feces, and thus represent percentage of radioactivity absorbed. The rates of excretion of radioactivity after administration of butyric-1-C^{14} or caprylic-1-C^{14} acids were significantly higher (at the 95% level of confidence) than that of the longer-chain acids during 4 of the 5 collection periods covering the first 4 hours. The values for samples collected during the third period showed large within-group variation and were not significantly different. The values of all samples collected between 4 to 8 hours were not significantly different, although the lauric acid group showed a higher $C^{14}O_2$ excretion rate than the other three acids. The rate during 8 to 12 hours was highest for palmitic acid and this was significantly higher than the butyric acid and caprylic acid groups, but not the lauric acid group. The lauric acid rate was significantly greater than the rates of butyric or caprylic acid, but not palmitic acid, during the 12- to 16-hour period. No significant differences were noted during the 16- to 24-hour and 24- to 48-hour periods. The results show that butyric and caprylic acids were metabolized at the same rate throughout the 48-hour period. The maximum hourly respiration rates of the $C^{14}O_2$, and the time intervals at which these maxima occurred were as follows: butyric acid (17.0%, 1 to 2 hours), caprylic acid (17.7%, 1 to 2 hours), lauric acid (7.0%, 2 to 4 hours) and palmitic acid (4.8%, 4 to 8 hours).

TABLE 4

Average percentage of radioactivity recovered in colon and feces of rats during 48 hours following stomach tube feeding of C^{14}-tagged glycerides

Labeled fatty acid fed	Carbon chain length	Average radioactivity recovered
		%
Butyric-1-C^{14} acid	4	0.5
Caprylic-1-C^{14} acid	8	0.6
Lauric-1-C^{14} acid	12	1.8
Palmitic-1-C^{14} acid	16	13.5

The absorption of these fatty acids occurred mostly within 4 to 8 hours (fig. 1), and they were either metabolized or deposited in the tissues within 12 hours. Thus, the activity recovered in the breath after about 16 hours represents that which was first deposited in the tissues and later remobilized and metabolized.

Bloom et al. ('50, '51) have reported that when long-chain (14C, 15C, 16C, 18C) fatty acids, or their triglycerides, are dissolved in corn oil and fed to rats, they are absorbed almost exclusively via the lymph. In contrast the major portion of the shorter-chain (10C, 12C) fatty acids are absorbed directly into the blood stream. The results reported here may be explained on the basis that the shorter-chain

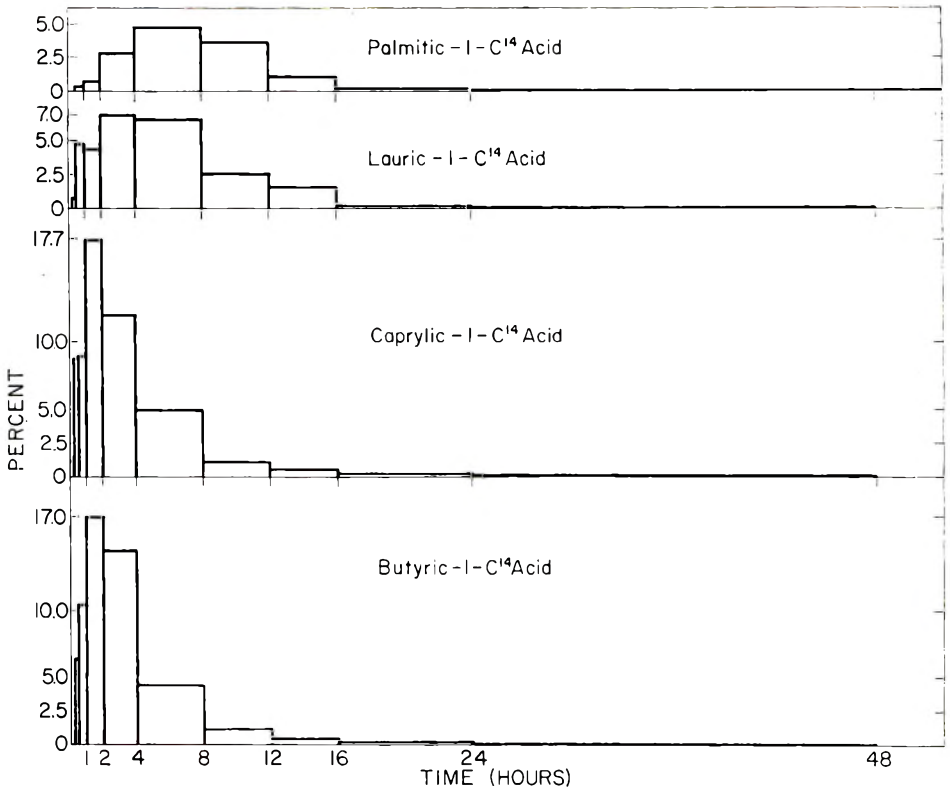

Fig. 1 Average percentage of radioactivity recovered per hour in the breath of rats administered C^{14}-tagged fatty acid glycerides.

TABLE 5

Cumulative percentage of the administered radioactivity recovered as $C^{14}O_2$ in the breath of rats after stomach tube feeding of C^{14}-tagged glycerides

Time interval	Butyric-1-C^{14} acid	Caprylic-1-C^{14} acid	Lauric-1-C^{14} acid	Palmitic-1-C^{14} acid
	Av. ± S.D.	Av. ± S.D.	Av. ± S.D.	Av. ± S.D.
	%	%	%	%
0–15 min.	0.4 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.0 ± 0.0
0–30 min.	2.0 ± 0.1	3.9 ± 2.6	0.2 ± 0.2	0.0 ± 0.0
0–60 min.	7.2 ± 4.5	8.4 ± 0.9	2.6 ± 2.6	0.1 ± 0.0
0–2 hours	24.2 ± 6.5	26.1 ± 3.4	7.0 ± 2.0	0.9 ± 0.5
0–4 hours	53.5 ± 8.0	50.3 ± 4.8	20.9 ± 0.4	6.5 ± 1.8
0–8 hours	70.9 ± 3.4	70.1 ± 3.0	47.6 ± 5.7	25.5 ± 5.2
0–12 hours	75.8 ± 4.4	74.4 ± 2.4	57.9 ± 2.8	40.3 ± 4.3
0–16 hours	77.6 ± 1.6	76.5 ± 3.0	64.2 ± 1.0	45.1 ± 2.7
0–24 hours	79.3 ± 1.8	77.9 ± 3.1	66.3 ± 1.6	46.8 ± 2.4
0–48 hours	81.7 ± 2.0	80.3 ± 4.3	68.6 ± 2.1	49.5 ± 3.2

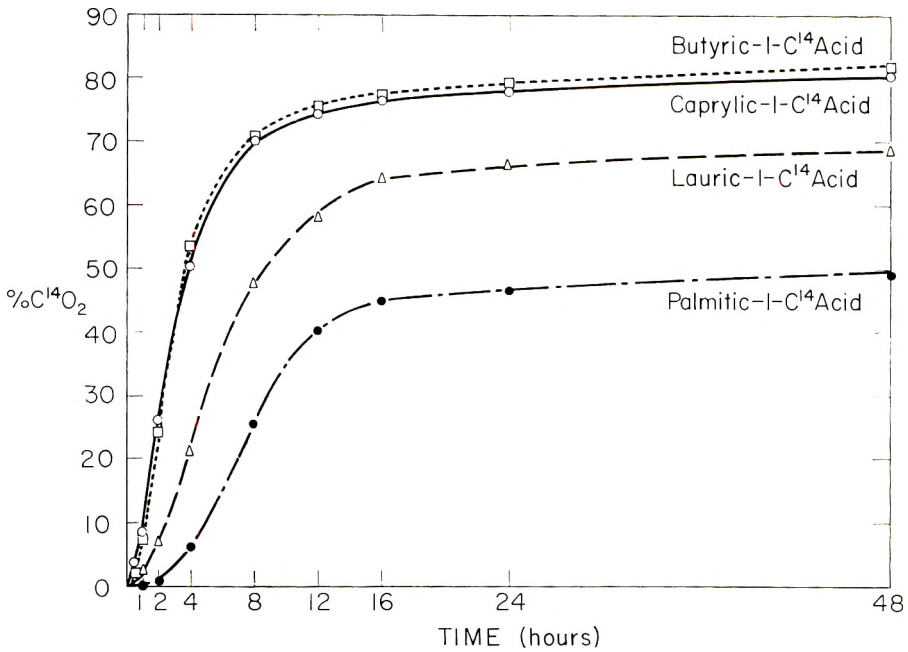

Fig. 2 Average cumulative percentage of recovery of $C^{14}O_2$ from the breath of rats administered C^{14}-tagged fatty acid glycerides.

acids were metabolized more rapidly partly because they were transported to the liver more rapidly via the portal system rather than the lymphatic system.

Gould et al. ('49) have presented evidence which indicates that all the carbons of a fatty acid molecule are converted to CO_2 at about the same time. This would mean that $C^{14}O_2$ recovered in the breath represents the complete conversion of an administered tagged fatty acid.

The cumulative radioactivity recovered in the breath of rats during the 48 hours following the stomach tube feeding of tagged triglycerides is shown in table 5 and figure 2. The $C^{14}O_2$ expired following the administration of either butyric-1-C^{14} or caprylic-1-C^{14} acids was higher at all times than that of lauric-1-C^{14} or palmitic-1-C^{14} acids. The palmitic acid values were significantly lower than those of the other three groups at the 95% confidence level. Similarly, the lauric acid values were significantly lower than those of butyric and caprylic acid, which were the same.

It is evident that the short-chain fatty acids (butyric and caprylic) are metabolized more rapidly than the longer-chain fatty acids (lauric and palmitic).

The radioactivity of the urine excreted by these rats during the 48-hour test period ranged between 0.5 and 1.5% of the administered dose, with no evidence of differences between groups. Thus, the absorbed fatty acids appear to have been either metabolized to carbon dioxide and water, or deposited in the tissues with little loss by excretion in the urine.

SUMMARY AND CONCLUSIONS

Four randomly mixed triglycerides containing either butyric-1-C^{14}, caprylic-1-C^{14}, lauric-1-C^{14} or palmitic-1-C^{14} acid were administered to young adult rats by stomach tube. Radioactivity measurements were made on samples of breath collected at timed intervals during 48 hours and on samples of urine, feces and colon contents collected during 48 hours.

Activity measurements on fecal samples indicated that the efficiency of absorption of palmitic acid (86%) from the intestinal tract was significantly lower than that of the three shorter-chain fatty acids (98 to 100%).

The rates of metabolism of butyric and caprylic acids to respired $C^{14}O_2$ were highest and similar, that of lauric acid was

intermediate, and that of palmitic acid was lowest.

The differences observed in the expired $C^{14}O_2$, an end product of fatty acid metabolism, were probably due, at least in part, to differences in the route of transport of the fatty acids to the tissues following absorption from the gastrointestinal tract. The rapid excretion of $C^{14}O_2$ observed when the shorter-chain fatty acids were fed indicates that they were oxidized instead of entering the fat depots.

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Alpha-Glycerophosphate and Lactic Dehydrogenase Activities in Tissues of Thiamine-Deficient Rats[1]

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At present it is difficult to interpret the thiamine deficiency syndrome entirely in terms of the biochemical defect of a decrease in thiamine pyrophosphate. The severity of the metabolic derangement and the peculiar tissue predilection associated with the deficiency are not in agreement with the degree of diminution in activity of such enzymes as pyruvic oxidase. In fact, despite considerable decrease in activity of this enzyme in induced deficiency, there remains activity almost adequate to meet the physiologic demands (Shils et al., '41). It has further been reported that the decrease is as much a loss of apoenzyme as of coenzyme (Oshima et al., '60) and that there is little change in the ability of the thiamine-deficient rat to oxidize C^{14}-labeled pyruvic and lactic acids (Jones and de Angeli, '60).

Similarly one can question that the decrease in activity of other thiamine pyrophosphate dependent enzymes (such as α-ketoglutaric dehydrogenase and transketolase) is primarily responsible for observed specific biochemical lesions. There are observations which cannot, at present, be interpreted on the basis of a decrease in thiamine pyrophosphate concentrations. Among these are the accumulation of methylglyoxal as a consequence of vitamin B_1 deficiency, which has been consistently reported (Salem, '54, '55), and the discrepancy in the deficiency syndromes provoked by oxythiamine or pyrithiamine. These latter differences prompted Woolley and Merrifield ('54) to propose a new role of thiamine beyond the presently known functions.

Recently a new thiamine derivative, thiamic acid, was isolated from the diphosphopyridine nucleotide (DPN)-dependent crystalline rabbit muscle α-glycerophosphate dehydrogenase.[3] Although the enzymatic function of this compound is not clear, it seemed of interest to investigate the effect of thiamine deficiency on this and other dehydrogenases. This paper describes the results of this investigation.

EXPERIMENTAL

Female Wistar rats with initial weights of around 100 gm were fed a commercial thiamine-deficient diet.[4] Thiamine and thiamine antimetabolites were administered by inclusion of the compounds in the drinking water, which was changed daily and supplied ad libitum.

To obtain material for enzymatic assay, the rats were decapitated and the tissues quickly excised and frozen until assay. When required, the organs were homogenized in a Potter-Elvehjem homogenizer in ice cold 0.154 M KCl. For muscle, a 10% homogenate was used, whereas for liver, a 20% homogenate was prepared. The latter was diulted ten-fold before assay.

α-Glycerophosphate dehydrogenase activity was assayed by following spectrophotometrically the reduction of the pyridine nucleotide in the following system in a final volume of 3.0 ml: disodium-DL-α-glycerophosphate, 20 μmoles; DPN, 2 μmoles; mercaptoethanol, 10 μmoles; and trishydroxymethyl - aminomethane buffer, pH 9.3, 300 μmoles. The reaction was initiated with enzyme. Lactic dehydrogenase was assayed similarly, but with the

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[2] Investigator in the Howard Hughes Medical Institute.
[3] van Eys, J. 1960 Thiamic acid, the nonprotein component of α-glycerophosphate dehydrogenase. Federation Proc., *19*: 26 (abstract).
[4] Nutritional Biochemicals Corporation, Cleveland.

omission of mercaptoethanol and substitution of lithium-DL-lactate for the glycerophosphate. For both enzymes one unit of activity is defined as an increase in optical density of 0.001 per minute under the conditions specified. Aldolase was assayed chemically (Taylor, '55).

Protein was estimated with the biuret test (Weichselbaum, '46), using crystalline ovalbumin as standard.

RESULTS

Growth of the experimental groups. Thiamine deficiency was induced by three devices: (1) omission of the vitamin from the diet, or by administration of either (2) oxythiamine or (3) pyrithiamine. The antimetabolites were fed in conjunction with a thiamine intake equivalent to half the amount required to counteract the level of antimetabolites: ratios of 50/1 for oxythiamine to thiamine and 2/1 for pyrithiamine to thiamine. This design was adopted to avoid a purely dietary deficiency in the rats fed the antimetabolites. In figure 1 is shown the growth obtained with the three levels of antimetabolites. At the higher level oxythiamine appears to be the more effective growth inhibitor, even though the effect at low concentrations of

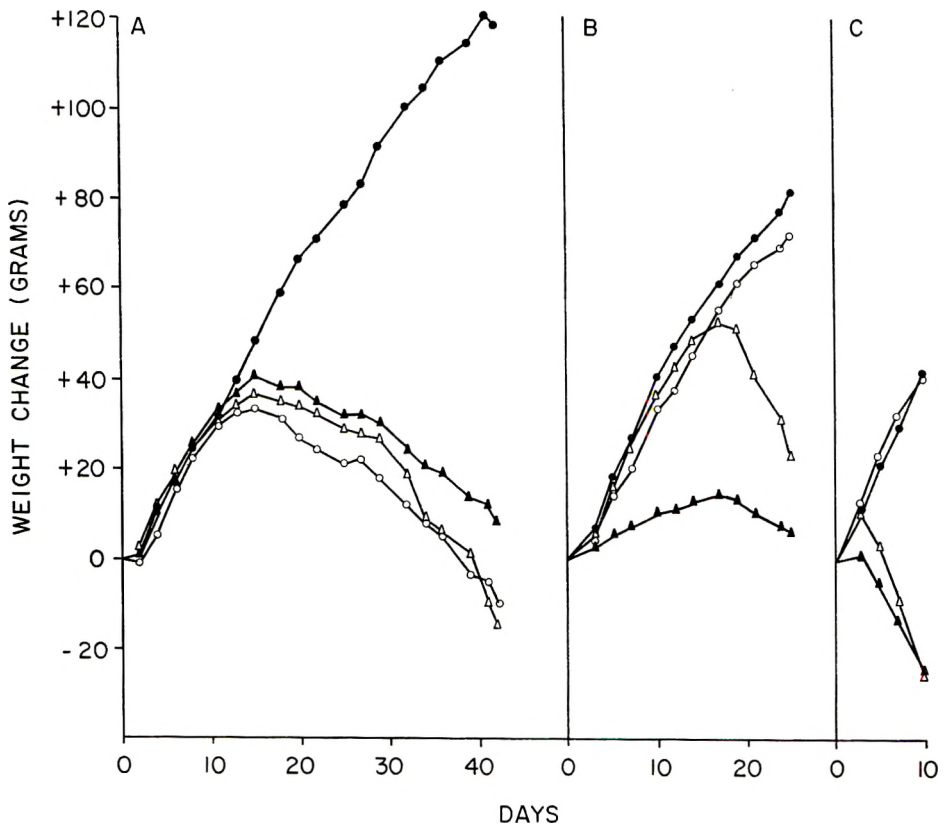

Fig. 1 Growth curve of thiamine-deficient animals treated with antimetabolites. Code: solid circles, control; open circles, animals receiving the dose of thiamine of the treated groups; closed triangles, oxythiamine-treated; open triangles, pyrithiamine-treated.

Group A Five micrograms of oxythiamine plus 0.1 μg of thiamine or 0.2 μg of pyrithiamine plus 0.1 μg of thiamine administered per milliliter of drinking water.

Group B Fifty micrograms of oxythiamine plus 1 μg of thiamine or 2 μg of pyrithiamine plus 1 μg of thiamine administered per milliliter of drinking water.

Group C Two-hundred-and-fifty micrograms of oxythiamine plus 5 μg of thiamine or 10 μg of pyrithiamine plus 5 μg of thiamine administered per milliliter of drinking water.

Control animals received at all times 10 μg of thiamine per milliliter of drinking water.

oxythiamine and pyrithiamine are comparable. Oxythiamine never provoked polyneuritis, however, whereas pyrithiamine did so at all levels. This was true even when the symptoms were produced acutely (table 1) and is in agreement with observations in the mouse (Eusebi and Cerecedo, '49).

α-Glycerophosphate dehydrogenase activity. The level of α-glycerophosphate dehydrogenase activity dropped markedly as a consequence of thiamine deficiency in skeletal muscle, liver and brain. The level of the enzyme in heart is too low to measure accurately. Some results obtained in dietary deficiency are shown in table 2.

The effect was not due to the diminished food intake of the thiamine-deficient animals, since paired feeding did not result in lowered activity of the enzyme. The decrease in activity is a relatively late effect, since animals sacrificed at the initial onset of weight loss did not show it.

Additional evidence that this decrease is a reflection of thiamine nutriture was that animals made deficient in 7 days with antimetabolites showed a similar decrease in activity (table 3). The effect of oxythiamine at lower concentrations was less than that occurring in either dietary- or pyrithiamine-induced deficiency. This is in sharp contrast with the effect on lactic dehydrogenase, as will be described later.

The decrease in activity was proportional to the level of either antimetabolite fed (table 3), and was not associated with a concomitant loss in liver protein concentration (table 2). The decrease did coincide with a marked loss in liver weight so that, expressed on a basis of total liver, there is an even more striking difference.

Lactic dehydrogenase activity. The lactic dehydrogenase activity also decreased in thiamine deficiency, but not as strikingly as the α-glycerophosphate dehydrogenase activity (30 vs. 50%). Peculiarly enough, however, even at low oxythiamine levels, which had little effect on α-glycerophosphate dehydrogenase activity, the lactic dehydrogenase activity decreased to barely detectable levels. (In liver, 20 units per mg is barely detectable.) Also in contrast with the α-glycerophosphate dehydrogenase activity, lactic dehydrogenase was lower in oxythiamine-treated rats than in rats treated with equivalent amounts of pyrithiamine. These points are also illustrated in table 3.

The effect of starvation on dehydrogenase activity. Although paired feeding did not abolish the effect of thiamine deficiency on those enzyme levels, it has been reported (Weber, '60) that lactic dehydrogenase decreases in starvation. To determine the magnitude of this effect, rats weighing about 250 gm were starved for 5 or 10 days. Control animals were fed a commercial laboratory chow.[5] The lactic dehydrogenase of the liver decreased about 30% (table 4), in agreement with the ob-

[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 1

Incidence of symptoms in thiamine-deficient rats

Group	Series	Days fed diet	Thiamine administered	Antimetabolite administered	No. rats/ group	Incidence of polyneuritis[1]	No. of deaths[1]
			μg/ml drinking water	*μg/ml drinking water*			
Control	600[2]	42	10	—	9	0	0
Restricted			0.1	—	9	5	2
Oxythiamine			0.1	5	9	0	0
Pyrithiamine			0.1	0.2	9	8	4
Control	800[3]	7	10	—	10	0	0
Restricted			5	—	3	0	0
Oxythiamine			5	250	9	0	1
Pyrithiamine			5	10	9	9	3

[1] The numbers designate the incidence out of the total.
[2] Group A in figure 1.
[3] Group C in figure 1.

TABLE 2

α-Glycerophosphate dehydrogenase activity of diet-induced thiamine-deficient rats[1]

Group[2]	No. animals assayed	Weight change	Liver			Muscle	
			Weight	Protein	α-Glycero-phosphate dehydrogenase	Protein	α-Glycero-phosphate dehydrogenase
		gm	*gm*	*mg/mg liver*	*units/mg liver*	*mg/mg muscle*	*units/mg muscle*
Control, ad libitum	6	+109[3,4] ± 13.6	—	0.38 ± 0.033	120[5] ± 78.0	0.053 ± 0.013	28.2[3] ± 10.4
Pair-fed control	11	+14[3] ± 17.8	—	0.37 ± 0.0245	79[5] ± 28.2	0.044 ± 0.003	31.2[3] ± 6.96
Deficient	11	−12 ± 14.4	—	0.33 ± 0.029	48 ± 35.3	0.068 ± 0.010	11.3 ± 8.83
Pair-fed control	8	+14[3] ± 20.3	6.3[5] ± 0.96	0.30 ± 0.094	63[5] ± 23.2	—	—
Deficient	8	−34 ± 9.5	4.0 ± 0.45	0.32 ± 0.044	37 ± 19.1	—	—
Control, ad libitum	9	+119[3] ± 19.0	8.4[3] ± 1.05	0.24 ± 0.046	57[3] ± 13.6	—	—
Deficient	7	−9.5 ± 25.3	3.6 ± 0.71	0.25 ± 0.046	27.5 ± 10.7	—	—

[1] Figures indicate ± standard deviation.
[2] Pair-fed animals are compared in paired variance.
[3] Difference statistically significant from the deficient group ($P < 0.01$).
[4] Difference statistically significant from pair-fed control group ($P < 0.01$).
[5] Difference statistically significant from the deficient group ($P < 0.05$).

servations reported by Weber. A similar drop occurred in α-glycerophosphate dehydrogenase but in neither case was the decrease as great as that occurring in thiamine deficiency (table 4).

DISCUSSION

Many factors enter into the interpretation of studies such as these. Although paired feeding equalized the food intake between deficient and nondeficient groups, it does not necessarily equalize the food utilization. In fact, thiamine-deficient animals almost always lose more weight than their pair-fed controls (table 2). One may postulate that the effects of thiamine deficiency include a "metabolic starvation." This interpretation cannot be disproven, but the effect of high levels of thiamine antimetabolites, where the weight loss is less than in acute starvation but the enzymatic effects are greater, argues in favor of some specific thiamine effect. Other points are in favor of this concept. A decrease in serum lactic dehydrogenase in humans after pyrithiamine treatment has been reported (Wendel, '60), yet these subjects were not acutely deficient. Several enzymes are not affected by thiamine deficiency (Terroine, '60). Furthermore, protein biosynthesis is possible in extreme thiamine deficiency as evidenced by the unimpaired ability of deficient animals to make antibodies (Axelrod and Hopper, '60). Also in this study aldolase was found to be unaffected.

The peculiar differential effect between oxythiamine on the one hand and pyrithiamine and dietary deficiency on the other, which was seen in this investigation, may well be a metabolic basis for the difference in the clinical syndrome which results from these forms of deficiency. It is tempting to speculate that the difference is partially the result of a differential effect of these antimetabolites on thiamic acid levels or activity. Such interpretations must await further investigation of the role of this compound. The data presented here do not shed light on the function of thiamic acid on α-glycerophosphate dehydrogenase. It has not been possible to activate a deficient muscle with a supernatant from a control muscle.

TABLE 3

Effect of oxythiamine and pyrithiamine on dehydrogenase activities[1]

Dietary history	No. animals assayed	Anti-metabolite administered[2]	Weight change at sacrifice[3]	Liver α-glycero-phosphate dehydrogenase[3]	Liver lactic dehydrogenase
		μg/ml drinking water	*gm*	*units/mg liver*	*units/mg liver*
Control,[4] ad libitum	9	—	+119 ±19.0	57 ±13.6	53 ±10.8
Deficient	7	—	− 9.5±25.3	27.5±12.2	37 ± 5.0[5]
Oxythiamine	9	5	+ 9 ±17.7	44.5±11.6[5,8]	23.5± 4.5[6,7]
	6	50	+ 6.5±12.5	37 ± 5.5	24.5± 3.1[6,8]
	8	250	− 24 ± 4.7	27 ± 9.1	19 ± 4.1[6,7]
Pyrithiamine	5	0.2	− 17 ± 8.3	39 ±12.2	—
	6	2	+ 27 ±13.2	33 ± 9.5	27.2± 7.35[5]
	6	10	− 25 ± 8.0	26 ±11.3	25 ± 6.5[6]

[1] Figures indicate ± standard deviation.
[2] The three levels of antimetabolites correspond to the series described in figure 1.
[3] All figures in these columns are significantly different from the control group at the $P < 0.01$ level except α-glycerophosphate dehydrogenase activity at 5 μg of oxythiamine where the significance is at the $P < 0.05$ level.
[4] The control group is representative of all the control groups included with each level of oxythiamine and pyrithiamine.
[5] Significantly different from the control group at a level of $P < 0.05$.
[6] Significantly different from the control group at a level of $P < 0.01$.
[7] Significantly different from the dietary deficient group at a level of $P < 0.02$.
[8] Significantly different from the dietary deficient group at a level of $P < 0.01$.

TABLE 4

Effect of complete starvation on dehydrogenase activities[1]

	No. animals	Weight change	Liver weight	Liver protein	Liver lactic dehydrogenase	Liver α-glycero-phosphate dehydrogenase
		gm	*gm*	*mg/mg liver*	*units/mg liver*	*units/mg liver*
Control	9	+18.4 ±5.15	8.4 ±0.98	0.43±0.089	32± 7.2	45 ±8.8
Starvation, 5 days	9	−46.1[2] ±5.09	4.9[2] ±0.56	0.36±0.068	23±10.3	30[2]±8.0
Starvation, 10 days	4	−82.3[2,3]±9.15	3.9[2,3]±0.21	0.41±0.050	28± 6.5	32[4]±7.6

[1] Figures indicate ± standard deviation.
[2] Significantly different from control at a level of $P < 0.01$.
[3] Significantly different from short starvation at a level of $P < 0.01$.
[4] Significantly different from control at a level of $P < 0.05$.

SUMMARY

Thiamine-deficient rats have a striking decrease in liver and muscle α-glycerophosphate dehydrogenase activity. When the deficiency was induced with oxythiamine or pyrithiamine the decrease in enzyme was proportional to the dose administered. At equivalent levels, oxythiamine was less effective than pyrithiamine.

For lactic dehydrogenase the decrease in activity was less pronounced in dietary deficiency but oxythiamine was more effective in lowering this enzyme concentration.

It is concluded that this represents a true effect of thiamine deficiency and not of starvation. The results are discussed in the light of the recently discovered thiamic acid.

ACKNOWLEDGMENTS

The technical assistance of Marlene J. Greene and Russell Root is gratefully acknowledged.

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Influence of Dietary Calcium, Phosphorus and Vitamin D_3 on Ca^{45}, P^{32} and Sr^{85} Uptake by Chicks[1]

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Hamilton ('47) reported that Sr^{89} and Sr^{90} are available in contaminated food and water and are deposited primarily in the skeleton. MacDonald and co-workers ('55) reported a clear-cut reduction of the Sr^{90} burden in rats from an increase of ingested calcium which became more pronounced when dietary phosphate was also increased. Ray et al. ('56) stated that a phosphorus-deficient diet seemed to cause selective demineralization of the rat skeleton resulting in a definite acceleration of strontium removal from the bone. Palmer et al. ('58) reported that a 20-fold increase in dietary calcium decreased skeletal Sr^{89} deposition twofold and that effects of similar magnitude were obtained by varying the phosphate and carbonate levels in the rat diet. Migicovsky and Emslie ('50) showed that vitamin D increased the uptake of orally administered Ca^{45} by tibiae of chicks but had no effect on intramuscularly administered Ca^{45}. Mraz and Bacon ('60) demonstrated with rats that vitamin D_3 increased Sr^{89} absorption from the gut, decreased fecal and increased urinary Sr^{89}, provided that either calcium or strontium supplements were present in the diet. Patrick and Bacon ('57) found that vitamin D increased the efficiency of utilization of Ca^{45} and Sr^{89} in both the organic and inorganic forms in both the rat and the chick.

The present study was devised to measure the effect of varying levels of vitamin D_3, calcium and inorganic phosphorus on Ca^{45}, P^{32} and Sr^{85} deposition in tibiae of chicks.

EXPERIMENTAL

Two-hundred-and-twenty-five day-old New Hampshire cockerel chicks were divided into 15 groups of 15 chicks and fed ad libitum the natural feedstuffs basal diet shown in table 1, with the levels of calcium, inorganic phosphorus and vitamin D_3 adjusted to those shown in table 2. Organic phosphorus remained rather constant at 0.26% of the diet. The sources for calcium and inorganic phosphorus were calcium carbonate and potassium acid phosphate, respectively. Adjustments for minerals and vitamin D_3 were made at the expense of corn. After following these dietary regimens for three weeks, the following doses were orally administered to 10 cockerels from each group: 20 μc of Ca^{45} (containing 0.8 mg of cal-

TABLE 1

Basal diet

	%
Corn[1]	53.38
Soybean oil meal (44%)[1]	33.00
Methionine	0.20
NaCl	0.50
$MnSO_4 \cdot H_2O$	0.02
Supplements[2]	0.90
Dietary variables plus corn	12.00

[1] Thirty per cent of the phosphorus of corn and soybean oil meal or 0.11% of the diet was considered as non-phytin phosphorus and, therefore, as part of the inorganic phosphorus (National Research Council, '54).

[2] Supplements added per pound of diet: (in milligrams) menadione, 0.20; vitamin B_{12}, 0.04; riboflavin, 2; Ca pantothenate, 3; niacin, 4; choline chloride, 300; KI, 0.65 (0.5 mg I); $ZnCO_3$, 22.5; and α-tocopheryl succinate, 10 I.U.; vitamin A, 2000 I.U.

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TABLE 2

Levels of calcium, inorganic phosphorus and vitamin D_3 observed in experimental diets

Group	Ca	P	Vitamin D_3
	%	%	*I.C.U./ pound diet*
1	1.0	0.6	100
2	2.0	0.6	100
3	1.0	1.2	100
4	2.0	1.2	100
5	1.0	0.6	10,000
6	2.0	0.6	10,000
7	1.0	1.2	10,000
8	2.0	1.2	10,000
9	1.5	0.9	1,000
10	0.5	0.9	1,000
11	2.5	0.9	1,000
12	1.5	0.3	1,000
13	1.5	1.5	1,000
14	1.5	0.9	10
15	1.5	0.9	100,000

cium as the chloride), 30 μc of P^{32} (containing 0.0003 mg of phosphorus as the phosphate) and 5 μc of Sr^{85} (containing 0.0005 mg of strontium as the nitrate). The remaining 5 cockerels received this dosage intraperitoneally. All birds were sacrificed 48 hours after dosing and a tibia removed from each. Tibiae were dry ashed at 650°C and dissolved in hydrochloric acid. Ca^{45} and P^{32}, both beta emitters, were counted with an end-window Geiger-Müller tube being separated before counting by precipitating Ca^{45} out of solution as the oxalate. Sr^{85}, a gamma emitter, was counted in a scintillation well-type counter.

A three-dimensional central composite design was used to assign the 15 treatments shown in table 2 (Box, '54). Treatments 1 through 8 formed a 2^3 factorial experiment and the additional treatments, 9 through 15, formed a fractional 3^3 factorial experiment. With this design, good estimates can be made of the main effects, the two-factor interactions and the quadratic effects. It is assumed that the quadratic × quadratic (4th degree) effects found in a complete 3^3 factorial experiment are negligible, as also may be the linear × quadratic and quadratic × linear (third degree) effects (Cragle et al., '55).

RESULTS

The heaviest chicks were obtained at 1% of calcium, 0.6% of phosphorus and 100 I.C.U. of vitamin D_3 per pound (fig. 1) which corresponds closely to the nutrient requirements for starting chicks suggested by the National Research Council ('54), even though they were not significantly ($P > 0.05$) heavier than those in groups 1, 5, 9, 11, and 14. The extreme dietary levels of phosphorus, the lowest level of calcium and the highest level of vitamin D_3 reduced growth rate in chicks ($P < 0.05$). An interaction between calcium and phosphorus on growth rate was found ($P < 0.01$), emphasizing the importance of the Ca:P ratio. At the dietary levels used, phosphorus contributed slightly more

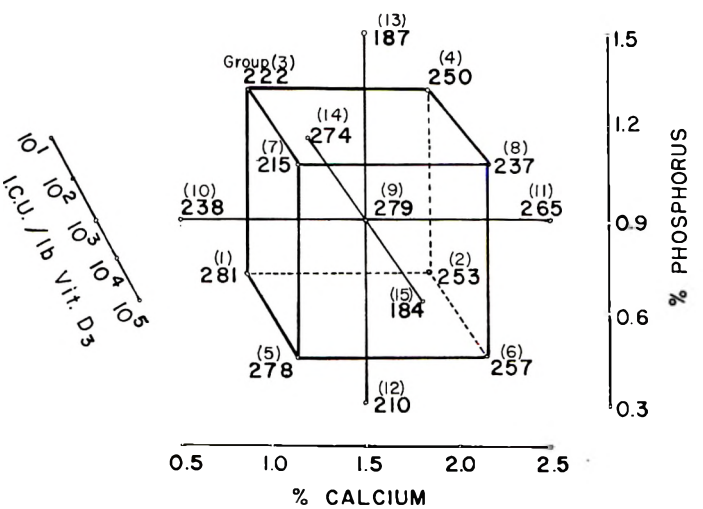

Fig. 1 Body weight (expressed in grams) of 3-week-old chicks fed varying levels of calcium, inorganic phosphorus and vitamin D_3.

than vitamin D_3 and twice as much as calcium to the quadratic effect on growth rate ($P < 0.01$).

Increasing dietary calcium (fig. 2) lowered ($P < 0.01$) deposition of orally administered Ca^{45} in tibiae but had no effect ($P > 0.05$) on intraperitoneally administered Ca^{45}. Increasing vitamin D_3 raised oral Ca^{45} and lowered intraperitoneal Ca^{45} ($P < 0.01$) deposition in tibiae. Within the dietary levels used, phosphorus contributed more to the quadratic effect ($P < 0.01$) on oral Ca^{45} deposition than calcium or vitamin D_3, whereas both phosphorus and vitamin D_3 contributed more than calcium to this effect on intraperitoneal Ca^{45}. No interactions were apparent ($P > 0.05$).

Increasing dietary phosphorus (fig. 3) lowered ($P < 0.01$) oral or intraperitoneal P^{32} deposition in chick tibiae. As dietary calcium increased, deposition of intraperitoneal P^{32} in tibiae increased ($P < 0.01$). Feeding vitamin D_3 at a level of 100,000 I.U. per pound in the diet reduced intraperitoneal P^{32} deposition in tibiae ($P < 0.05$). The greatest contribution to the quadratic effect ($P < 0.01$) in intraperitoneal P^{32} deposition came from vitamin D_3, whereas in oral P^{32}, it came from phosphorus. No interactions between dietary variables on P^{32} depositions were observed ($P > 0.05$).

In most instances (fig. 4) increasing calcium and phosphorus reduced ($P < 0.01$) the uptake of orally administered Sr^{85} by tibiae but did not appreciably influence intraperitoneal Sr^{85} ($P > 0.05$). At the median-to-high levels of dietary phosphorus supplementation, vitamin D_3 increased ($P < 0.01$) the uptake of oral Sr^{85}, whereas at the low-to-median level of calcium supplementation it reduced ($P < 0.01$) intraperitoneal Sr^{85} in tibiae. Within the limits of the dietary variables used, calcium contributed more than twice as much to the oral Sr^{85} quadratic effect ($P < 0.01$) as phosphorus and vitamin D_3, whereas vitamin D_3 and phosphorus contributed twice as much as calcium to the intraperitoneal Sr^{85} quadratic effect ($P < 0.01$). A Ca × vitamin D_3 interaction was observed ($P < 0.05$) in intraperitoneal Sr^{85} uptake with calcium reducing the effect of vitamin D_3.

DISCUSSION

The increase in uptake of orally administered Sr^{85} and Ca^{45} due to high vitamin D_3 feeding may be explained by increased absorption from the gut and is in agreement with the findings of Mraz and Bacon ('60) in rats. The reduction in Ca^{45}, Sr^{85} and P^{32} content in tibiae of chicks receiving the highest level of vitamin D_3 supplementation (100,000 I.C.U. per pound) is

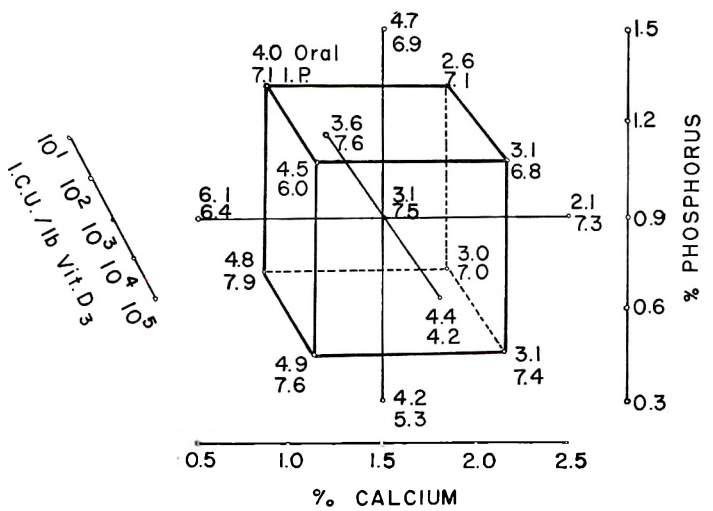

Fig. 2 Ca^{45} content of chick tibiae (expressed as percentage of administered dose per tibia).

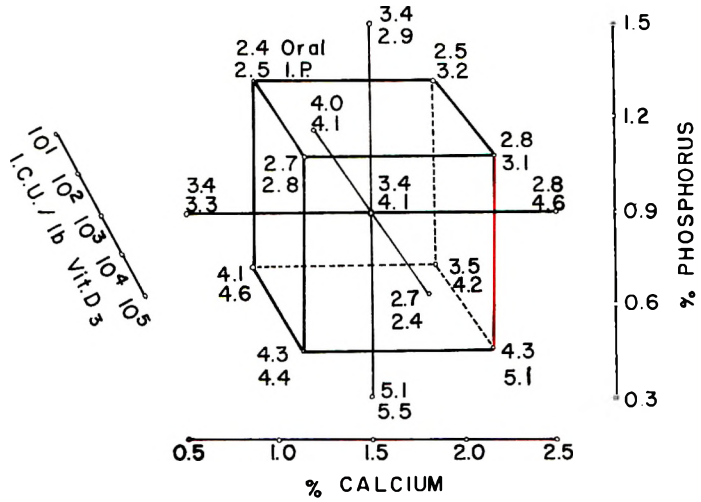

Fig. 3 P^{32} content of chick tibiae (expressed as percentage of administered dose per tibia).

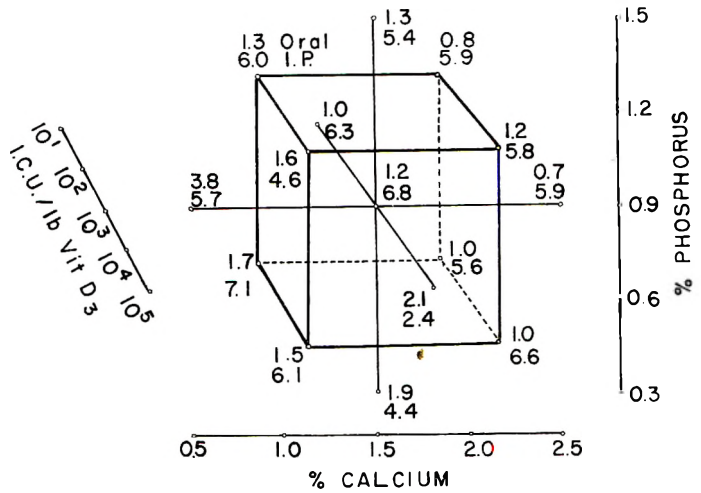

Fig. 4 Sr^{85} content of chick tibiae (expressed as percentage of administered dose per tibia).

a reflection of hypervitaminosis D which is described by Shelling ('32) and Steck et al. ('37) as leading to a mobilization of calcium from the bone, and as a result, an excretion of phosphorus. Shelling ('32) suggested that excessive vitamin D feeding expresses itself in a removal of calcium salts from the bone. In the presence of optimal amounts of both calcium and phosphorus in the diet, the excessive calcium and phosphorus in the blood are derived both from the bones and the diet. The excess of the solubility of these ions in blood is excreted in urine or feces and that which escapes excretion is deposited in the soft tissues. In the absence of both dietary calcium and phosphorus, the calcium salts liberated by the dissolution of bone, as a result of excessive doses of vitamin D, are excreted mainly through the kidneys. This hypervitaminosis would mean a more rapid turnover of Ca^{45}, Sr^{85} and P^{32} in tibiae. The intraperitoneally administered dose would enter the tibia much more rapidly than the oral dose and might, therefore, be available to be removed

sooner. The absorption of oral Ca^{45} and Sr^{85}, on the other hand, was enhanced by the high levels of vitamin D_3 enabling more of the radionuclides to enter the tibia. The delay in entering the tibia experienced on oral dosing might well be sufficient to give the appearance that the intraperitoneal dose is removed at a more rapid rate than the oral dose.

The ratio of Ca^{45}:Sr^{85} in tibiae of the chicks dosed orally varied from 1.6 to 3.7:1, whereas in those dosed intraperitoneally it was from 1.1 to 1.7:1. This would suggest a preferential absorption of Ca^{45} over Sr^{85} from the gut. The ratio of Ca^{45}:P^{32}, however, seemed primarily dependent on the ratio of calcium-phosphorus in the diet with no appreciable effect attributable to mode of administration of the two radionuclides. In the growing chick fed an optimal dietary level of calcium, phosphorus and vitamin D, the equilibrium at the site of deposition is toward the positive side and the slight excess of the solubility product of calcium and phosphate in the blood occasioned by injection is deposited in the growing osseous tissue. The path of excretion of the unused calcium and phosphorus is determined by the ratio of these ions (Shelling, '32). Excess calcium is excreted through the kidneys until its concentration in the urine reaches a maximum and the remainder is excreted through the bowel as the insoluble phosphate salt, thus robbing the body also of phosphorus. Excess phosphorus follows a similar path, first through the kidneys, which are able to excrete phosphorus to a limited concentration, and then the remainder through the bowel as the calcium salt, thus removing calcium from the body. In the growing chick, although the bones are receptive to calcium salt deposition, the constant loss of these elements by excretion prevents their reaching the bones in the proper concentrations for deposition. Little influence on the Ca^{45}:P^{32} ratio by vitamin D_3 was seen, except for their oral administration at the level of 100,000 I.C.U. of vitamin D_3 per pound of diet when this ratio increased.

The great decrease in the deposition of intraperitoneally administered Sr^{85} in tibiae of chicks receiving the 100,000 I.C.U. per pound level of vitamin D_3 would suggest the technique of massive vitamin D_3 might be useful as a means of reducing the radiostrontium burden of bones. Steck et al. ('37) stated that if certain precautions were carried out, massive doses of vitamin D might be utilized as safely as many other agents administered daily. One would, of course, have to be careful to discontinue the treatment at the first sign of toxicity. High calcium alone would appear, however, to be more satisfactory for reducing the uptake of radiostrontium than would either of the other two dietary variables.

SUMMARY

Fifteen groups of 15 New Hampshire cockerel day-old chicks were fed ad libitum, diets containing varying levels of calcium (0.5 to 2.5%), phosphorus (0.3 to 1.5%) and vitamin D_3 (10 to 100,000 I.C.U. per pound). The experimental design was a three dimensional central composite type. Three weeks later, Ca^{45}, P^{32} and Sr^{85} were administered orally to 10 birds from each group and intraperitoneally to the remaining 5. All birds were sacrificed 48 hours after dosing and tibiae removed for radioisotope assay. A significant interaction, Ca × P was observed on growth rate emphasizing the importance of the Ca:P ratio. As the level of dietary vitamin D_3 increased, deposition in tibiae of orally administered Ca^{45} and Sr^{85} increased but that of intraperitoneally administered Ca^{45} and Sr^{85} decreased. Deposition in tibiae of intraperitoneally administered P^{32} was reduced at the highest level of vitamin D_3 feeding. As the level of dietary calcium increased, deposition of orally administered Sr^{85} and Ca^{45} decreased and that of intraperitoneally administered P^{32} increased. As dietary phosphorus increased, deposition of orally administered Sr^{85} and P^{32} and intraperitoneally administered P^{32} decreased.

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Pulpal Response of Scorbutic Guinea Pigs to Injury[1]

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The biological responses of connective tissue to ascorbic acid deficiency are striking and dramatic. Since Zilva and Wells ('19) first described the effects of this deficiency on the dentition of guinea pigs, numerous studies as reviewed by Bolden[2] have been reported.

Although experimental studies concerning the effects of local injury to the odontoblasts have been conducted in the teeth of dogs, monkeys, rats and man (Massler, '55), such experiments have been performed on well animals. The reaction of pulps of nutritionally deficient animals to injury has not previously been reported, and it was the purpose of the present investigation to study the effects of cavity preparation, as a means of injury, on the pulp of scorbutic guinea pigs.

MATERIALS AND METHODS

This study in based on complete serial sections through the mandibular incisors of 70 guinea pigs. The animals weighed an average of 280 gm at the start of the experiment and were divided into the following groups:

Group A. Fifteen animals were fed a basal ascorbic acid-deficient diet.[3] These animals developed symptoms of scurvy within 14 days. The symptoms were evidenced by the loss of weight and appetite, lessening of activity and the loss of luster of eyes and hair. Following the operative procedure mentioned below, some of these animals were given replacement therapy which consisted of a single dose of 3 mg of ascorbic acid.

Group B. Ten animals were fed the basal diet in addition to which they received orally a daily dose of 0.2 mg of ascorbic acid. These animals developed symptoms of scurvy within 4 to 6 weeks.

Group C. Twenty-five animals were used as pair-fed controls for the animals of groups A and B. These animals received only as much of the basal diet as their experimental partner, in addition to which they received orally a daily dose of 3 mg of ascorbic acid, which prevented the development of scorbutic symptoms.

Group D. Twenty animals served as ad libitum-fed controls. These animals were fed the basal diet ad libitum in addition to which they received orally a daily dose of 3 mg of ascorbic acid.

Two weeks following the beginning of the experiment, when symptoms of scurvy became apparent, cavities were prepared in the right mandibular incisors of the animals in group A, in 15 animals in group C and 10 animals in group D.

The cavities were prepared in an area just anterior to the mental foramen according to the method described by Mohammed and Schour ('55). Although this method was developed for use in rat incisors, the procedure for guinea pigs is almost identical. In all cases the cavities were filled with zinc oxide and eugenol.

When signs of chronic scurvy evidenced by loss of weight and reduced activity became apparent, approximately 5 weeks after the beginning of the experiment, cavities were prepared in the mandibular right incisors of the animals in group B and the remaining animals in groups C and D.

The animals were sacrificed by ether anesthesia 4 days following the operative procedure. The mandibles were excised,

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[2] Bolden, T. E. 1951 The effect of chronic vitamin C deficiency upon the mandibular incisor and alveolar bone of the guinea pig. Master's thesis, Graduate School of Professional Colleges, University of Illinois.

[3] Vitamin C-Deficient Guinea Pig Test Diet, General Biochemicals, Inc., Chagrin Falls, Ohio.

fixed in formalin (10%) neutralized, demineralized with the disodium salt of ethylenediamine tetraacetic acid (EDTA) at pH 7.4 to 7.6 and embedded in paraffin. Both incisors were cut serially at 10 μ and sections were stained with hematoxylin and eosin and, for ribonucleic acid, with azure B at 0.25 mg per ml concentration, pH 4.0, for two hours at 40°C according to Flax and Himes ('52). Two rows of 5 sections each were placed on a slide. The bottom row was covered by a silicon grease recommended by Feder and Sidman ('57). Three sections of the upper row were flooded with a ribonucleate solution of 1 mg per ml in glass-distilled water. The other two sections were flooded with distilled water and served as controls. The sections were digested for two hours at 37°C, dehydrated and the grease removed with xylol, hydrated and stained with azure B.

The effectiveness of the methods described above is apparent in figures 1 to 4.

RESULTS

Group A. In animals with symptoms of acute scurvy, there was almost a complete cessation of dentin formation in both mandibular incisors (figs. 7 and 8). The pulp of the incisors in which cavities were prepared, however, showed a marked decrease in number of cells as compared with the adjacent unoperated teeth (figs. 7 and 8). No recognizable odontoblasts could be identified, and the majority of cells in the pulp of the unoperated incisor were large and oval shaped containing large nuclei with one or more nucleoli. The cytoplasm and the nucleoli exhibited a metachromatic staining reaction with azure B which was removed by digestion with ribonuclease.

The reaction of the pulp to the injury was apparently a generalized one and not localized to the area immediately underlying the cavity floor.

In animals which received replacement therapy of ascorbic acid following the operative procedure, there was an increase in cellular constituents in the pulp of the operated teeth (figs. 5 and 9). Large stellate cells, the cytoplasm of which exhibited a metachromatic staining reaction which was removed by digestion with ribonuclease, appeared in the central area of the unoperated pulp (figs. 6 and 10). In the pulps of the unoperated adjacent teeth many mitotic figures were present in the central area of the pulp (fig. 12). Some of the cells at the periphery of the pulp were more odontoblast-like in morphology, unlike the cells seen in the pulp of animals with no replacement therapy.

Group B. The pulps of the operated incisors of animals with chronic scurvy showed no essential difference as compared with the unoperated teeth (figs. 13 and 14). There was an abundance of osteodentin in both operated and unoperated incisors. The osteodentin was characterized by irregularly arranged dentinal tubules and projected into the pulp chamber to give it a distorted outline.

An interesting point was the intense staining reaction with azure B of the odontoblasts located at the periphery of the pulp (figs. 3 and 4). Cells in the odontoblastic layer stained more intensely than any other area of the pulp.

Groups C and D. No essential difference was observed between the pair-fed and ad libitum-fed controls. The operated teeth in both of these groups exhibited a marked increase of dentin deposition (figs. 15 and 16). Within 4 days the amount of dentin formed was so great that it almost completely filled the pulp chamber. The type of dentin formed was quite regular although many calciotraumatic lines were evident. This was more apparent in the pair-fed than the ad libitum-fed controls.

DISCUSSION

In animals receiving the optimum amount of ascorbic acid the characteristic reaction of the pulp to cavity preparation was a greatly increased amount of dentin formation. Although a filling of zinc oxide and eugenol has been shown to produce secondary dentin (Weider, Schour and Mohammed, '56), it is doubtful that the amount of dentin seen in the guinea pig incisor could be attributed to this material. The guinea pig odontoblast apparently will react to the slightest stimulus by an overproduction of dentin. The reaction is not localized to the odontoblasts that have been injured by cavity preparation but is a generalized one.

In animals receiving only enough ascorbic acid to produce chronic scurvy, there was the characteristic osteodentin formation. Injury to the pulps of such teeth evinced no discernible reaction (figs. 13 and 14). It is possible that the amount of irregular osteodentin previously laid down as a result of chronic scurvy acted as a protective covering to the pulpal cells. The large amount of osteodentin formed prior to the operation made this area comparable, in some respects, to the normal incisal end of the tooth. This suggests that future operative procedures should be performed earlier than 5 weeks after the beginning of the experiment.

In animals with acute scurvy, the pulpal reaction was characterized by a marked decrease in the number of cells as compared with the unoperated incisors. The reaction to the cavity preparation appeared to be generalized. No identifiable odontoblasts were seen in these specimens. Where replacement therapy was given, however, large cells rich in ribonucleic acid (figs. 5 and 9) appeared in the central area of the pulp. These cells were readily seen in the operated incisors. They appear to be undifferentiated mesenchymal cells and it is possible that under the influence of ascorbic acid may become odontoblasts to form new dentin. In the unoperated incisors numerous mitotic figures were seen. Such mitotic figures have been mentioned by Boyle and Irving ('52) who saw mitosis among odontoblasts and adjacent pulp cells in well developed acute scurvy. These workers concluded that proliferation of cells appeared to occur as a compensating phenomenon where normal differentiation and function of the cell is inhibited. It is possible, however, that these cells undergoing mitosis, seen mostly in the central area of the pulp, are undifferentiated mesenchymal cells which are destined to become odontoblasts under the influence of ascorbic acid. This is particularly demonstrated in the operated incisors in which many of the cells have disappeared due to cavity preparation, but where in the central pulpal area large, undifferentiated cells are present.

The odontoblasts of the control guinea pig incisors in the regions studied in this investigation were rich in ribonucleic acid. The presence of ribonucleic acid was demonstrated by the removal of the metachromatically stained cellular material after digestion with ribonuclease. This further confirms the work of Mardfin and James ('57) concerning the possibility of staining chelated material for nucleic acids. Of all the pulpal cells the odontoblasts appear to contain the greatest amount of ribonucleic acid.

The odontoblasts adjacent to the osteodentin in animals with scurvy are rich in ribonucleic acid (figs. 3 and 4) and closely resemble the young odontoblasts described by James et al. ('54). In these animals the odontoblasts stain more intensely for ribonucleic acid than any other cells of the pulp.

In animals with acute scurvy the pulpal cells of unoperated teeth that stained most intensely were located centrally rather than at the periphery. The peripheral cells were not morphologically like odontoblasts, having lost their columnar characteristic. Upon the administration of a single feeding of ascorbic acid, however, the peripheral cells became somewhat more elongated and stained more intensely with azure B. Previous work (Yale et al., '59) has indicated that these peripheral cells also show an intense staining reaction for ascorbic acid after a single feeding of this vitamin.

This suggests that there may be a close relationship between ribonucleic acid and ascorbic acid metabolism. The findings in unoperated teeth would tend to further confirm the work of Thorell and Wilton ('45), who concluded that the peripheral cells are odontoblasts which have reverted to a more immature cell type. In chronic scurvy the cells revert to a stage comparable to that of a young odontoblast. In acute scurvy, however, the regression is to a much earlier stage of odontoblastic development. These cells have lost their morphology but not the potentiality to produce dentin. There is good evidence, however, that new cells also differentiate from the undifferentiated mesenchymal cells of the pulp to become odontoblasts, as evidenced in animals receiving replacement therapy.

SUMMARY

1. This study was based on serial sections of the mandibular incisors of guinea pigs having acute and chronic scurvy, with pair-fed and ad libitum-fed controls. When symptoms of scurvy, acute or chronic, became apparent, cavities were prepared in the area just anterior to the mental foramen. Some of the guinea pigs having acute scurvy were given a single dose of 3 mg of ascorbic acid following cavity preparation. After sacrifice both mandibular incisors were processed for histologic staining with hematoxylin and eosin and azure B.

2. The operated incisor of the guinea pigs with acute scurvy showed almost a complete cessation of dentin deposition, no recognizable odontoblasts, and a decrease in pulpal cell population. The reaction to the insult was generalized. The unoperated pulp differed in that the cell population of the pulp was greater and the cells more viable.

3. No essential difference was noted between the operated and unoperated incisor in animals with chronic scurvy.

4. The characteristic reaction of both the pair-fed and the ad libitum-fed controls was a greatly increased deposition of regular dentin following cavity preparation.

5. In the controls and in chronic scurvy the odontoblasts stained most intensely for ribonucleic acid. In acute scurvy, however, the centrally located cells of the pulp were the most intensely stained.

6. The possible correlation between ribonucleic acid content and ascorbic acid metabolism is discussed.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Photomicrograph of a cross section through the operated right mandibular incisor of an ad libitum-fed control animal sacrificed soon after cavity preparation. Note the intense cytoplasmic staining reaction of the odontoblasts. Azure B stain.

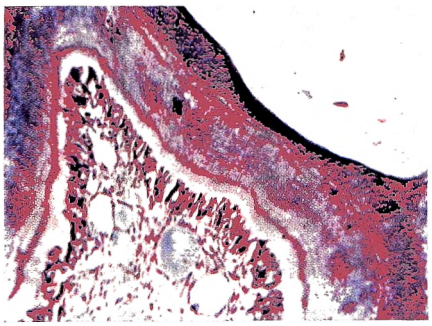
2 Photomicrograph of a cross section from the same specimen described in figure 1. Note the lack of the cytoplasmic staining reaction seen in figure 1. Azure B stain after ribonuclease digestion.

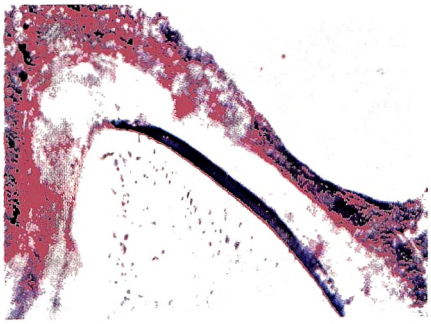
3 Photomicrograph of a cross section through the operated right mandibular incisor of an animal with chronic scurvy. Note the intense cytoplasmic staining reaction and morphology of the odontoblasts. Azure B stain.

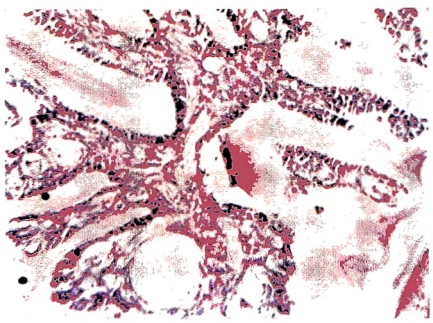
4 Photomicrograph of a cross section from the same specimen described in figure 3. Note the lack of cytoplasmic staining reaction seen in figure 3. Azure B stain after ribonuclease digestion.

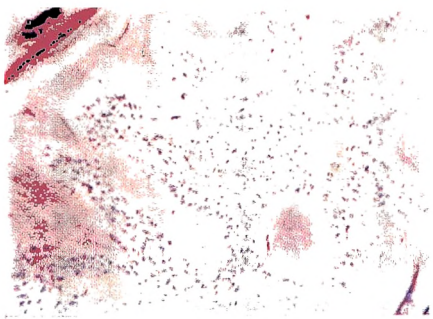
5 Photomicrograph of a cross section through the operated right mandibular incisor of an animal with acute scurvy which received a single feeding of ascorbic acid following the cavity preparation. Note the large centrally located pulpal cells rich in ribonucleic acid. Azure B stain.

6 Photomicrograph of a cross section through the unoperated left mandibular incisor of the same animal described in figure 5. Note the density of the cell population as compared with figure 5. Azure B stain.









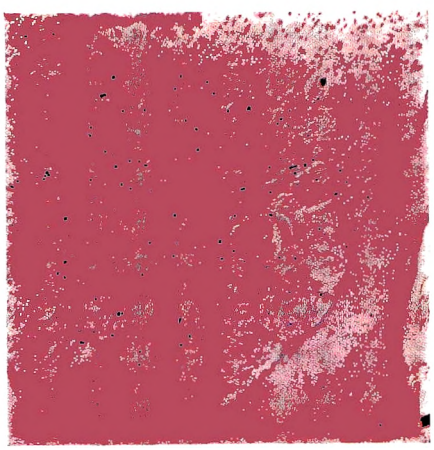


PLATE 2

EXPLANATION OF FIGURES

7 Photomicrograph of a cross section through the operated right mandibular incisor of an animal with acute scurvy sacrificed 4 days following the operative procedure. Note the lack of identifiable odontoblasts and the decrease of cell population. H. and E.

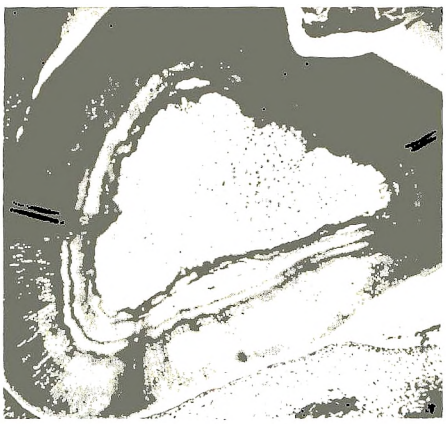
8 Photomicrograph of a cross section through the unoperated left mandibular incisor of the same animal described in figure 7. Note the number of pulpal cells as compared with figure 7. H. and E.

9 Photomicrograph of a cross section through the operated right mandibular incisor of an animal with acute scurvy which received a single feeding of ascorbic acid following the operative procedure. Note the large cells located in the central area of the pulp. H. and E.

10 Photomicrograph of a cross section through the unoperated left mandibular incisor of the same animal described in figure 9. Note the number of pulpal cells and the cells at the periphery (arrow) which have elongated. H. and E.

11 Higher magnification of the area marked in figure 9.

12 Higher magnification of the area marked in figure 10. Note the mitotic figure in the center of the field.



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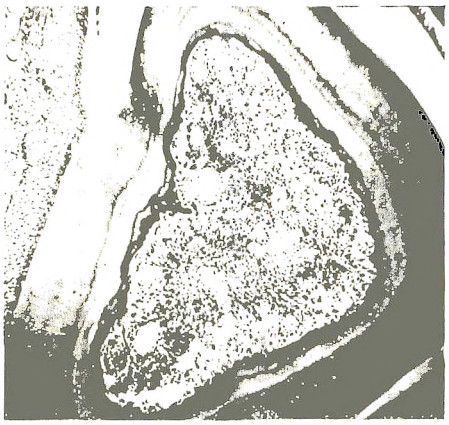

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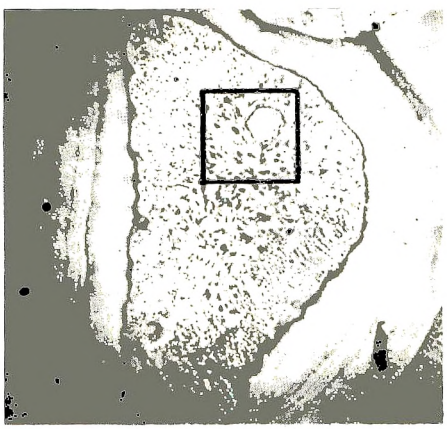

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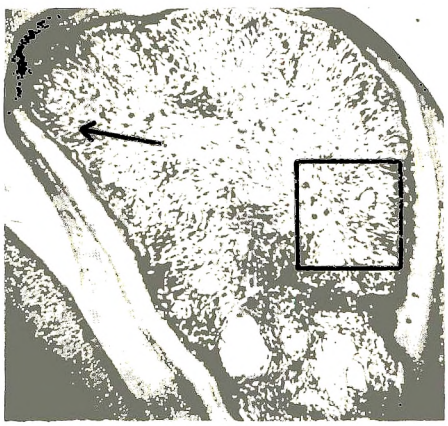

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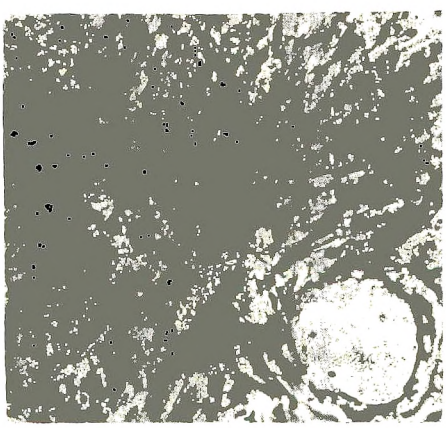

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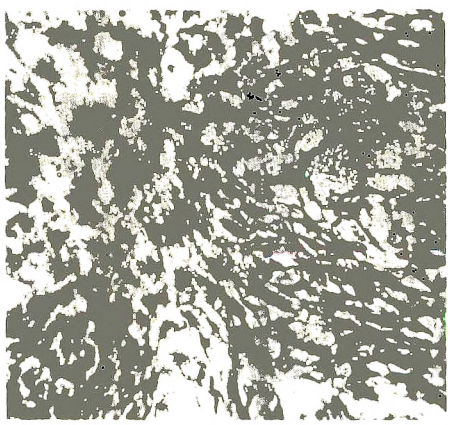

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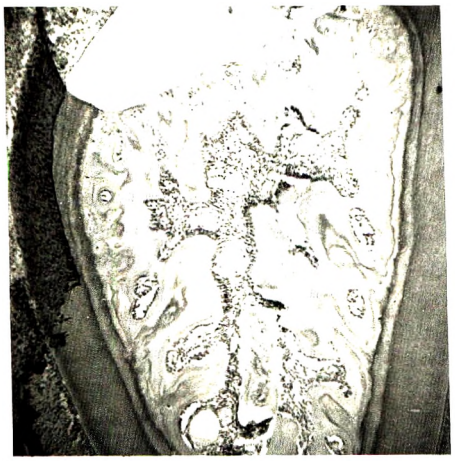

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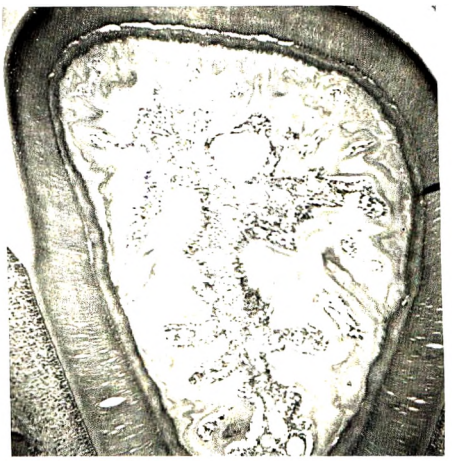

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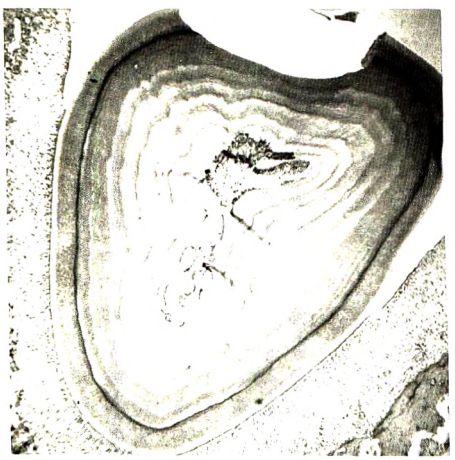

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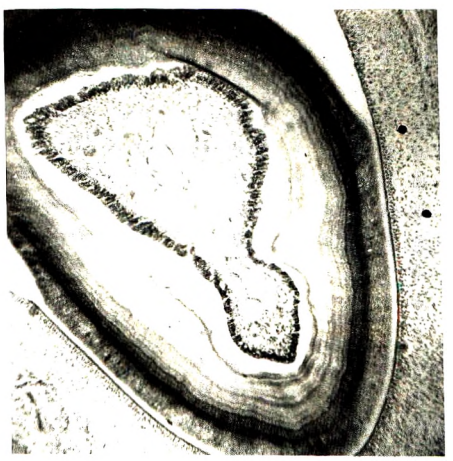

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13 Photomicrograph of a cross section through the operated right mandibular incisor of an animal with chronic scurvy. Note the amount of osteodentin and the relatively small pulpal area. H. and E.

14 Photomicrograph of a cross section through the unoperated left mandibular incisor of the same animal described in figure 13. H. and E.

15 Photomicrograph of a cross section through the operated right mandibular incisor of a pair-fed control sacrificed 4 days following operative procedure. Note the abundance of dentin formation. H. and E.

16 Photomicrograph of a cross section through the unoperated left mandibular incisor of the same animal described in figure 15. H. and E.

Studies on Copper and Iron Deficiencies in Growing Chickens[1,2]

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Shortly after Hart et al. ('28) demonstrated the importance of copper, as well as iron, in correcting the anemia of rats fed milk diets, Elvehjem and Hart ('29) extended these findings to chicks. Since that time little work has been conducted on the effects of copper and/or iron deficiencies in this species. In a recent report, however, Gallagher ('57) was unable to produce a pure copper-deficiency anemia in chicks.

The work presented in this report was undertaken to investigate the effects of copper and/or iron deficiencies on the blood hemoglobin level and the general hematological picture of growing chicks and to ascertain the chicks' requirements for these elements.

MATERIALS AND METHODS

The chicks used in these studies, Rhode Island Reds obtained from a commercial hatchery, were weighed and distributed into experimental lots at one day of age. Forty chicks were used per treatment in the first three experiments, 25 in the 4th, and 20 in the 5th. The chicks were housed in electrically heated battery brooders with raised wire floors. In all but experiment 4 the feed was placed in galvanized iron feeder troughs but in the 4th experiment stainless steel feeding troughs were used. Demineralized water fed in glass jars with plastic bottoms or stainless steel watering troughs were used in all experiments. Feed and water were supplied ad libitum.

The basal diet used throughout these studies is presented in table 1. It is based on skim milk supplemented to contain all the necessary vitamins, minerals and amino acids necessary for chick growth. For those groups receiving copper, a solution of $CuSO_4 \cdot 5H_2O$ was mixed into the feed, and a solution of $FeSO_4 \cdot 7H_2O$ was used for those receiving the iron supplementation. Both solutions were made from reagent grade chemicals. Copper and iron analyses of the diets were conducted by the method of Parks et al. ('43).

TABLE 1
Basal diet

	%
Sucrose[1]	31.7
Dried skim milk	60.0
Vegetable oil[2]	5.0
DL-Methionine	0.3
L-Arginine·HCl	0.5
Glycine	0.5
NaCl (reagent grade)	0.5
$MgSO_4 \cdot 7H_2O$ (reagent grade)	0.5
Vitamin mixture[3]	1.0
	mg/pound
$MnSO_4 \cdot H_2O$ (reagent grade)	100

[1] Cerelose, Corn Products Refining Company, New York.
[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.
[3] Supplied per pound of diet: (in milligrams) thiamine, 1.6; riboflavin, 2.6; Ca pantothenate, 8.4; niacin, 24; α-tocopheryl acetate, 10; menadione sodium bisulfite, 0.36; and vitamin B_{12}, 8 μg; vitamin A, 2400 USP units; and vitamin D, 180 I.C.U.

In order to minimize contamination, the mixing bowl, mixing paddle and any feed scoops to be used were thoroughly washed with a detergent and rinsed with deionized water before use. In addition the mixer was washed with deionized water, particular care being taken with the under surfaces from which contaminants might have dropped into the bowl during mixing.

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[1] Published with the approval of the Director of the Experiment Station as paper no. 1240 of the journal series.
[2] Supported in part by a grant from the Herman Frasch Foundation.

Hemoglobin determinations were made at intervals on 10 chicks from each of the experimental groups. When hemoglobin analysis was to be made at less than weekly intervals, no chick was bled more than once a week. The blood for the hemoglobin determination was obtained by wing vein puncture. In the first three experiments the hemoglobin values were obtained using the acid hematin method; in the 4th and 5th experiments, the oxyhemoglobin method was used.

In experiment 4 erythrocyte counts and hematocrit values were obtained by the standard laboratory procedures on 8 chicks from each treatment at stated intervals.

Cytochrome oxidase was determined by the method of Cooperstein and Lazarow ('51).

RESULTS AND DISCUSSION

The first experiment was undertaken to determine the effect of copper and iron additions to the basal diet on the hemoglobin content of the blood. The results of this study, presented in table 2, show that as either the copper or iron was increased, the concentration of hemoglobin increased. Furthermore, there was an apparent interaction between copper and iron. That is, as iron was increased less copper was needed for a given concentration of hemoglobin and vice versa.

Two other similar studies were conducted to test further the validity of these observations. The results of these studies are also presented in table 2. Although the effect of the evident interaction is less pronounced in these two studies, a clear-cut copper-deficiency anemia was obtained in each experiment. A similar interaction between copper and iron on hemoglobin levels of rats was obtained by Mitchell and Miller ('31).

The apparent interactions observed lend credence to a tacit assumption by many investigators that copper contributes to hemoglobin synthesis. Iodice et al.[3] reported that δ-aminolevulinic acid dehydrase contained 0.1% of copper. Since this enzyme is active in the pathway to protoporphyrin and thus presumably to hemoglobin syn-

[3] Iodice, A. A., D. A. Richert and M. P. Schulman 1958 Copper content of purified δ-aminolevulinic acid dehydrase. Federation Proc., *17*: 248 (abstract).

TABLE 2

Effect of iron and copper levels on chick hemoglobin concentration[1]

Fe	Experiment 1, day 22				Experiment 2, day 28					Experiment 3, day 56			
	Cu ppm				Fe	Cu ppm				Fe	Cu ppm		
	1.8	2.2	3.2	5.2		1.7	2.7	4.7	8.7		1.9	2.9	4.9	8.9
ppm					*ppm*					*ppm*				
10	4.08	3.18	5.18	5.50	12.3	5.22	5.89	9.07	5.10	7	3.50	5.67	8.81	11.39
15	3.98	6.50	5.84	6.50	22.3	5.93	8.95	7.12	8.40	17	4.86	9.19	8.40	10.35
20	5.08	5.79	6.84	10.54	42.3	6.47	7.36	10.21	9.97	37	5.69	8.00	8.14	9.35
40	6.93	7.17	10.53	10.68										

[1] Indicated as the mean for 10 chicks/treatment, in grams of hemoglobin/100 ml.

thesis, copper might have acted at this point in hemoglobin formation. Later, however, these workers reported that removal of the copper did not affect the activity of the enzyme.[4] Anderson and Tove ('58) observed that heme synthesis by copper-deficient chick blood was stimulated by the addition of copper *in vitro*. Failure to consistently produce copper deficiency, however, prevented these workers from pursuing this investigation further.[5] If copper actually contributes to hemoglobin synthesis, the site at which it acts remains obscure.

The appearance of anemia as shown by decreased hemoglobin concentration does not necessarily mean that hemoglobin formation has been directly depressed. The concentration of hemoglobin in the blood stream actually depends upon the concentration of hemoglobin in the erythrocytes and the number of erythrocytes. Although it is generally agreed that iron anemia results from both a depression in the number of cells and a depression in the hemoglobin concentration within the cells, there is less unanimity about the morphological picture of a copper-deficiency anemia. Lahey et al. ('52) reported that copper deficiency in swine led to a hypochromic anemia with a reduced number of cells. Van Wyk et al. ('53) and Maas et al. ('44) presented evidence that in the dog a copper deficiency results in a normochromic anemia with a reduced number of cells. Ruminants grazing in copper-deficient pastures have been observed to have a slightly hypochromic anemia (Cunningham, '46).

Since the hematological picture of copper deficiency in chicks has not been reported, a study was conducted in which dietary levels of iron and copper were studied in a factorial experiment involving 9 treatment combinations. At 19 days of age, when the anemia had become apparent, the blood of 8 chicks from each group was examined for hemoglobin level, erythrocyte count and hematocrit values. The results in terms of hemoglobin content, erythrocyte numbers, cell volume and mean cell hemoglobin concentration are presented in table 3.

In this experiment, as in the previous ones, either copper or iron partially corrected the anemia as measured by decreased hemoglobin concentration. Analysis of variance indicated that the effect of copper or iron was highly significant. This analysis, however, also indicated no significant interaction of these two minerals on the hemoglobin concentration of the blood.

The analysis of the results of the mean cell hemoglobin concentration determinations showed that only iron had a significant effect on this parameter. Likewise, only iron had a significant effect on the mean cell volume. In neither of these measurements was there an interaction between the two minerals. In contrast with these results, copper alone had a significant effect on erythrocyte numbers. Iron deficiency showed no effect and no interaction was observed between iron and copper.

The effect of copper deficiency on cell numbers is not unexpected when other investigations on the effect of copper on reticulocytes are considered. The administration of copper to rats (Schultze and Elvehjem, '33; Smith and Medlicott, '44; Stein and Lewis, '33), rabbits (Smith and Ellis, '44) and swine (Lahey et al., '52) made anemic as a result of a copper and iron deficiency has been shown to result in a marked reticulocyte response. The administration of iron had little effect. In dogs and swine suffering from copper anemia alone, the administration of copper also resulted in a marked reticulocyte response (Baxter and Van Wyk, '53; Lahey et al., '52). It appears, therefore, that the main function of copper in maintaining a normal hematological picture is related to the production of erythrocytes and may have little or nothing to do with the production of hemoglobin *per se*.

During the course of these studies many of the erythrocytes from the iron-deficient chicks were observed to be deformed. The abnormal shapes of such cells compared

[4] Wilson, T. L., A. A. Iodice, M. P. Schulman and D. A. Richert 1959 Studies on liver δ-aminolevulinic acid dehydrase. Federation Proc., *18*: 352 (abstract).

[5] Anderson, R. L. 1959 The effect of copper on *in vitro* heme synthesis in blood from chickens deficient in copper. M.S. thesis, North Carolina State College.

TABLE 3

Effect of iron and copper on hemoglobin, mean cell hemoglobin, mean cell volume and cell members

Level of Fe	Level of copper, ppm			$\bar{x}$	Analysis of variance		
	1.47	3.47	5.47		Source	df	M.S.
ppm	*Hb gm/100 ml*						
7	5.96	5.98	7.21	6.38	Fe	2	22.17000[1]
					Cu	2	8.24000[1]
47	7.08	8.15	8.18	7.80	Fe × Cu	4	2.14800
					Error	63	1.29167
$\bar{x}$	6.80	7.70	7.90				
L.S.D. means within table (8 obs.) 1.1364; border means (24 obs.) 0.656.							
	Mean cell Hb μμg						
7	27.3	25.8	29.5	27.5	Fe	2	317.35[1]
					Cu	2	46.40
47	36.8	33.5	32.5	34.3	Cu × Fe	4	23.32
					Error	63	50.45
87	35.0	31.5	33.2	33.2			
$\bar{x}$	33.0	30.3	31.7				
L.S.D. means within table (8 obs.) 7.104; border means (24 obs.) 4.100.							
	Mean cell volume μ^3						
7	74.05	80.98	82.05	79.03	Fe	2	1373.58[2]
					Cu	2	409.98
47	102.03	88.10	90.69	93.61	Fe × Cu	4	421.25
					Error	63	313.47
87	100.20	83.29	85.94	89.81			
$\bar{x}$	92.10	84.12	86.23				
L.S.D. means within table (8 obs.) 17.7061; border means (24 obs.) 10.22.							
	Mean cell no. $10^6/mm^3$						
7	2.24	2.42	2.64	2.43	Fe	2	0.1087
					Cu	2	1.6142
47	2.06	2.51	2.53	2.37	Fe × Cu	4	0.0782
					Error	63	0.2234
87	2.10	2.88	2.52	2.50			
$\bar{x}$	2.13	2.60	2.56				
L.S.D. means within table (8 obs.) 0.522; border means (24 obs.) 0.310.							

[1] Statistically highly significant ($P \leq 0.01$).
[2] Statistically significant ($P \leq 0.05$).

with those in the blood from animals receiving adequate iron are shown in figure 1. In general the cells are elongated and have many tail-like protuberances. In order to ascertain the degree of this abnormality, 500 erythrocytes were counted in the blood of 4 26-day-old chicks receiving either copper-and-iron (combined), iron (alone), copper-deficient (alone), or control diets. The results of that study are presented in table 4.

Copper deficiency alone had little effect on the number of abnormal cells. The combined copper and iron deficiency resulted in the greatest number of abnormal cells. Overall, the iron deficiency resulted in approximately a threefold increase in the poikilocytes.

Another observation in these studies was that chicks receiving the copper-deficient diets were lighter in color than those fed adequate copper. The chicks receiving the iron-deficient diets were somewhat lighter in color than the controls. This effect on coloring is shown in figure 2. The depressed pigmentation due to copper deficiency has been observed in the rat (Keil and Nelson, '31), rabbit (Smith and Ellis, '47) and sheep (Marston, '49). It has also been shown that when high levels of molybdenum and sodium thiosulfate were fed to chicks, feather pigmentation was depressed and the depression could be prevented by the addition of copper to the diet (Miller and Denton, '59). As far as known, however, this is the first observation of

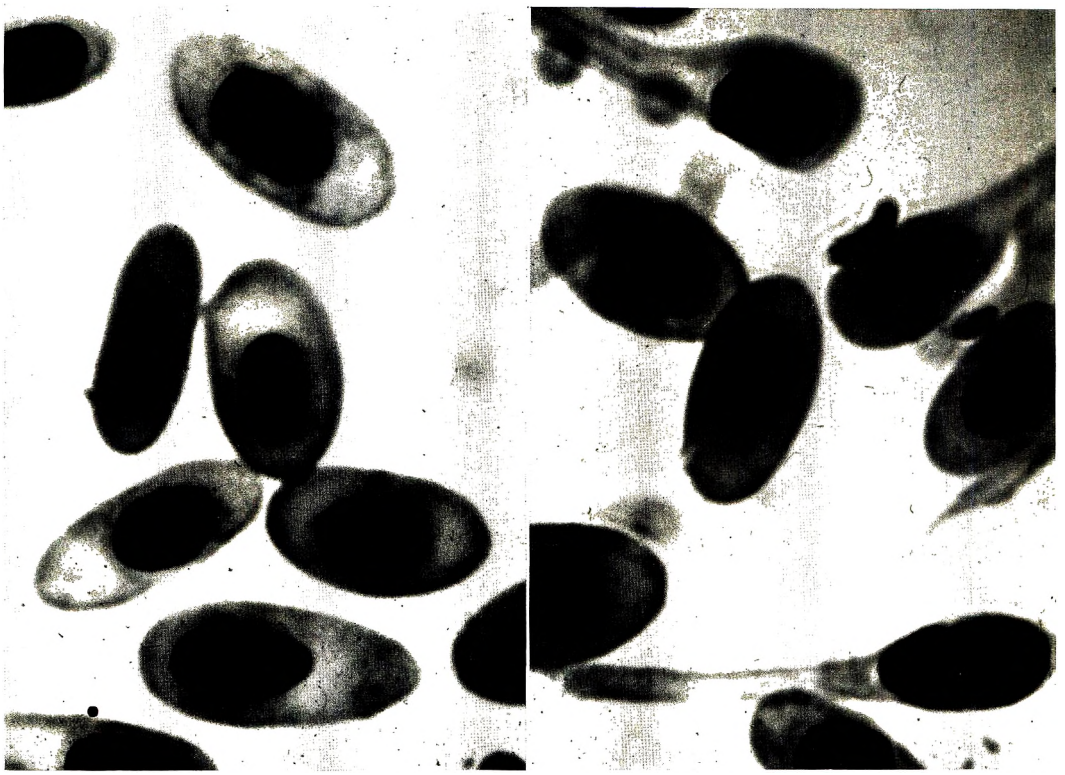

Fig. 1 Effect of iron deficiency on erythrocyte shape; left, from control animal; right, from iron-deficient chick.

TABLE 4

Effect of copper and iron deficiency on percentage of poikilocytes in chick blood

Fe	Cu ppm	
	1.47	5.47
ppm	%	%
7	6.85[1]	4.20
87	2.20	1.50

[1] Mean of 4 samples/treatment.

decreased feather pigmentation in uncomplicated copper deficiency in the chick.

Reference has been made to the failure of Gallagher ('57) and Anderson[6] to produce a copper-deficiency anemia in chicks. Several studies during the course of these investigations have also resulted in normal hemoglobin levels despite low dietary copper levels. These failures occurred even though extreme care was taken to keep the dietary content low by using a chelating agent in the water used to rinse and wash the mixing utensils. In these studies the copper content of the basal diet was as low as 0.5 ppm. In one study the chicks were kept in an isolated room and housed in cardboard cages to keep them from any contact with metal. Even under these conditions the chicks did not become anemic from copper deficiency although they became anemic from the iron deficiency. In all experiments in which the copper deficiency diets did not result in anemia, however, mortality was heavy compared with that of the control groups. An example of hemoglobin concentration and mortality data of such an experiment is presented in table 5.

Those chicks in the copper-deficient groups which die, die suddenly, without apparent symptoms. It has been found that cattle grazing on copper-deficient pasture develop fibrosis of the heart and die suddenly (Bennetts et al., '48). This may be the result of a failure of the heart cyto-

[6] See footnote 5.

Fig. 2 Effect of copper and iron deficiencies on feather pigmentation. From left to right: pair 1, copper- and iron-deficient; pair 2, copper-deficient; pair 3, iron-deficient; pair 4, control.

TABLE 5

Effect of copper and iron deficiency on mortality and hemoglobin levels in chicks[1]

Fe	Cu ppm			
	0.78		4.78	
	Hb[2]	Mortality	Hb	Mortality
ppm		%		%
8	3.24	75	5.16	10
48	7.02	50	7.40	0

[1] Chicks 5 weeks of age, 20 chicks/treatment.
[2] Gm/100 ml of blood.

TABLE 6

Effect of iron and copper deficiencies on cytochrome oxidase of chick hearts

Fe	Cu ppm	
	0.7	4.7
ppm	*O.D. change/min./mg protein*	
10	3.07[1]	5.68
40	3.55	4.19

[1] Each value represents the mean of 4 determinations.

chrome oxidase which has been observed to be depressed in copper-deficient animals (Schultze, '39; Gubler et al., '57).

An experiment was conducted in which the cytochrome oxidase was determined of heart muscle obtained from chicks fed combined copper and iron, copper, or iron-deficient diets, as well as the control diet. The results of this study are presented in table 6. Copper deficiency resulted in a definite lowering of the cytochrome oxidase activity, whereas iron deficiency did not, even though the copper-deficient chicks were not anemic.

Although heavy mortality occurred in the groups fed the low-copper diets, these chicks did not die before those in other experiments became anemic. It is not a case of the chicks dying before they can become anemic. The mechanism by which this phenomenon takes place is unknown and requires further study.

The requirements of the chick for copper and iron have been set at approximately 2 ppm of copper and 20 ppm of iron (National Research Council, '60). If hemoglobin concentration is used as the criteria of requirement, these figures are evidently too low. Since there is an apparent interaction between copper and iron for this function, no requirement can be stated for either element without specifying the level of the other. In the first experiment, for example, 20 ppm of iron was sufficient if the diet contained 5.2 ppm of copper.

On the other hand, 40 ppm of iron was needed if the diet contained only 3.2 ppm of copper. In the second experiment, 42.3 ppm of iron and 4.7 ppm of copper were needed to produce maximum hemoglobin concentration. In the third experiment, only 7 ppm of iron was needed if the diet contained 8.9 ppm of copper. Although the results varied between experiments, in no instance was maximum hemoglobin concentration reached with a combination of 20 ppm of iron and 2 ppm of copper. According to the evidence presented here, a more realistic estimate of requirements would be 4 ppm of copper and 40 ppm of iron.

SUMMARY

Studies were conducted in which copper-deficiency and/or iron-deficiency anemias were produced in the chick. The initial effect of copper deficiency was a decrease in the number of erythrocytes, whereas the initial effect of iron deficiency was a decrease in the hemoglobin content of the erythrocytes. Copper deficiency decreased the feather pigmentation of the chick and reduced the heart cytochrome oxidase activity, and iron deficiency affected pigmentation only slightly and did not reduce cytochrome oxidase activity. The requirement of the chick for these two minerals is discussed.

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