EFFECT OF NITROGEN FERTILIZATION ON AMINO ACIDS IN WHOLE WHEAT [1]

HANS GUNTHARDT [2] AND JAMES McGINNIS
Departments of Agronomy and Poultry Science,
State College of Washington, Pullman

(Received for publication May 11, 1956)

Variations in environment produce spectacular differences in protein content of wheat. Little is known about the effect of such variations on the relative proportions of the amino acids of the total protein. Some researchers (Schweigert, '48 and Miller et al., '50) have suggested that lysine (expressed as a percentage of protein) decreased as the total protein of whole wheat increased. Other amino acids have been found to vary from sample to sample of wheat. On the other hand, Pence et al. ('50) found that the amino acid pattern of the gluten isolated from 17 straight-grade, unbleached flours of widely varying baking properties, representing wheat from a wide range of type and sources, was essentially uniform.

Barton-Wright ('46) determined the concentrations of essential amino acids in whole wheat, patent flour (inner endosperm), outer endosperm (portion abutting the aleurone layer), bran and germ. The germ contained a much higher concentration of arginine, lysine, threonine and histidine than did the inner endosperm. The bran was particularly rich in arginine. Phenylalanine, isoleucine, and leucine showed a slightly reversed trend, in that they were more concentrated in the inner endosperm and were at a minimum in the bran and the germ.

[1] Scientific paper no. 1214, Washington Agricultural Experiment Station, Pullman. Project no. 952.

[2] Present address: General Foods Corporation, Central Laboratories, Hoboken, N. J.

The purpose of this investigation was to study the effects of nitrogen fertilization on the amino acid composition of wheat samples differing widely in crude protein content.

MATERIALS

The wheat samples analyzed were grown on Palouse Silt Loam in 1949 and 1950. They were arbitrarily selected on the basis of protein content from two extensive fertilizer and irrigation experiments to suit the purpose of this study.

The wheat was threshed and cleaned, and yield measurements were made. Approximately 200 gm from single plots of the selected treatments were finely ground and stored in glass bottles.

All of the high-protein samples were produced by a combination of nitrogen (120 lbs., as ammonium nitrate, per acre) and water applications as late as the boot, flowering or milk stages of growth. The low-protein samples had received much less nitrogen (from 0 to 40 lbs. N) at seeding time only. The comparatively much higher yields of the two 1950 wheat samples were obtained by sprinkler irrigation throughout the season, regardless of nitrogen application. Yield and protein content of the samples are given along with the amino acid composition in tables 1, 2 and 3.

METHODS

Hydrolysis. One gram of finely ground wheat was hydrolyzed with 25 ml of 3N HCl for 8 hours at 15 pounds steam pressure (120°C.). Subsequently most of the HCl was evaporated on a steam bath. The remaining HCl was neutralized with NaOH, or preferably a mixture of NaOH and KOH, since a number of microorganisms used in the microbiological assays are more tolerant to potassium ions than to sodium ions. The pH was adjusted to 6.8 and the hydrolysates were made up to volume, preferably 100 ml and filtered through several layers of filter paper on a Buchner funnel. If the hydrolysates were not promptly used in assay work, they were

stored in large Erlenmeyer flasks at minus 10°C. after addition of a drop of toluene. Before use the high-protein hydrolysates were further diluted 1:4 and the low-protein hydrolysates 1:2.5. This fact becomes of some importance in the light of recent studies made by Horn et al. ('53) who found that filtering at pH 6.8 does not remove all the humin formed during hydrolysis. Humin has, particularly in the presence of high quantities of pyridoxamine, a stimulating effect, so that the following problems arise:

1. All hydrolysates used in this study probably contained small amounts of humin; thus values for the amino acids lysine, isoleucine, arginine, valine, . . . may be slightly high.
2. The low-protein samples (diluted 1:2.5) may show slightly higher values for the above mentioned amino acids than the high-protein samples (diluted 1:4) because of a somewhat greater concentration of humin.

Microbiological assay. The amino acids present in the wheat hydrolysates were determined by microbiological assays based on procedures described by Henderson and Snell ('48) and Henderson et al. ('48) with certain modifications. A Cannon dispenser and an electrometric titration were used, but a 10 ml microburette was substituted for the electrical counter and was found to be considerably more accurate for titration. The basal medium of Steele et al. ('49) was found better suited than that of Henderson and Snell ('48). *Leuconostoc mesenteroides* P-60 and occasionally *Leuconostoc citrovorum* 8081 [3] were used for inoculation. Twelve different levels of each amino acid were used in triplicate tubes for making up the standard curves. Recoveries of pure amino acids ranged from 93 to 104%. The results from replicate assays (different days) replicate protein hydrolysates, and from all the assay levels within the usuable portion of the standard curve were averaged and reported as percentages of the total crude protein of the whole wheat samples.

[3] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

TABLE 1

Amino acid contents of 4 high-protein and 4 low-protein whole wheat samples (Idaed wheat)

YEAR	PROTEIN CONTENT	YIELD	AMINO ACIDS AS PERCENTAGE OF CRUDE PROTEIN											
			Leucine	Phenylalanine	Isoleucine	Tyrosine	Aspartic acid	Arginine	Lysine	Glycine	Proline	Valine	Threonine	Histidine
	%	*bu./ac.*												
1950	10.44	92.5	6.24	4.15	4.38	2.47	4.43	4.85	3.81	6.15	11.5	4.52	3.25	1.77
1949	10.00	39.0	6.51	4.64	4.53	2.57	4.80	4.55	3.55	6.32	12.53	4.67	3.46	1.78
1949	10.32	60.0	6.52	3.98	4.42	2.52	5.25	4.81	3.51	5.92	11.5	4.39	3.21	1.68
1949	10.36	15.1	6.82	4.02	4.85	2.46	5.06	5.45	3.53	6.13	11.6	4.65	3.25	1.74
Average	10.3		6.52	4.20	4.55	2.50	4.88	4.91	3.60	6.13	11.66	4.56	3.29	1.74
1950	15.78	135.6	6.54	4.17	4.69	3.12	4.41	6.71	3.34	6.69	12.1	4.36	3.11	1.78
1949	17.30	21.0	6.99	4.63	4.56	2.49	4.98	4.85	3.24	6.17	10.72	4.61	3.19	1.84
1949	16.04	31.1	6.51	4.53	4.80	2.84	4.23	6.60	3.25	4.78	12.3	4.71	3.48	1.66
1949	16.82	24.8	6.21	4.37	4.45	2.62	3.72	5.49	3.19	5.57	11.6	4.70	3.18	1.76
Average	16.5		6.56	4.42	4.63	2.77	4.33	5.91	3.26	5.80	11.68	4.60	3.24	1.76
Over-all mean			6.54	4.31	4.59	2.64	4.66	5.41	3.43	5.96	11.67	4.58	3.26	1.75

The crude protein percentages were obtained by multiplying the percentage values for total nitrogen by 6.0 instead of the conventional 6.25, because Jones ('26) has shown that the percentage of nitrogen in whole wheat is approximately 17% instead of 16%.

RESULTS

The amino acid contents of 4 low-protein and 4 high-protein whole wheat (Idaed) samples were determined in several independent experiments. The statistical analyses of the data had to be based upon those individual experiments. For

TABLE 2

Average amino acid content of high-protein and low-protein whole wheat

AMINO ACIDS	HIGH-PROTEIN SAMPLES	LOW-PROTEIN SAMPLES	AMINO ACIDS	HIGH-PROTEIN SAMPLES	LOW-PROTEIN SAMPLES
	% of N×6 [1]	% of N×6		% of N×6 [1]	% of N×6
Leucine	6.55	6.54	Phenylalanine	4.42	4.20
Isoleucine	4.46	4.48	Tyrosine	2.77	2.50
Valine	4.65	4.55	Arginine	5.91	4.91
Threonine	3.23	3.30	Proline	11.75	11.77
Histidine	1.76	1.73	Lysine	3.27	3.61

[1] The factor 6.0 was used instead of the conventional 6.25 because Jones ('26) has shown that the percentage of nitrogen in whole wheat is approximately 17% instead of 16%.

reasons of space only two representative experiments, together with the respective analysis of variance, are presented in tables 2 and 3, and in 4 and 5. The amino acid contents obtained by the various experiments are summarized in table 1. Amino acid contents are reported as percentages of total crude protein.

Tables 2 and 3 show that lysine and arginine varied significantly with protein content of the samples. In order to check this finding, another method of hydrolysis (Kofranyi, '50) was employed, which involved removing most of the carbohydrates before acid hydrolysis of the protein. The assays of hydrolysates produced by this method failed to show a significant difference in the arginine content of low-

and high-protein wheats. A significant difference in the content of lysine was also obtained with these hydrolysates. This confirms the findings of McElroy et al. ('49) and Price ('50) who found that high-protein wheat had relatively lower lysine contents.

TABLE 3

Analysis of variance of 10 amino acids contained in low-protein and high-protein whole wheat

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Samples	7	4.129
High-protein vs low-protein samples	1	0.798
Hydrolyzates within samples: error (a)	16	0.128
Amino acids (A.A.)	9	211.140
A.A. × samples	63	0.377 [2]
A.A. × high-protein vs low-protein samples [1]	9	0.697 [2]
Lysine vs other A.A. × high- vs low-protein	1	1.069 [3]
Arginine	1	5.250 [3]
Proline	1	0.139
Leucine	1	0.071
Isoleucine	1	0.110
Valine	1	0.000
Threonine	1	0.214
Histidine	1	0.026
Phenylalanine	1	0.010
Tyrosine	1	0.002
A.A. × hydrolyzates within samples: error (b)	144	0.126
Coefficients of variation: error (a): 7.80% error (b): 7.75%		

[1] Interaction is broken down into non-orthogonal comparisons.
[2] 1% level of significance.
[3] 1% level of significance.

In a second experiment (tables 4 and 5) a more detailed analysis of the interaction "Amino Acids × Whole Wheat Samples" suggests that yield and year as well as protein content affected the lysine percentages. Thus, within the "low-protein" as well as the "high-protein" groups the sample grown in 1950 was outstanding for its significantly

higher lysine content. In 1950 much higher yields of wheat (due to sprinkler irrigation whenever necessary throughout the season) were obtained than in 1949.

This second experiment is of special importance in the light of the recent study by Horn et al. ('53) discussed at some length under "Methods." Isoleucine and lysine behaved exactly alike in the presence of humin and varying amounts of

TABLE 4

Leucine, isoleucine, and lysine content of 4 low-protein and 4 high-protein whole wheat samples

YEAR	PROTEIN CONTENT	YIELD	LEUCINE	ISOLEUCINE	LYSINE
	%	*bu./ac.*	% of N × 6 [1]	% of N × 6	% of N × 6
1950	10.44	92.5	6.25	4.43	3.81
1949	10.00	39.0	6.43	4.64	3.54
1949	10.32	60.0	6.49	4.48	3.51
1949	10.36	15.1	6.85	4.86	3.47
Average	10.3		6.51	4.60	3.58
1950	15.78	135.6	6.53	4.69	3.38
1949	17.30	21.0	7.03	4.56	3.22
1949	16.04	31.1	6.60	4.80	3.24
1949	16.82	24.8	6.18	4.45	3.12
Average	16.5		6.57	4.62	3.24
Over-all mean			6.54	4.61	3.41

[1] The factor 6.0 was used instead of the conventional 6.25 because Jones ('26) has shown that the percentage of nitrogen in whole wheat is approximately 17% instead of 16%.

pyridoxamine, while leucine apparently was not affected. Yet, only the comparison "Lysine vs Isoleucine + Leucine × Low- vs High-Protein" is statistically significant while the comparison "Isoleucine vs Lysine + Leucine × Low- vs High-Protein" is not. This greatly discounts any possibility that the differences in lysine content found between high-protein and low-protein samples were due to different amounts of a stimulating factor formed by hydrolysis.

DISCUSSION

Despite large differences in protein content only minor differences in lysine content of whole wheat samples have been established. There are indications that other amino acids may also vary slightly with protein content or with certain

TABLE 5

Analysis of variance of amino acids, isoleucine, leucine and lysine

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Samples	7	0.281
High-protein vs low-protein samples	1	0.235
Hydrolysates within samples: error (a)	16	0.034
Amino acids (A.A.)	2	150.337
A.A. × samples	14	0.297 [2]
Lysine vs other A.A. × samples	7	0.426 [2]
Lysine vs other A.A. × high- vs low-protein	1	1.213 [2]
Lysine vs other A.A. within low-protein	3	0.447 [2]
1949 vs 1950	1	0.985 [2]
Remainder	2	0.355 [2]
Lysine vs other A.A. within high-protein	3	0.141 [1]
1949 vs 1950	1	0.270 [2]
Remainder	2	0.154 [1]
A.A. × high-protein vs low-protein samples	2	0.619 [2]
Lysine vs leucine plus isoleucine	1	1.213 [2]
A.A. × hydrolysates within samples: error (b)	32	0.031
Determinations	71	0.039
Coefficients of variation: error (a): 3.9% error (b): 3.8%		

[1] 5% level of significance.
[2] 1% level of significance.

environmental conditions (year, yield), but the methods of hydrolysis available for this kind of study were not precise enough to establish statistical significance in other cases.

There are two possible explanations for the significant difference in lysine contents of high-protein and low-protein whole wheat samples:

1. Changes in the individual protein molecules.
2. Changes in the proportions of the different proteins.

On the basis of known species and organ specificity of the proteins, the first possibility appears to be unlikely. On the other hand, varying proportions of the different proteins in the wheat kernel might be ascribed to morphological differences, which in turn would be caused by such environmental factors as nitrogen and moisture availability. It might thus be possible that in certain years, under certain conditions (irrigation), the germ portion of the kernel is comparatively larger than in other years and the lysine content might thus be increased, together with other amino acids known to be richer in the germ part of the kernel.

In view of the analytical data presented in this paper, showing that the ratio of amino acids in wheat of different crude protein content remains essentially the same, it is suggested that the use of such high protein wheat might cause some nutritional problems due to amino acid imbalances in the diet. This suggestion is strengthened by the report of Dobbins et al. ('50) which showed that corns of differing protein content gave markedly different growth results when fed to swine in diets of uniform total protein content. In contrast, when the high-protein and low-protein corns were included in the diets to supply equal amounts of protein essentially the same growth results were obtained.

SUMMARY

Four low-protein (10.3%) and 4 high-protein (16.5%) wheat samples (Idaed) were analyzed by microbiological procedures for their content of different amino acids to determine the influence of protein content on amino acid composition of whole wheat.

The high-protein samples contained significantly ($P < 0.01$) less lysine (expressed in percentage of crude protein) than did the low-protein wheat samples. The lower lysine content of crude protein resulted when nitrogen applications were made

as late as the boot, flowering, or milk stages of wheat growth. The lysine content also appeared to vary with year or yield or both.

Other changes in amino acid composition were indicated, but were not statistically significant.

The data show that great differences in protein content of wheat were produced by nitrogen application, with only slight differences in the amino acid content of the total crude protein being observed. Even though some of the differences were statistically significant they are of doubtful practical importance.

LITERATURE CITED

BARTON-WRIGHT, E. C. 1946 The microbiological assay of amino acids. II. The distribution of amino acids in the wheat grain. Analyst, *71:* 278.

DOBBINS, F. A., J. L. KRIDER, T. S. HAMILTON, E. B. EARLEY AND S. W. TERRILL 1950 Comparison of high and low protein corn for growing-fattening pigs in dry lot. J. An. Sci., *9:* 625.

HENDERSON, L. M., W. L. ERICKSON AND E. E. SNELL 1948 A micromethod for the microbiological determination of amino acids. J. Biol. Chem., *172:* 31.

HENDERSON, L. M., AND E. E. SNELL 1948 A uniform medium for the determination of amino acids with various organisms. Ibid., *172:* 15.

HORN, M. J., A. E. BLUM, E. F. GERSDORFF AND H. W. WARREN 1953 Sources of error in microbiological determinations of amino acids on acid hydrolysates. I. Effects of humin on amino acid values. Ibid., *203:* 907.

JONES, D. B. 1926 A new factor for converting the percentage of nitrogen in wheat into that of protein. Cereal Chem., *3:* 194.

KOFRANYI, E. 1950 Proteinhydrolyse bei Anwesenheit grosser Kohlenhydratmengen. Naturwissenschaften, *37:* 91.

MCELROY, L. W., D. R. CLANDININ, W. LOBAY AND S. I. PETHYBRIDGE 1949 Nine essential amino acids in pure varieties of wheat, barley and oats. J. Nutrition, *37:* 329.

MILLER, B. S., J. Y. SEIFFER, J. A. SHELLENBERGER AND G. D. MILLER 1950 Amino acid content of various wheat varieties. Cereal Chem., *27:* 96.

PENCE, W. J., D. K. MECHAN, A. H. ELDER, J. C. LEWIS, N. S. SNELL AND H. S. OLCOTT 1950 Characterization of wheat gluten. II. Amino acid composition. Ibid., *27:* 335.

PRICE, S. A. 1950 The amino acid composition of whole wheat in relation to its protein content. Ibid., *27:* 73.

SCHWEIGERT, B. S. 1948 The value of various feeds as sources of arginine, histidine, lysine, and threonine for poultry. Poultry Sci., *27:* 223.

STEELE, B., H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMAN 1949 Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem., *177:* 533.

GRAVIMETRIC DETECTION OF RESORPTION OF EMBRYOS IN RATS ON LATHYRUS ODORATUS DIET

ZOLTON T. WIRTSCHAFTER

The Research Laboratory, Veterans Administration Hospital and the University of Oregon Medical School, Portland

(Received for publication August 13, 1956)

In a previous communication it was reported (Walker and Wirtschafter, '56) that there were no instances of fetal resorption in lathyrus-fed rats prior to the 16th day after conception. It was found that the embryos were uniform in size and form, and exhibited a normal crown-rump length for their respective embryonic ages. Further observations and additional experiments indicate that in lathyrus-fed rats evidence of fetal resorption is present as early as the 12th day of gestation.

The failure to detect the presence of the resorptive process in embryos of the pea-fed rats from the 12th to the 15th day is overcome by means of weighing the embryos immediately after removal by Caesarian section. The edema that is present in the early stages of the resorptive process cannot be determined macroscopically and the stage of resorption that is characterized by diminution in size again cannot be detected macroscopically, hence gravimetric methods are employed.

METHODS

Forty adult female rats were subjected to the same environment, diets and autopsy procedures, as reported previously, except that the total weight of every individual embryo and placenta of the entire litter was determined. The diet consisted of one part, each, of the control diet and the Lathyrus

odoratus pea. This 1 : 1 mixture was ground into a fine powder and then made up into hard pea pellets. Four pregnant rats on a Lathyrus odoratus diet and 4 pregnant rats on a control diet were autopsied for each day between the 12th through 16th day after conception. Following Caesarian section, each embryo, together with its placenta was weighed immediately on a Roller-Smith torsion balance.

RESULTS

Figure 1 presents the weights of normal embryos and embryos of lathyrus-fed rats. The weight recorded includes the weight of the embryo, its amniotic fluid, and the placenta. The uniformity in weight of the normal controls will be noted. In contrast to this is the wide range of the weights of the embryos of the lathyrus-fed rats and their higher median values through the 14th day.

The resorptive process in the rat is not uniform within a given litter. Embryos from the same uterus demonstrate a wide range of resorption, from edema of the embryo, through resorption of the embryo, and finally to complete resorption of the placenta. Similar observations have been made by Nelson, Asling and Evans ('52) and Nelson, Lyons and Evans ('53) studying pteroylglutamic acid-deficient and pyridoxine-deficient rats, respectively. The marked variations in the weight of embryos from the same animal fed a pea-pellet diet demonstrates that the onset of the resorptive process occurred prior to the 12th day after conception. The higher median weights observed in the experimental animals prior to the 15th day are probably due to the edema initially characteristic of resorption.

The normal embryos of the 12th through 16th day after conception and the most severe examples of the resorptive process that we have observed in the embryos of lathyrus-fed animals of the same ages are presented in plate 1. The failure of macroscopic observations to detect the presence of edema in the 12- to 15-day embryos is overcome by means of weighing the embryos immediately following removal by Caesarian section.

SUMMARY

The onset of the resorption process in rat embryos is difficult to determine macroscopically. Because differences in severity are a characteristic of the resorptive process, it is possible to detect its onset by means of weighing the individual embryos of a litter after their removal by Caesarian section.

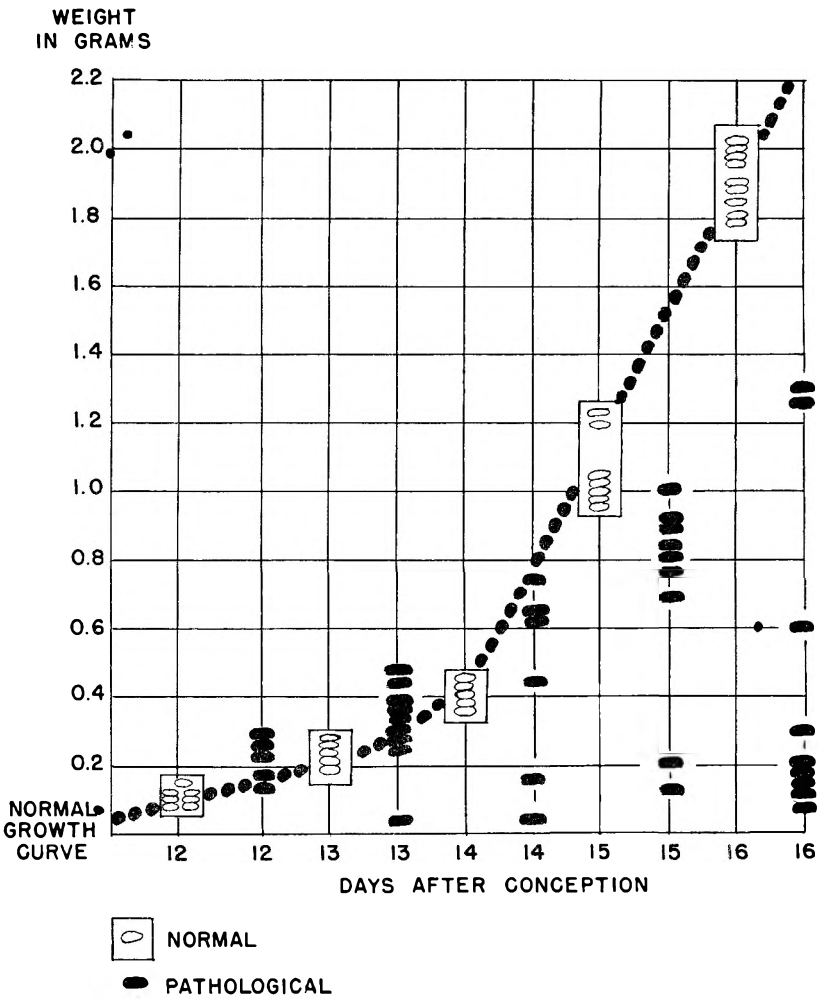


Fig. 1 Comparison of weights of embryos of litters of normal and pea-fed rats (fed from day of conception).

The weights of resorbing embryos show wide variation within a litter as compared to those of normally developing embryos. By this gravimetric method resorption in embryos of lathyrus-fed animals can be detected on the 12th day after conception.

LITERATURE CITED

NELSON, M. M., C. W. ASLING AND H. M. EVANS 1952 Production of multiple congenital abnormalities in young by maternal pteroylglutamic acid deficiency during gestation. J. Nutrition, *48:* 61–79.

NELSON, M. M., W. R. LYONS AND H. M. EVANS 1953 Comparison of ovarian and pituitary hormones for maintenance of pregnancy in pyridoxine-deficient rats. Endocrinology, *52:* 585–589.

WALKER, D. G., AND Z. T. WIRTSCHAFTER 1956 Resorption of embryos in rats on Lathyrus odoratus diet. J. Nutrition, *58:* 147–160.

PLATE

PLATE 1

EXPLANATION OF FIGURES

2 Normal rat embryos 12 days after conception.

3 Resorbing rat embryos 12 days after conception.

4 Normal rat embryos 13 days after conception.

5 Resorbing rat embryos 13 days after conception.

6 Normal rat embryos 14 days after conception.

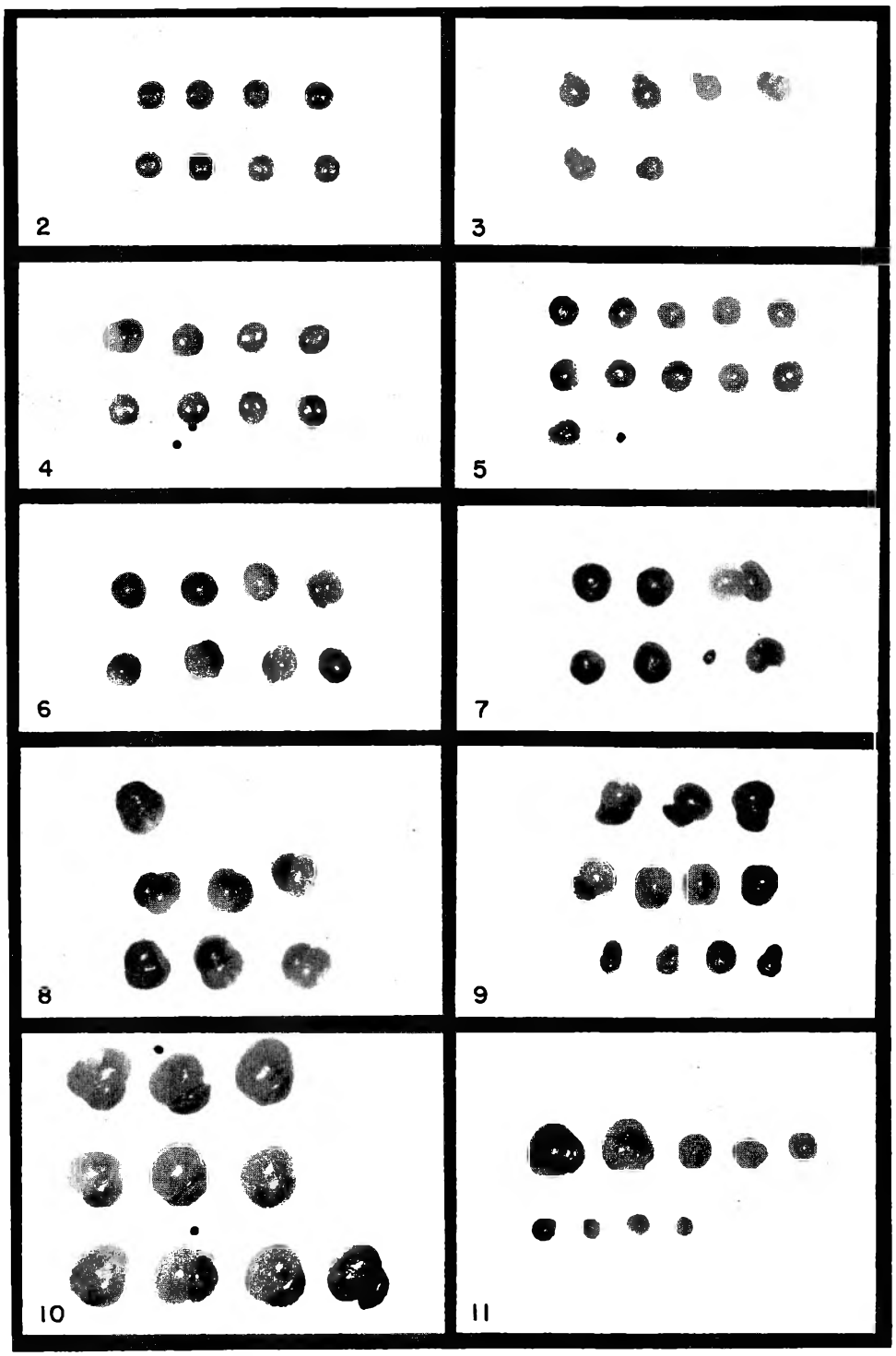
7 Resorbing rat embryos 14 days after conception.

8 Normal rat embryos 15 days after conception.

9 Resorbing rat embryos 15 days after conception.

10 Normal rat embryos 16 days after conception.

11 Resorbing rat embryos 16 days after conception.


2
3
4
5
6
7
8
9
10
11

COMPARATIVE REPRODUCTION AND LACTATION OF RATS FED BUTTER OR VEGETABLE FAT INCLUDED IN A BASAL RATION FREE OF ANIMAL PRODUCTS

LESLIE P. DRYDEN, JOSEPH B. FOLEY,[1] PAUL F. GLEIS, LANE A. MOORE AND ARTHUR M. HARTMAN

Dairy Husbandry Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

(Received for publication June 18, 1956)

The comparative effects of butterfat and various vegetable fats in regard to reproduction and lactation of rats have been studied by several investigators. Schantz et al. ('40) reported that although normal litters were obtained and reared by rats fed mineralized skimmed milk into which butterfat had been homogenized, young failed to survive when their mothers were fed similar diets containing corn oil or coconut oil instead of butterfat. Viswanatha and Liener ('56) also found a greater mortality of young from mothers fed corn oil than from those fed butterfat but only when the experimental rations contained sulfathalidine. On the other hand, most investigators (Deuel et al., '44, '45, '50; Sure, '41; v. Euler et al., '46a, b, '47, '51) failed to find significant differences among the fats tested in regard to reproduction and lactation.

The present investigation differed from the above experiments in that a basal ration devoid of any animal product was used whereas all of the above workers, in making their comparisons, used basal rations containing animal protein and, in some cases, other animal products also. Moreover, the conditions of reproduction and lactation were generally more rigorous than those used by the earlier investigators.

[1] Present address: National Institutes of Health, Bethesda, Maryland.

Such stress conditions included mating at an early age, omitting a rest period for the female between weaning a litter and remating, and refraining from reducing the number of young in a litter during lactation.

The primary comparisons were made between butter and a hydrogenated vegetable fat product. The butter diets contained either whole butter (WB) or the butterfat rendered from it (RBF). The vegetable fat diets contained either the whole hydrogenated vegetable fat product (WHVFP) or the fat rendered from this product (RHVF). WHVFP was a commercial product made to simulate butter (approximately 80% fat) and was composed primarily of hydrogenated cottonseed oil or hydrogenated soybean oil or both. It contained no animal fat except traces of butterfat (not $> 0.02\%$ of the total fat) contained in the skim milk added to the product during manufacture. Two experiments were carried out. They differed from each other in that in the second experiment, sulfathalidine was incorporated in all the rations and two additional vegetable fats, corn oil (CO) and cottonseed oil (CSO) were included in the comparisons.

EXPERIMENTAL PROCEDURE

The experimental animals used were albino rats (primarily of the Wistar strain) derived from a colony maintained in this laboratory for many years.

The stock colony mothers (F_0 generation) of the litters fed 10% fat were divided into two groups. The first group (60% of the total) was placed at parturition on the experimental ration containing RBF or the one containing RHVF. At weaning, 4 female young (F_1 generation) from each of these litters were distributed among the 4 rations containing WB, RBF, WHVFP or RHVF. The second group of mothers (40% of the total) was placed at parturition on the same ration which the young were to receive after weaning (containing WB, RBF, WHVFP or RHVF). In the case of the litters fed 30% fat, all mothers at parturition likewise received the same ration as their young were to receive after weaning.

When the female young (F_1 generation) reached 11 weeks of age, they were mated to stock males and carried through a number of successive pregnancies and 21-day lactation periods. Where possible, the rats were carried through three pregnancies on the 30% fat diets and 4 on the 10% fat diets. With the 30% fat diets, the tests were discontinued with the F_1 generation. With the 10% fat diets, female descendants of the F_1 generation rats were continued on their mothers' diets through the F_2, F_3 and F_4 generations and subjected to the same procedure as in the F_1 generation.

In the second experiment (with sulfathalidine) the comparisons were made at only one fat level, namely, 10%. The litters used were derived from stock colony mothers placed at parturition on the experimental ration containing CO and the young from each of their litters were distributed at weaning among the 6 different rations. The procedure used for reproduction and lactation was the same as that used in the first experiment, but the experiment was discontinued after the weaning of the second litter in the F_1 generation.

Each mother was kept in an individual cage, on wood shavings during late pregnancy and lactation but on raised screen floors at all other times.

WB used in experiment 1 was unsalted and was made from pasteurized sweet cream derived from milk collected from Holstein and Jersey cows in the Beltsville herd. The cows used had been for at least two months on a pasture ration consisting of blue grass or mixed orchard grass and ladino clover, grain and occasional hay supplements. WB used in experiment 2 was salted and consisted of a mixture of equal parts of 4 brands purchased on the retail market. WHVFP used in both experiments was a mixture of equal parts of 5 or, later, 4 brands of margarine purchased on the retail market. RBF and RHVF were prepared from their respective whole products under as mild conditions as possible, the temperature not being allowed to rise above 48°C. The 4 test fats and fat products were stored at —29°C. until used.

The 10% fat basal ration had the following percentage composition: sucrose, 46.86; soy protein,[2] 38.10; DL-methionine, 0.25; salt mixture,[3] 4.50; vitamin mixture,[4] 0.29; and fat, 10.00. The 30% fat basal ration had the following composition: sucrose, 17.00; soy protein,[2] 46.79; DL-methionine, 0.31; salt mixture,[3] 5.50; vitamin mixture,[4] 0.36; and fat 30.00. Thus, on a calorie basis, the rations at both fat levels contained the same amounts, respectively, of soy protein, methionine, minerals and vitamins. In preparing the rations containing WB or WHVFP, enough of the whole product was added to supply the fat level indicated, the diet being adjusted to allow for the small amounts of protein and carbohydrate supplied by the non-fat solids in the whole product. Where necessary, extra sodium chloride was added so that all rations in a particular experiment contained equal amounts of this salt per calorie of diet. In experiment 2, 1% sulfathalidine replaced 1% sucrose in the rations.

Fresh diets were made at approximately weekly intervals and stored in sealed Mason jars at — 3°C. or below between feedings. Glass feeders were used and a complete change of food was made each week. The rations and distilled water were supplied ad libitum.

RESULTS AND DISCUSSION

The data on the reproduction and lactation of the females used in these experiments are summarized in table 1.

With regard to number of young born per litter, there were no significant differences in experiment 1. In experiment 2,

[2] Orthoprotein #220, The Drackett Products Co., Cincinnati, Ohio.

[3] Salt mixture #12 (Jones and Foster, '42), modified to contain 92 μg $K_2Al_2(SO_4)_4 \cdot 24H_2O$ and 506 μg NaF per gram of total salt mixture.

[4] Micrograms per 10 gm of 10% fat diet: thiamine hydrochloride, 120; riboflavin, 120; pyridoxine hydrochloride, 120; calcium pantothenate, 750; choline chloride, 18,000; nicotinic acid, 750; inositol, 3000; *p*-aminobenzoic acid, 3000; biotin, 3; folic acid, 45; vitamin B_{12}, 0.5 ascorbic acid, 750; α-tocopherol acetate, 2000; 2-methyl-1,4-naphthoquinone, 40; calciferol, 2. Synthetic vitamin A acetate, dissolved in α-tocopherol, was fed separately by syringe at approximately weekly intervals at the rate of 100 I.U. per day.

TABLE 1

Reproduction and lactation in rats fed butter or vegetable fat

FAT IN RATION SUPPLIED BY	NO. OF FEMALES MATED	MATINGS RESULTING IN PREGNANCIES	PREGNANCIES RESULTING IN LITTERS	NO. OF LITTERS BORN	AVERAGE NO. OF YOUNG PER LITTER FOUND AT BIRTH		AV. BIRTH WT. OF LIVING YOUNG	SURVIVAL OF LIVING YOUNG AT WEANING	AVERAGE BODY WEIGHT AT WEANING	
					Living	Dead			Male young	Female young
		%	%				*gm*	%	*gm*	*gm*
Experiment 1 — 30% fat diets — F_1 generation										
WB[1]	6	88	100	10	9.0	0.7	5.6	91	47	46
RBF	6	96	100	15	9.5	0.3	5.4	61	34	34
WHVFP	6	76	100	14	6.9	0.9	5.6	75	38	38
RHVF	6	93	100	15	7.8	0.4	5.4	87	44	42
(F)[2]	..	(2.5)	...	..	(1.5)	(1.5r)	(2.9r)	(2.0)	(9.8**)	(7.0**)
L.S.D.[3]	..	..	...	..	..	..	..	..	5.0	6.5
Experiment 1 — 10% fat diets — F_1 generation										
WB	15	91	100	52	7.9	0.4	5.7	75	40	37
RBF	15	90	99	56	8.4	0.7	5.5	69	39	37
WHVFP	13	88	100	41	7.5	1.0	5.6	71	41	39
RHVF	14	90	100	39	7.9	0.8	5.8	74	41	40
(F)[2]	..	(1.0)	...	..	(2.8r)	(1.4)	(1.2)	(2.7r)	(1.2r)	(1.1)
Experiment 1 — 10% fat diets — F_2–F_4 generations										
WB	30	84	97	88	7.2	0.5	5.8	78	46	43
RBF	32	90	99	104	7.7	0.5	5.8	85	45	44
WHVFP	28	89	100	97	8.0	0.5	5.9	85	44	42
RHVF	24	85	99	79	8.5	0.9	5.8	83	42	40
(F)[2]	..	(1.4)	...	..	(2.1)	(1.5)	(15.7r*)	(1.0r)	(5.1**)	(4.2**)
L.S.D.[3]	..	..	...	..	..	..	..	..	2.2	2.2
Experiment 2 — 10% fat diets — F_1 generation										
WB	10	91	97	16	8.4	0.4	6.0	95	42	40
RBF	10	85	100	19	8.4	0.5	6.0	96	45	44
WHVFP	10	81	96	19	7.2	0.5	5.8	86	46	42
RHVF	10	72	100	17	9.8	0.5	6.0	90	45	42
CO	10	85	94	17	9.8	0.8	6.1	92	41	40
CSO	10	84	97	19	9.7	0.6	5.7	92	42	40
(F)[2]	..	(1.2r)	...	..	(2.7*)	(4.4r)	(1.1)	(1.2)	(1.2)	(1.5r)
L.S.D.[3]	..	..			1.86	..	..	..	..	..

[1] The diets used are designated as: WB (whole butter); RBF (butterfat rendered from whole butter); WHVFP (whole hydrogenated vegetable fat product); RHVF (fat rendered from whole hydrogenated vegetable fat product).

[2] The symbol ** adjacent to an F or t value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference; "r" indicates that the error mean square was greater than the treatment mean square.

[3] Least significant mean difference at the 5% level.

the rats fed RHVF, CO or CSO had a somewhat larger average number of living young in their litters than did those fed WHVFP. This difference was significant at the 5% level. There was no significant difference in this respect between the rats fed WB or RBF and those fed the other fats.

In the litters from the F_0 [5] and F_1 generation mothers of both experiments, the differences in weaning weights between the butter diets and the vegetable fat diets were either not significant or (in the case of the 30% fat diets, F_1 generation) not consistent. In the F_2 to F_4 generations (10% fat diets, experiment 1), the average body weights at weaning were slightly higher for the groups fed WB or RBF as compared to the groups fed WHVFP or RHVF. These differences, although small, were significant for both male and female young when the two fat groups (RBF and RHVF) were compared but only for the male young when the whole products (WB and WHVFP) were compared. The figures [5] for the three generations taken separately show this same tendency in 10 of the 12 comparisons (male and female young, three generations, whole products and rendered fats). The average number of young nursed per litter,[5,6] however, was significantly larger during the F_2 to F_4 generations for WHVFP as compared to WB and for RHVF as compared to RBF. It seemed possible that this difference in number of nursing young might explain the larger average weaning weights of the young from the mothers fed the butter diets (WB or RBF). Accordingly, the weaning weights were adjusted by covariance for this factor. The average differences between the butter (WB or RBF) and vegetable fat (WHVFP or RHVF) diets which previously had ranged from 2 to 10% for the 4 comparisons in the F_2 to F_4 generations combined were decreased by this adjustment to from 1 to 8% for the same comparisons. The differences between RBF and RHVF

[5] Not shown in table.

[6] Computed as an average of litter means, which were calculated by taking for each litter the mean of the three weekly averages of the number of young surviving at the beginning and end of each week of lactation.

were still significant but those between WB and WHVFP were not.

With regard to most of the criteria shown in table 1, it is clear that there were no significant differences among the various fats in any experimental group. Although not shown in the table, there was likewise no difference among the various fats in regard to the change in weight of the mother rats during lactation.

In experiment 1, comparisons of 4-week post-weaning weight gains were made on littermate pairs of rats of the same sex. One member of a litter from a female fed WB or WHVFP was put at weaning on WB while the other was put on WHVFP. Similarly, one young from a female on RBF or RHVF was put on RBF while its littermate was put on RHVF. When all of these littermate pairs were considered together, regardless of generation, level of fat or whether rendered fat or whole product was involved, 138 male and 132 female comparisons of littermate pairs were obtained. The average weight gains for the rats fed WB or RBF (155 gm for males, 110 gm for females) were slightly less than those for rats fed WHVFP or RHVF (158 gm for males, 114 gm for females). Considering individual pairs, the rats fed WB or RBF gained less than those fed WHVFP or RHVF in 54% of the male comparisons and 55% of the female comparisons. Although the average differences were quite small (2% to 3%) they were statistically significant ($t = 2.43$ for males, significant at 5% level; and $t = 2.88$ for females, significant at 1% level).

Food consumption was followed during the F_1 generation in experiment 1 for the rats fed RBF or RHVF and the average 4-week post-weaning gain in weight per gram of food consumed was calculated, as a measure of the efficiency of food utilization during this period. A comparison of the two diets showed practically no difference between the rations at a particular fat level.

Females on the 10% fat diets in experiment 1 were on experiment longer than any of the other groups. When a 4th litter from a mother rat in this group had been weaned or

failed to survive, the rat was discarded. Some of these females, however, died or were discarded prior to that time for failure to conceive. When all generations were considered together, it was found that 16% of the females started on WB or RBF died of natural causes before the weaning of a 4th litter, while 9% of those started on WHVFP or RHVF

TABLE 2

Comparative weights of organs and glands of F_1 generation male rats [1]

ORGAN OR GLAND	NO. OF RATS PER DIET [2]	ORGAN OR GLAND WEIGHT [3]					
		30% fat level		10% fat level		F [4]	
		WB or RBF [5]	WHVFP or RHVF	WB or RBF	WHVFP or RHVF	Kind of fat	Level of fat
		mg/100 gm body weight		*mg/100 gm body weight*			
Seminal vesicles	9	58	59	66	69	2.8r	6.1*
Testes	9	821	845	861	899	1.1r	1.2
Adrenals	7	6.5	6.8	6.8	6.4	68.2r	20.6r
Kidneys	9	832	901	864	908	3.0	1.9r
Spleen	8	176	202	228	244	1.5	7.5*
Liver	8	4295	4571	4623	4804	4.5*	7.0*
Heart	8	302	291	304	299	1.1r	52.0r
Thyroid	9	5.1	5.4	5.1	5.6	2.4	41.0r
Brain	8	505	510	530	547	1.7r	2.5
Pituitary [6]	8	1.9 [7]	1.9 [7]	2.0	2.1	14.3r	1.9

[1] Seventy-seven to 85 days of age.

[2] One rat from a given litter on each diet. Littermates killed at same age.

[3] The average body weights were 359, 365, 336 and 335 gm for the respective groups.

[4] See footnote 2, table 1.

[5] See footnote 1, table 1.

[6] Anterior lobe.

[7] Seven rats in group.

failed to survive. Similarly, after a number of trials, 22% of the rats fed WB or RBF were discarded because of failure to conceive, while 28% of those on WHVFP or RHVF were disposed of for this cause. In the case of both fats, 62% of the females had 4 complete pregnancies.

In table 2 is shown a comparison of the weights of certain organs and glands of a group of 11- to 12-week-old F_1 generation male young, in experiment 1, who had been on their

respective diets from birth. The weights have been expressed on the basis of 100 gm of body weight. On this basis, the weights of the seminal vesicles, spleen and liver were significantly higher on diets containing 10% fat than on those containing 30% fat, regardless of the type of fat involved. So far as differences between the various types of fat are concerned, only in the case of the liver was there a statistically significant difference. The liver weight per unit of body weight was somewhat smaller for the rats fed WB or RBF than for those fed WHVFP or RHVF.

As far as experiment 2 was carried (through two litters in the F_1 generation), the inclusion of sulfathalidine in the soy protein rations containing 10% fat failed to bring about any differences between WB or RBF on the one hand and WHVFP or RHVF on the other, or between WB or RBF and CO or CSO (table 1).

Thus despite the fact that all sources of animal products were eliminated from the basal ration and the conditions of reproduction and lactation were rigorous, the results did not differ markedly from those obtained by the majority of investigators. In most respects, no significant differences were found among the various fats and such differences as were obtained were not large.

SUMMARY

The effects of butter or butterfat have been compared with those of a hydrogenated vegetable fat product or the fat rendered from it in regard to reproduction and lactation of rats fed these fats over several generations in a basal ration free of any animal product. Fairly rigorous conditions of reproduction and lactation were used. In most respects, no significant differences were found among the various fats in regard to the various aspects of reproduction and lactation. Such differences as were observed were not large. In the F_2 to F_4 generations combined, average weaning weights of the young were found to be significantly higher for butterfat as

compared to the hydrogenated vegetable fat but not for whole butter as compared to the whole vegetable fat product. Post-weaning weight gains of rats fed the two different fats or fat products differed by only 2 or 3% on the average. A comparison was also made between the weights of certain organs and glands of 11- to 12-week-old male rats fed the butter or vegetable fat rations. The inclusion of sulfathalidine in the rations failed to bring about any differences among these and other fats tested.

LITERATURE CITED

DEUEL, H. J., JR., S. GREENBERG, E. E. SAVAGE AND L. A. BAVETTA 1950 Studies on the comparative nutritive value of fats. XIII. Growth and reproduction over 25 generations on Sherman Diet B where butterfat was replaced by margarine fat, including a study of calcium metabolism. J. Nutrition, *42:* 239.

DEUEL, H. J. JR., L. F. HALLMAN AND E. MOVITT 1945 Studies on the comparative nutritive value of fats. VI. Growth and reproduction over ten generations on Sherman Diet B where butterfat was replaced by margarine fat. Ibid., *29:* 309.

DEUEL, H. J., JR., E. MOVITT AND L. F. HALLMAN 1944 Studies on the comparative nutritive value of fats. IV. The negative effect of different fats on fertility and lactation in the rat. Ibid., *27:* 509.

V. EULER, B., AND H. V. EULER 1951 Influence of fat nutrition upon the growth the fertility and the longevity of rats. IV. Arkiv. Kemi, *3:* 31.

V. EULER, B., H. V. EULER AND I. RÖNNESTAM-SÄBERG 1946a Influence of fat nutrition upon the growth, the fertility and the longevity of rats. I. Arkiv. Kemi, Mineral Geol., *22A(8):* 1.

——— 1946b Influence of fat nutrition upon the growth, the fertility and the longevity of rats. II. Ibid., *24A(15):* 1.

——— 1947 Influence of fat nutrition upon the growth, the fertility and the longevity of rats. III. Ibid., *24A(20):* 1.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

SCHANTZ, E. J., C. A. ELVEHJEM AND E. B. HART 1940 The comparative nutritive value of butter fat and certain vegetable oils. J. Dairy Sci., *23:* 181.

SURE, B. 1941 Dietary requirements for fertility and lactation. XXVIII. The lactation-promoting properties of cystine when added to casein diets. J. Nutrition, *22:* 491.

VISWANATHA, T., AND I. E. LIENER 1956 Reproduction and lactation of rats receiving corn oil or butterfat in the presence of sulfathalidine. Ibid., *59:* 197.

PANTOTHENIC ACID DEFICIENCY IN THE GROWING CALF

A. J. SHEPPARD AND B. CONNOR JOHNSON
Division of Animal Nutrition, University of Illinois, Urbana

(Received for publication August 20, 1956)

In previous communications it has been shown that the ruminant, as exemplified by the young calf or lamb, requires thiamine (Johnson et al., '48; Draper and Johnson, '51), riboflavin (Wiese et al., '47), nicotinic acid-tryptophan (Hopper and Johnson, '56), pyridoxine (Johnson, Pinkos and Burke, '50), vitamin B_{12} (Draper, Sime and Johnson, '52), biotin (Wiese, Johnson and Nevens, '46), choline (Johnson et al., '51), and folic acid (Draper and Johnson, '52), of the B vitamin series. Preliminary reports have indicated that the young dairy calf also has a requirement for pantothenic acid (Johnson et al., '47; Johnson, Nevens and Mitchell, '51). However, the syndrome originally produced appeared to be non-specific and unsatisfactory. More recent work with an improved type of ration (Hopper and Johnson, '55) has now been carried out and is reported here.

Pantothenic acid deficiency has previously been described in the young pig (Wintrobe et al., '43), the dog (Schaefer, McKibbin and Elvehjem, '42), and the guinea pig (Reid and Briggs, '54), as well as in rats, mice and poultry. Recently Bean and Hodges ('54) induced symptoms in the human by the use of omega-methyl-pantothenic acid. This antimetabolite was also tried in the present study with calves.

EXPERIMENTAL

Twenty-five calves of the various major dairy breeds have been used in two experiments. When placed on experiment

the calves ranged in age from one to 5 days, with a mean of 2.5 days. The calves were housed as previously reported by Draper, Sime and Johnson ('52) and fed from nippled pails.

Vitamin A, 100,000 units, and vitamin E, 200 mg, were given weekly by capsule. Vitamin D, 10,000 units, was given monthly by capsule. To control scours, terramycin, 13 mg per liter of diet (or sulfathalidine, 2% of the dry matter of the diet) was used. The drug was given only during periods of actual scouring.

The composition of the pantothenic acid-free synthetic milk ration used is given in table 1 and the composition of the vitamin supplement in table 2. The calves were fed the diet ad libitum.

Two experiments were carried out. In the first experiment 9 calves were fed the pantothenic acid-free diet and 5 others fed the same ration with pantothenic acid supplementation. In the second experiment 11 calves were fed the pantothenic acid-deficient diet but instead of a positive control group, recovery following therapy was used as an index of previous deficiency. The therapeutic treatment consisted of the intramuscular injection of calcium pantothenate at the rate of 0.5 gm the first day, followed by 0.1 gm daily thereafter. The animals in this experiment were permitted to develop symptoms until they lost weight for at least two consecutive weeks before therapy was begun. Also in this experiment omega-methyl-calcium-pantothenate was tried as a metabolic antagonist with two calves.

Feed consumption records were kept on all calves and all calves were weighed at weekly intervals. All calves which died were autopsied and examined grossly as well as histologically.[1]

[1] We wish to express our appreciation to Drs. R. M. Thomas, D. R. Lingard, and J. R. Pickard of The Illinois State Department of Agriculture Diagnostic Laboratory and Dr. R. D. Hatch of the Department of Veterinary Clinical Medicine for carrying out these examinations.

In the first experiment one-day urine collections were made at weekly intervals from two positive control calves and from 6 calves on the pantothenic acid-free diet. These samples were analyzed for pantothenic acid by the *L. arabinosus* assay procedure (Cheldelin et al., '51). The blood picture of these

TABLE 1

Basal synthetic diet

INGREDIENT	AMOUNT IN GRAMS FOR 100 GAL.	
$Ca(OH)_2$	908.48	Dissolve successively in 65 gal. ice water.
Casein [1]	14,757.60	
DL-Methionine	98.38	
KOH	363.39	Mix in 5 gal. cold water before adding.
NaOH	352.04	
Allow to mix for 30 minutes, then add:		
Glucose [2]	13,896.74	
$CaCl_2 \cdot H_2O$	807.53	Mix in 7 gal. cold water before adding.
MgO	113.56	
KH_2PO_4	340.68	Dissolve in 7 gal. hot water before adding.
Citric acid	832.77	
NaH_2PO_4	1,851.03	
$CuSO_4$	0.88	
KI	2.51	
$MnSO_4$	4.43	
$ZnCl_2$	0.74	
CaF_2	1.48	
Fe citrate	103.53	
Lard emulsion, 60%	24,596.00	

[1] Labco.

[2] Cerelose.

TABLE 2

Vitamin solution

(8 ml of this solution was fed per 1 liter of synthetic milk diet)

INGREDIENT	QUANTITY PER 9 LITER VOLUME	INGREDIENT	QUANTITY PER 9 LITER VOLUME
Thiamine	0.9 gm	Folic acid	360 mg
Riboflavin	1.8 gm	Ascorbic acid	22.5 gm
Pyridoxine	1.8 gm	Vitamin K	360.0 mg
Nicotinic acid	3.6 gm	Choline	180.0 gm
Vitamin B_{12}	20 mg	Ethyl alcohol (95%)	3 liters
Biotin	22.5 mg	Distilled water	6 liters

calves was also examined at regular intervals. The blood in some of these calves was also assayed for pantothenic acid.

In the case of two calves, intestinal contents were taken for pantothenic acid assay at post mortem in order to determine the extent of intestinal synthesis of this vitamin.

TABLE 3

Data on calves in experiment 1

CALF NO.	BREED	WEEKS SURVIVED	GAIN	URINARY PANTOTHENIC ACID		
				At start of experiment	Min. value	Week at which min. value first reached
			lb./week	*mg/day*	*mg/day*	
		(Pantothenic acid-deficient ration)				
18	Ayrshire	11	3.1	47.5	0.14	9
19	Guernsey	16	4.6	13.7	0.67	9
34	Holstein	20	1.5	31.5	0.1	8
41	Jersey	12	7.3	3.0	0.5	12
49	Holstein	14	1.6	66.2	1.1	10
54	Holstein	14	1.75	7.7	0.03	8
		(Pantothenic acid-supplemented ration)				
					Value at 8 weeks	
24	Holstein		5.2			
33	Jersey		4.0	18.9	11.6	
37	Holstein		5.9			
40	Holstein		9.7	4.7	14.0	

RESULTS

Experiment 1. Of the 9 calves fed the pantothenic acid-deficient diet, two died after one day and two days respectively from other causes, and one inadvertently received pantothenic acid for two weeks and was thus perfectly normal after 20 weeks, having gained 112 lb. The other 6, however, all succumbed to the deficiency in times ranging from 11 to 20 weeks, as indicated in table 3.

The blood picture was normal in all calves with regard to hemoglobin, red cell count, white cell count, and differential, the mean values being 10 gm/100 ml, 11 million/mm^3, and 11 thousand/mm^3 for hemoglobin, red blood cells, and white blood cells, respectively. With regard to blood pantothenic

acid, two deficient calves showed essentially constant values of approximately 15 μg per 100 ml from 3 to 13 weeks. A third calf also on this pantothenic acid-free diet had a blood level of 450 μg per 100 ml at one day of age. This level fell to approximately 15 μg at 3 weeks, but then continued to decline to 3 μg per 100 ml after 9 weeks on experiment. The intestinal contents of the two calves assayed contained 3 and 4 μg pantothenic acid per gram wet sample, which would definitely indicate some pantothenic acid synthesis and may indicate why calves 19 and 41 grew satisfactorily on the deficient ration. However, histological examination of calf 19 revealed a patchy, mild to moderate, proliferation of the Schwann cells and a moderate demyelination of the peripheral nerves. The deficient calves (in particular calves 34, 49 and 54) showed gross symptoms of the deficiency in addition to a poor growth. These symptoms included diarrhea, poor haircoat, and later weakness of the legs with inability to stand.

Experiment 2. In view of the rather unsatisfactory nature of this first experiment, a second series of 11 calves has recently been used in a further elucidation of the deficiency. Deficiency symptoms of varying degrees of severity were demonstrated in all 11 of these calves. Five calves recovered following pantothenic acid therapy, while 6 of the calves died, three without pantothenic acid treatment, usually from pneumonia, which progressed rapidly in all the deficient calves. One calf died of bloat caused by a hair ball lodged in the pylorus.

Typical of the deficient calves in this experiment are the following two case histories. Calf 349, a Brown Swiss, was two days old when placed on the experimental pantothenic acid-free synthetic milk diet. The animal started scouring at 3 days and scoured intermittently throughout the period on the deficient diet. After 22 days the haircoat was rough and starry and lacrimation was evident. The calf went "off feed" at 47 days and from then on feed consumption was rather poor. A this time, the calf also developed a "cold." By 55 days there was a heavy scaly dermatitis about the eyes,

a typical "spectacle eye" condition. This dermatitis also appeared about the muzzle, and the calf had been losing weight, as can be seen from figure 1. On the 57th day pantothenic acid therapy was started. At this time the spectacle eye appearance was very marked due to the scaly dermatitis and accumulation of fluid around the eyes. The haircoat was rough and there were some scaly areas in the skin. The skin

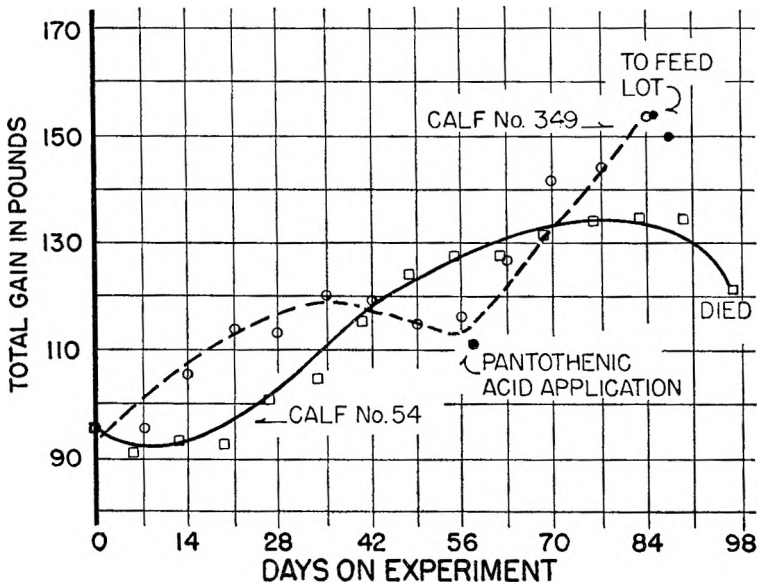


Fig. 1 Growth curves for two calves on the pantothenic acid-free diet.
Calf 54. A pantothenic acid-deficient calf from experiment 1.
Calf 349. A pantothenic acid-deficient calf from experiment 2.

was also denuded under the lower jaw. These symptoms can be seen in the photographs (fig. 2). Excess salivation and nasal mucous discharge was also evident. By the 59th day the nasal discharge had cleared up and the animal was eating well, although quivering all the time he ate. By the 83rd day all visible symptoms had disappeared and the calf was sent to the feed lot.

Animal 396, a Holstein, was 5 days old when started on experiment. It was noted throughout the experiment that

a considerably longer time was required to develop the dificiency symptoms in calves started on experiment at 4 to 5 days of age than in those 1 to 2 days of age. This calf also started scouring after 3 days on experiment and this was a constant symptom during the deficiency. By 43 days he had developed a "cold" and cough, and by 53 had reduced his feed consumption. At 71 days the typical dermatitis about the eyes (spectacle eye) was evident. By 91 days the animal was nervous and unsteady when standing. By 105 days these symptoms were all much more severe. The animal exhibited

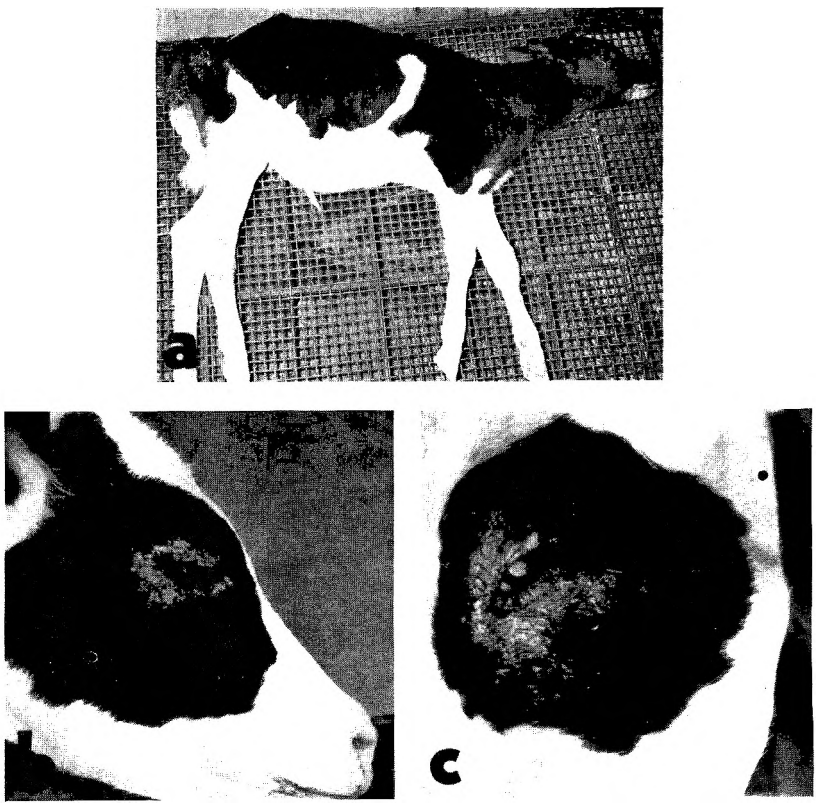


Fig. 2 Illustrations of pantothenic acid deficiency in the growing calf.
(a) This calf was unable to stand due to weakness.
(b) Typical example of the "spectacle eye" condition.
(c) Another example of the eye lesion.

incoordination of movement, was nervous, appeared emaciated. The scaly dermatitis around the eyes was cracked and moist, and there was a light denuded area under the chin. At this time pantothenic acid therapy was begun. By the 113th day the exudate had begun to clear from around the eyes and the calf was again eating well. By the 126th day the symptoms were no longer visible and the animal was sent to the feed lot.

In the three cases where no therapeutic treatment was attempted, post mortem examination revealed much inflammation and hemorrhage in the mucosa throughout the intestinal tract. In all other animals examined, whether cured or treated at an advanced stage of development of the syndrome and also where treatment failed to bring about recovery of the animal (2 calves), there was no visible evidence of hemorrhage occurring in the intestinal tract.

Histopathological examination of the cerebrum, cerebellum, pons, and spinal cord revealed a moderate hyperemia accompanied by hemorrhages. A demyelination of the sciatic nerve was noted in several cases. A softening in the cerebrum along with some congestion and minute extravasations of blood was occasionally noted in the depleted animals. Examination of the sciatic nerve revealed extensive edema in the fascial planes of the surrounding musculature even when the nerve was intact.

A large percentage of the calves showed pneumonic changes in the lung tissue. It involved, in many cases, both cardiac lobes and the central aspects of the apical lobes.

In several instances the renal cortices were very pale and the medullae congested.

In one extremely advanced case the calf showed tonic-clonic convulsions, was unable to stand, showed a running movement while down, and some opisthotonos.

Figure 2 shows the dermatitis around the eye and the denuded area under the lower jaw which were found typical of pantothenic acid deficiency.

Animals injected with 100 mg daily of omega-methyl-pantothenic acid made excellent weight gains and showed none of

the symptoms noted in the other calves fed pantothenic acid-free diets. These results would lead one to believe that perhaps the compound omega-methyl-pantothenic acid is not an antagonist for the calf.

DISCUSSION

The characteristics of this deficiency as demonstrated in the growing calf are in general supported by the descriptions previously reported for other species.

Wintrobe et al. ('43) reported diarrhea, loss of appetite, very marked impairment of growth, cough and excessive nasal secretion, and abnormal gait in the young pig. Convulsions and an enteritis similar to that reported in this experiment were reported in the pantothenic-deficient dog by Schaefer, McKibbin and Elvehjem ('42). Wiese et al. ('51) reported that pantothenic acid deficiency in the young pig is characterized by poor growth, loss of appetite, scours, lacrimation, dermatitis, coughing, a dark brown exudate around the eyes, spastic gait, "goose-stepping," alopecia, and low urinary excretion of pantothenic acid; in general the same syndrome as noted in the calf. Luecke et al. ('49) also reported symptoms of locomotor incoordination and a degeneration of the myelin sheath in the pig.

Myelin degeneration was found in the sciatic nerve and in the spinal cord and desquamative dermititis similar to that reported by Lippincott and Morris ('41) for pantothenic acid-deficient CBH mice.

The deficiency symptoms reported for the calf are very similar to those reported by Reid and Briggs ('54) for young guinea pigs supplied a "synthetic" diet lacking pantothenic acid.

It is apparent from this experiment that pantothenic acid is required for the proper function of the myelin sheath of the sciatic nerve and of the spinal cord. This may explain the bicycle motion noted in the calf prior to death and the "goose-stepping" reported in other species.

We were unable to induce an abnormal metabolic state in the calf using the metabolic antagonist omega-methyl-pantothenic acid as Bean and Hodges ('54) did in the human. The two calves given the antagonist made excellent gains and showed no signs of deficiency symptoms. Symptoms did occur some two weeks following the discontinued use of the antagonist in a pantothenic acid-free diet. However, these data were obtained only in two calves, thus need further investigation.

SUMMARY

Pantothenic acid deficiency was produced in calves fed an improved pantothenic acid-free synthetic milk diet. Major symptoms of the deficiency noted include rough haircoat, spectacle eye, dermatitis under lower jaw, excessive nasal mucous, loss of appetite, reduced growth rate, eventual loss of weight followed by death. The internal symptoms included pneumonic changes in lung tissue, demyelination of sciatic nerve and spinal cord, extensive edemas in the fascial planes of muscle tissue, and softening and congestion of the cerebrum.

It was possible to cure the calves with calcium pantothenate therapy.

LITERATURE CITED

BEAN, W. B., AND R. E. HODGES 1954 Pantothenic acid deficiency induced in human subjects. Proc. Soc. Exp. Biol., *86:* 693–698.

CHELDELIN, V. H., A. P. NYGAARD, O. M. HALE AND T. E. KING 1951 Pantothenic acid involvement in fatty acid oxidation. J. Am. Chem. Soc., *73:* 5004–5005.

DRAPER, H. H., AND B. C. JOHNSON 1951 Thiamine deficiency in the lamb. J. Nutrition, *43:* 413–422.

———— 1952 Folic acid deficiency in the lamb. Ibid., *46:* 123–132.

DRAPER, H. H., J. T. SIME AND B. C. JOHNSON 1952 A study of vitamin B_{12} deficiency in the calf. J. Animal Sci., *11:* 332–340.

HOPPER, J. H., AND B. C. JOHNSON 1955 The production and study of an acute nicotinic acid deficiency in the calf. J. Nutrition, *56:* 303–310.

———— 1956 Transmethylation reactions, *in vivo* and *in vitro* in the young calf. Proc. Soc. Exp. Biol. Med., *91:* 497–499.

JOHNSON, B. C., H. H. MITCHELL, T. S. HAMILTON AND W. B. NEVENS 1947 Vitamin deficiencies in the calf. Fed. Proc., *6:* 410.

JOHNSON, B. C., T. S. HAMILTON, W. B. NEVENS AND L. E. BOLEY 1948 Thiamine deficiency in the calf. J. Nutrition, *35:* 137–146.

JOHNSON, B. C., J. A. PINKOS AND K. A. BURKE 1950 Pyridoxine deficiency in the calf. Ibid., *40:* 309–322.

JOHNSON, B. C., H. H. MITCHELL, J. A. PINKOS AND C. C. MORRILL 1951 Choline deficiency in the calf. Ibid., *43:* 37–48.

JOHNSON, B. C., W. B. NEVENS AND H. H. MITCHELL 1951 B. Vitamin nutrition of the dairy calf studied with "synthetic milk" diets. J. Dairy Sci., *34:* 506.

LIPPINCOTT, S. W., AND H. P. MORRIS 1941 Morphologic changes associated with pantothenic acid deficiency in the mouse. J. Nat. Cancer Inst., *2:* 39–46; Chem. Abs., *35:* 8044.

LUECKE, R. W., F. THORP, JR., W. N. MCMILLEN AND H. W. DUNNE 1949 Pantothenic acid deficiency in pigs fed diets of natural feedstuffs. J. Animal Sci., *8:* 464–469.

REID, M. E., AND G. M. BRIGGS 1954 Nutritional studies with the guinea pig. II. Pantothenic acid. J. Nutrition, *52:* 507–517.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942 Pantothenic acid deficiency studies in dogs. J. Biol. Chem., *143:* 321–330.

WIESE, A. C., B. C. JOHNSON AND W. B. NEVENS 1946 Biotin deficiency in the dairy calf. Proc. Soc. Exp. Biol. Med., *63:* 521–522.

WIESE, A. C., B. C. JOHNSON, H. H. MITCHELL AND W. B. NEVENS 1947 Riboflavin deficiency in the dairy calf. J. Nutrition, *33:* 263–270.

WIESE, A. C., W. P. LEHRER, JR., P. R. MOORE, O. F. DAHNISH AND W. V. HARTWELL 1951 Pantothenic acid deficiency in baby pigs. J. Animal Sci., *10:* 80–87.

WINTROBE, M. M., R. H. FOLLIS, JR., P. ALCAYAGA, M. PAULSON AND S. HUMPHREYS 1943 Pantothenic acid deficiency in swine. With particular reference to the effects on growth and on the alimentary tract. Bull. Johns Hopkins Hosp., *73:* 313–341.

INVESTIGATIONS ON THE METABOLISM OF FLUORIDE

IV. FLUORIDE BALANCE STUDIES AT HIGH LEVELS OF INTAKE IN RABBITS [1]

DWIGHT E. GARDNER, THOMAS G. SCHARFF, FRANK A. SMITH AND HAROLD C. HODGE

Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

(Received for publication August 29, 1956)

There is, at the moment, a lively interest in the skeletal storage of fluoride. The widespread fluoridation of community water supplies in this country makes the metabolism of fluoride a matter of public health import. That fluoride is a bone seeker, all agree. There is an equally clear-cut disagreement on storage rates. Some hold that at a given level of fluoride ingestion, a steady state is established such that excretion essentially equals absorption. Others believe that at any save trace levels of ingestion there is a continuing storage that is greater with greater fluoride absorption. Only a few experimental approaches have been made; the present investigation explores the bone storage when a wide range of fluoride doses was given rabbits for a relatively short period.

EXPERIMENTAL

Ten young male albino rabbits weighing 2.9 to 3.8 kg were distributed randomly into 5 groups of two animals each. The

[1] This paper is based on work performed in part under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York, and supported in part by a grant from the E. I. duPont de Nemours and Co.

animals were housed individually in metabolism cages for one week prior to and during the 19-day experimental period. A commercial pellet ration (fluoride content, 38 ppm) was fed ad libitum. The drinking water contained 0 (control group given distilled water), 15, 60, 90, or 150 ppm fluoride as sodium fluoride and was supplied ad libitum. Daily measurements of food and water consumption were recorded for each animal. Pooled urine and feces samples collected in 4 intervals of 4 or 5 days each were analyzed for fluoride content. Fluorides were determined by the modified Willard-Winter distillation (Flagg, '49) and titration procedure described by Smith and Gardner ('50).

RESULTS AND DISCUSSION

All rabbits showed normal weight gains. The absence of acute damage to kidney tubule cells was indicated by normal urinary protein and sugar percentages.

Briggs and Phillips ('52) put the upper limit of fluoride ingestion for normal growth in young rabbits at 11 mg of fluoride per kilogram of body weight per day. At the highest ingestion level in this investigation, two young adult rabbits tolerated 9 and 15 mg/kg/day respectively.

Fluoride balance. The average fluoride intake (food and water separately) and the average fluoride excretion via urine and feces for each rabbit are presented graphically in figure 1. The average total amounts of fluoride ingested by the 5 groups were as follows: 3.7, 7.4, 18, 26, and 36 mg fluoride per day; of this, 3.2 to 4.2 mg per day were dietary, the rest came from the drinking water. Balances were computed ($\frac{\text{ingested} - \text{excreted}}{\text{ingested}} \times 100$) for each interval (table 1). With a single exception, all of the balances were positive; the mean value for all groups was + 51%. Only 4 values less than + 40% and none over + 70% were found; most of the data were closely grouped: viz., 7 values in the range + 40 to 49%, 21 from + 50 to 59% and 8 from + 60 to 69%.

Slightly higher values were obtained at the higher levels of ingestion; perhaps water-borne fluorides are slightly more

available than dietary fluorides. The literature is not clear on this point. Using rats, McClure ('39) found no differences in availability, whereas Lawrenz and Mitchell ('41) and Weddle and Muhler ('54) found sodium fluoride more readily

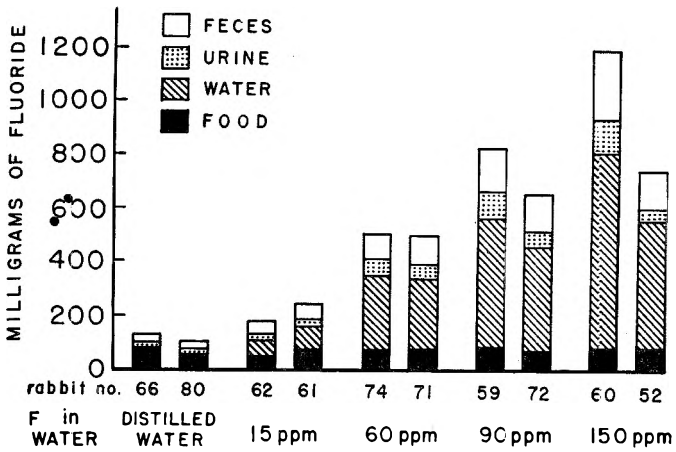


Fig. 1 Total fluoride intake and excretion for each rabbit during a 19-day period.

TABLE 1

Fluoride balances in rabbits

RABBIT NO.	WATER CONTENT OF F	FLUORIDE BALANCE, PER CENT [1]				
		Interval 1	Interval 2	Interval 3	Interval 4	Average
	ppm					
66	0	+ 65	+ 64	+ 21	+ 44	39
80	0	+ 57	— 11	+ 36	+ 34	
62	15	+ 56	+ 42	+ 42	+ 40	46
61	15	+ 50	+ 55	+ 44	+ 41	
74	60	+ 63	+ 55	+ 57	+ 50	54
71	60	+ 60	+ 50	+ 50	+ 45	
59	90	+ 60	+ 52	+ 50	+ 53	55
72	90	+ 56	+ 59	+ 53	+ 58	
60	150	+ 58	+ 51	+ 50	+ 51	59
52	150	+ 69	+ 58	+ 59	+ 69	

[1] The difference between ingested and excreted fluorides is expressed as a percentage of that ingested.

assimilated from water than from food. Perhaps the brief experimental period was too short to establish steady state conditions for rabbits on the higher doses. The average percentage balances for the 4 time intervals were 59, 54, 47, and 48; the decrease may indicate a saturation phenomenon in which the available skeletal surfaces fix fluoride by an exchange reaction (Neuman et al., '50). Longer periods of observation might well show lower rates of positive balances at the higher levels of intake. Jackson ('55) Zipkin and McClure ('52) and Savchuck and Armstrong ('51) have shown that the growing rat shows a diminishing ability to accumulate fluoride in the skeleton.

The percentages retained by these young rabbits confirm the reports of positive fluoride balances in rats (Savchuck and Armstrong, '51; Wallace-Durbin, '54; Jackson et al., '50; Weddel and Muhler, '54, '55), rabbits (Schulz, '51; Smith, Gardner and Hodge, '55) and sheep and cattle (Hobbs et al., '54), but fail to agree with the observation of McClure et al. ('45) that practically no storage occurred in humans ingesting 1.5 to 6 mg of fluoride daily. The results essentially are in agreement with those reported for humans by other workers. Ham and Smith ('54b) found young adults to retain 31 to 54% of ingested fluoride when the daily intake was 0.4 to 0.8 mg; at doses of 0.9 to 1.4 mg, the retention was 23 to 48%, and was 34 to 46% when 1.2 to 1.4 mg was ingested. The same authors ('54a) showed that young infants receiving 0.1 to 0.17 mg daily retained 6 to 29%, and when ingesting 0.5 to 0.7 mg, retention was 17 to 51%. Machle and Largent ('43) reported 63% retention when 6 mg of fluoride was ingested daily as sodium fluoride, while Largent and Heyroth ('49) found 48% retention of 19.4 mg of fluoride fed as sodium fluoride and 45.4% of 36.4 mg of fluoride daily as solid cryolite.

Skeletal storage at various levels of fluoride ingestion. Although the fluoride content in the bone was not measured directly, a reliable estimate of skeletal storage can be made from the intake-excretion differences. The validity of this assumption rests on the well-established tendency for fluorides

to be removed speedily from the blood stream (and soft tissues), either to be fixed in the bone or excreted mainly via the urine. The intake-excretion differences support a simple conclusion: for each rabbit and at each level of the daily fluoride ingestion, from one-third to one-half of the ingested fluoride was stored in the skeleton. The apparent increase in storage with fluoride dose (39% in the control group, 46% in the group given 15 ppm fluoride in the drinking water, 56% in the remaining groups) disappears when the retention is

TABLE 2

Relative absorption, retention and excretion of fluoride

RABBIT NO.	F IN WATER	TOTAL F INGESTED	% OF TOTAL F WHICH WAS ABSORBED	% OF ABSORBED F RETAINED	% OF ABSORBED F EXCRETED IN URINE
	ppm	*mg*			
66	0	80	57.5	82.4	17.6
80	0	65	47.8	55.5	44.5
62	15	110	55.6	79.2	20.8
61	15	180	65.8	71.5	28.5
74	60	350	72.3	77.5	22.5
71	60	335	68.0	74.8	25.2
59	90	535	70.9	75.4	24.6
72	90	455	68.4	82.2	17.6
60	150	810	68.1	76.5	23.5
52	150	580	73.2	90.2	9.81

calculated on the basis of "absorbed" fluoride (intake — fecal fluoride). These data are shown in table 2. Retention of the "absorbed" fluoride ranged from 55 to 90% and averaged 76.5%, no significant differences were apparent between groups. The extraordinary ability of the skeleton to fix fluoride, essentially to "detoxify" it, to remove it from the circulation, is plainly seen. The near constance of the fraction retained when the daily intake varied over a 10-fold range, viz., from 3.7 to 36 mg indicates that the saturation level of the skeletal mineral has not been exceeded in this range. The maintenance of the percentage retention over the three-week

period without a marked decline is additional support of this idea. If the deposition were accomplished by the conversion of hydroxylapatite to fluorapatite, 36 mg of fluoride would convert about 1 gm of bone mineral or roughly 1% of the total ash of the rabbit skeleton to fluorapatite daily. Other mechanisms, however, are available for fluoride fixation; Hendricks and Hill ('50) have described non-hydroxyl adsorption sites in the crystal lattice.

Extrapolation of the data from this brief study of rabbits to humans drinking fluoridated water for protracted periods obviously must be made with reservations. Assuming a 50% retention and assuming an average daily intake of a liter of water containing 1 ppm fluoride, the skeletal storage from this source would be 0.5 mg/day. If continued at this rate for 70 years, 12 gm of fluoride would be deposited and about 800 gm of fluorapatite would be formed, thus converting nearly one-third of the bone mineral to this form. There is no evidence that fluorapatite is any less useful as a bone mineral than is hydroxylapatite. Apparently healthy animals with two or three times this fluoride content in bone have been examined after programs of high fluoride feeding (Maynard, Downs and Hodge, '53). Furthermore, long time deposition would not proceed in the absence of mobilization and removal. A limited removal of fluoride from the skeletons of rats has been reported (Glock, Lowater and Murray, '41; Savchuck and Armstrong, '51; Miller and Phillips, '53) and a continuing mobilization from human bone *in vivo* has been demonstrated (Largent, '52; Hodge, '56). The fluoride content in the human skeleton after 70 years of drinking fluoridated water is therefore predicted to be considerably less than the 12 gm as calculated above and will probably not exceed a quarter of this amount. The total ash of 19 human cadavers has been analyzed for fluoride in this laboratory (Smith, Gardner and Wing, '49) and found to contain 0.85 to 4.97 gm of fluoride; the subjects ranged in age from 49 to 85 years. In our opinion, there is no danger of bone disease nor of any detectable ill effect from such quantities of fluoride in the skeleton.

The constancy of the urinary excretion suggests a similarity of retention of absorbed fluoride despite wide differences in the ratios of water-borne to dietary fluoride or in amounts ingested. This is in accord with the work of Wallace-Durbin ('54) who found that the percentages of urinary excretion and skeletal deposition of F^{18} in rats remained unchanged regardless of considerable variation in the size of the stable fluoride dose. Jackson et al. ('50) observed a retention of 30% of the

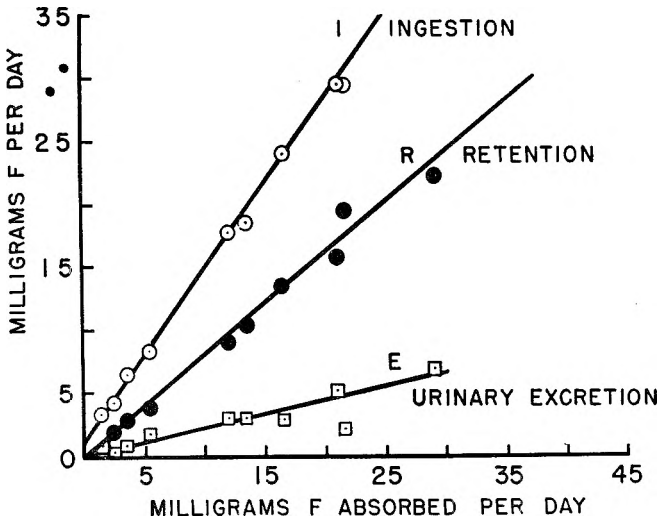


Fig. 2 Relations between fluoride absorbed and fluoride ingested, retained and excreted.

ingested fluoride in rats drinking water containing 2 to 16 ppm of fluoride as sodium fluoride.

If fecal fluoride is assumed to represent the unabsorbed portion of ingested fluoride the percentage of absorbed fluoride can be calculated. Absorption of ingested fluoride averaged 65% but was slightly greater at higher than at lower levels (table 2). Urinary excretion was more variable, but averaged 24% of the absorbed fluoride. Consequently, the percentage retention with a mean value of 76% reflects no increase with greater intake. Machle and Largent ('43), in balance studies of humans, found an average retention of 63%

at a daily ingestion level of 6 mg/day and 52% for experiments including larger fluoride doses. With increasing amounts of fluoride ingested, the absorption increased linearly (fig. 2). With increasing absorption, both the retention and the urinary excretion increased linearly (fig. 2). Considerable scattering of individual points can be seen but the linear tendencies for each curve are evident. One other linear relation was found: increasing the fluoride intake in the drinking water proportionately increased the fluoride concentration in the urine.

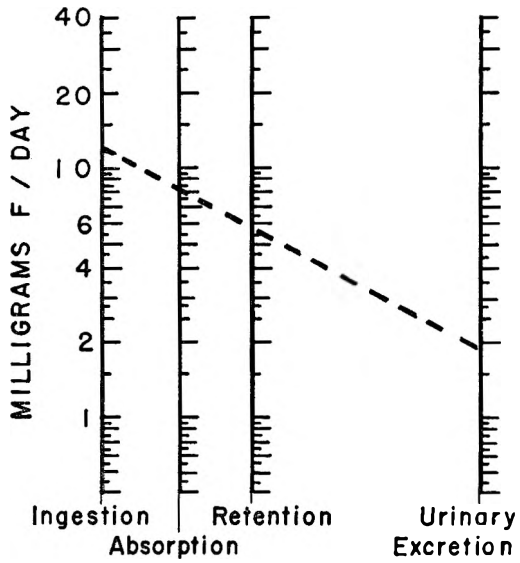


Fig. 3 Nomograph for interconversion of fluoride balance data.

When logarithmic plots of the type $y = ax^b$ are made of the data presented in figure 2, the relations are not only linear but of similar slope. Thus, for any logarithmic change in dose there is a proportionate logarithmic response in retention and excretion. These factors lead to a simple transformation of the data to nomographic form (fig. 3) in which the scales for independent and dependent variables are logarithmic and of similar modulus. An index line was computed from the mean slopes of best-fitting straight lines drawn through the 4

variables at each ingestion level; the spacing of scales is similar to the spacing of y-intercepts of the log-log plots. From a single value for ingestion, absorption, retention or urinary excretion any of the other variables is found by laying

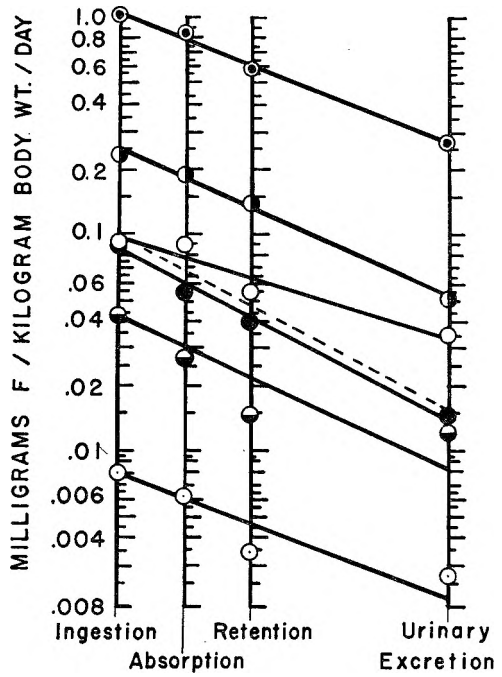


Fig. 4 Fluoride balance data from the literature fitted to the preceding figure. ○ Machle and Largent ('43): 6 mg F daily as NaF solution (man); ◑ Smith, Gardner and Downs (unpublished data): 3 ppm F as NaF in drinking water (rats); ◒ Weddle and Muhler ('55): low F ration, distilled H_2O (rats); ● Hobbs et al. ('54): pre-exp., no added F (lambs); ◉ Hobbs et al. ('54): 40 ppm F as NaF in ration (cattle); ⊙ Ham and Smith ('54b): normal diet (man).

a straight edge parallel to the index line through the known point and noting the intersected values on the appropriate scales. As long as the line intersects all 4 scales, the values will not fall outside the range of the emperical data.

Some of the data describing the ingestion of soluble fluorides by various species over a wide range of levels are tested in a

similar fashion in figure 4. In most cases the slopes of the lines agree well with that obtained in this experiment.

SUMMARY

Fuoride intake and excretion were measured in rabbits given drinking water containing from 0 to 150 ppm of fluoride over a 19-day period. About half of the ingested fluorides were retained, viz., positive balances averaged 51% of the ingested fluoride. An average of 76.5% of the absorbed fluoride was retained, presumably in the skeleton. More fluoride was excreted in the feces than in the urine. However, urinary excretion of fluoride increased in proportion to the increase in water fluoride concentration, the fluoride content of the food being constant. A nomograph has been constructed for the interconversion of ingestion, absorption, retention, and urinary excretion data. Data from literature sources tested on the nomograph agreed well with those obtained in this experiment.

LITERATURE CITED

BRIGGS, G. M., AND P. H. PHILLIPS 1952 Development of fluorine toxicosis in the rabbit. Proc. Soc. Exp. Biol. Med., *80:* 30.

FLAGG, J. F. 1949 Chapter 2 in *Pharmacology and Toxicology of Uranium Compounds, Part I,* edited by C. Voegtlin and H. C. Hodge. McGraw-Hill Book Co., Inc., New York.

GLOCK, G. E., F. LOWATER AND M. M. MURRAY 1941 The retention and elimination of fluorine in bones. Biochem. J., *35:* 1235.

HAM, M. P., AND M. D. SMITH 1954a Fluorine balance studies on four infants. J. Nutrition, *53:* 215.

———— 1954b Fuorine balance studies on three women. Ibid., *53:* 225.

HENDRICKS, S. B., AND W. L. HILL 1950 The nature of bone and phosphate rock. Proc. Nat. Acad. Sci., *36:* 731.

HOBBS, C. S., R. P. MOOREMAN, JR., J. M. GRIFFITH, J. L. WEST, G. M. MERRIMAN, S. L. HANSARD AND C. C. CHAMBERLAIN 1954 Fluorosis in Cattle and Sheep. Bull. no. 235. The University of Tennessee Agricultural Experiment Station.

HODGE, H. C. 1956 Fluoride metabolism: Its significance in water fluoridation. J. Am. Dent. Assn., *52:* 307.

JACKSON, S. H. 1955 The stabilization of the fluorine concentration of the total ash of rats. Can. J. Biochem. and Physiol., *33:* 93.

JACKSON, S. H., F. F. TISDALL, T. G. H. DRAKE AND D. WIGHTMAN 1950 The retention of fluorine when fed as bone and as sodium fluoride. J. Nutrition, *40:* 515.

LARGENT, E. J. 1952 Rates of elimination of fluoride stored in the tissues of man. Arch. Ind. Hyg. and Occupational Med., *6:* 37.

LARGENT, E. J., AND J. J. HEYROTH 1949 The absorption and excretion of fluorides. III. Further observations on metabolism of fluorides at high levels of intake. J. Ind. Hyg. Toxicol., *31:* 134.

LAWRENZ, M., AND H. H. MITCHELL 1941 Relative assimilation of fluorine from fluorine-bearing minerals and food (tea) and from water and food. J. Nutrition, *22:* 621.

MACHLE, W., AND, E. J. LARGENT 1943 The absorption and excretion of fluoride. II. The metabolism at high levels of intake. Ibid., *25:* 112.

MAYNARD, E. A., W. L. DOWNS AND H. C. HODGE 1953 Chapter 20 in *Pharmacology and Toxicology of Uranium Compounds, Part III,* edited by C. Voegtlin and H. C. Hodge. McGraw-Hill Book Co., Inc., New York.

MCCLURE, F. J. 1939 Fluorides in food and drinking water — a comparison of effects of water-ingested vs. food-ingested sodium fluoride. Nat. Inst. Health Bull., *172:* 53.

MCCLURE, F. J., H. H. MITCHELL, T. S. HAMILTON AND C. A. KINSER 1945 Balances of fluorine ingested from various sources in food and water by five young men. Secretion of fluorine through the skin J. Ind. Hyg. Toxicol., *27:* 159.

MILLER, R. F., AND P. H. PHILLIPS 1953 The metabolism of fluorine in the bones of the fluoride-poisoned rat. J. Nutrition, *51:* 273.

NEUMAN, W. F., M. W. NEUMAN, E. R. MAIN, J. O'LEARY AND F. A. SMITH 1950 The surface chemistry of bone. II. Flouride deposition. J. Biol. Chem., *187:* 655.

SAVCHUCK, W. B., AND W. D. ARMSTRONG 1951 Metabolic turnover of fluoride by the skeleton of the rat. J. Biol. Chem., *193:* 575.

SCHULZ, A. 1951 Fluorine metabolism in rabbits and a method of fluorine determination. Arch. Tierernahr., *1:* 329.

SMITH, F. A., AND D. E. GARDNER 1950 A comparison of the thorium nitrate back-tritration and the salt-acid thorium titration for the determination of fluoride. Arch. Biochem., *29:* 311.

SMITH, F. A., D. E. GARDNER AND H. C. HODGE 1955 Investigations on the metabolism of fluorides. III. Effect of acute renal tubular injury on urinary excretion of fluoride by the rabbit. Arch. Ind. Health, *11:* 2.

SMITH, F. A., D. E. GARDNER AND D. O'M. WING 1949 University of Rochester Atomic Energy Project Quarterly Technical Report. UR-103, pp. 61–63.

WALLACE-DURBIN, P. 1954 The metabolism of fluorine in the rat using F^{us} as a tracer. J. Dent. Res., *33:* 789.

WEDDLE, D. A., AND J. C. MUHLER 1954 The effects of inorganic salts on fluorine storage in the rat. J. Nutrition, *54:* 437.

———— 1955 Metabolism of small concentrations of fluorine in the albino rat. J. Dent. Res., *34:* 900.

ZIPKIN, I., AND F. J. MCCLURE 1952 Deposition of fluorine in the bones and teeth of the growing rat. J. Nutrition, *47:* 611.

THE EFFECT OF A COMBINED DEFICIENCY OF THIAMINE AND OF PANTOTHENIC ACID ON THE NERVOUS SYSTEM OF THE RAT

J. D. K. NORTH [1] AND H. M. SINCLAIR
Laboratory of Human Nutrition, University of Oxford, England

(Received for publication August 3, 1956)

It has been shown that chronic thiamine deficiency in the rat, if of sufficient duration and intensity, will cause degenerative lesions in the peripheral nerves which can be demonstrated histologically (Prickett et al., '39; Rodger, '53; North and Sinclair, '56). The present workers found that degenerative changes were most marked in rats which received the smallest daily allowance of thiamine (1.5 μg of thiamine daily). It was considered, for this reason, that the effect on the peripheral nerves of repeated acute deficiencies of thiamine should be investigated.

There is a species difference in the effect of thiamine deficiency on the nervous system; in the pigeon degenerative lesions are readily induced (Swank, '40; Shaw and Phillips, '45a) whereas in the pig the nervous system is intact after prolonged deficiency of thiamine (Follis et al., '43; Wintrobe et al., '44). In contrast, the nervous system of the pig is very susceptible to a deficiency of pantothenic acid and degenerative lesions develop both in the peripheral nerves and in the neurones of the dorsal-root ganglia (Follis and Wintrobe, '45; Swank and Adams, '48). There has been no investigation of the effect of pantothenic acid deficiency on the nervous system of the rat although Lippincott and Morris ('41) have reported myelin degeneration in both the spinal cord and in

[1] Formerly Walter Dixon Memorial Scholar, British Medical Association.

the sciatic nerve of deficient mice. Shaw and Phillips ('45b) and Ram ('49) have shown that this deficiency in the chick does not affect the peripheral nerves but causes degenerative changes in the fibre tracts of the spinal cord. The effect on the nervous system of a combined deficiency of thiamine and pantothenic acid has not been studied in any species.

EXPERIMENTAL METHODS

Two separate experiments were carried out during this study. The first was designed to investigate the effect on the nervous system of repeated acute deficiencies of thiamine, and for this purpose 20 young male hooded rats, 6 weeks old, were divided into 10 pairs of litter mates. The first 5 pairs contained a deficient and a pair-fed control rat, the remaining pairs consisted of a deficient and a weight-control animal. The purpose of the weight-control animals has been discussed in a previous communication (North and Sinclair, '56). In this experiment the deficient rats were given a diet free of thiamine and were injected intraperitoneally with thiamine each time they showed obvious signs of an acute deficiency of this vitamin. The dose of thiamine injected was:

Number of the injection	*μg of thiamine*
1 st	100
2 nd	400 (200 μg on two successive days)
3 rd	200
4 th	200
5 th	200

In this way the rats underwent repeated acute deficiencies of thiamine during the experiment.

Thirty male hooded rats, 6 weeks old, were employed in the second study. Twenty of these animals were divided into 10 pairs of litter mates; these pairs comprised, as before, 10 deficient rats, 5 pair-fed control rats and 5 weight-control animals. The 10 deficient rats were deprived of both thiamine and pantothenic acid. Thiamine deficiency developed more rapidly and these animals were given several injections of thiamine (dosage as above) before they showed signs of

pantothenic acid deficiency. Five rats were deprived only of pantothenic acid and the remaining 5 animals were fed ad libitum a diet containing adequate quantities of all known nutrients (whole ground wheat, 50 parts; whole ground barley, 25 parts; fish meal, 7 parts; meat and bone meal, 6 parts; dried grass meal, 5 parts; dried brewers' yeast, 5 parts; cod-liver oil, 1 part; sodium chloride, 1 part). The rats were kept in individual cages with wire-mesh floors and had a liberal supply of water. All rats except the 5 stock-diet control rats were fed a pure diet the constituents of which were: sucrose, 65 parts; casein (fat- and vitamin-free), 20 parts; arachis oil, 10 parts; salt mixture (Hegsted et al., '41) 5 parts.

The animals on the pure diet were supplied with water-soluble vitamins in a sucrose solution on alternate days (the daily allowance being: riboflavin, 150 μg; nicotinic acid, 1 mg; pyridoxine hydrochloride, 100 μg; *p*-amino-benzoic acid, 1 mg; inositol, 2 mg; choline chloride, 10 mg; folic acid, 20 μg; biotin, 2 μg; vitamin K, 100 μg). The following oil-soluble vitamins were given in 4 drops of arachis oil each week: vitamin A_1 acetate, 1000 I.U.; vitamin D_2, 50 I.U.; α-tocopherol, 5 mg. Details of the quantities of thiamine and of pantothenic acid fed to each group of rats are given in table 1.

The rats were weighed and examined twice weekly and also prior to each injection of thiamine. The examination included a test for incoordination which consisted of making the animals walk along a narrow "L"-shaped runway. The heart-rate was measured by recording electrocardiographic tracings of the action of the heart using the method described by Hundley et al. ('45). Electrocardiographic records were taken in the third, 4th, 5th and 6th acute deficiencies of thiamine and also 7 days after the third injection of thiamine. This last record gave the heart-rates of the rats while in a temporary remission as a result of the injected thiamine.

Each pair of rats was killed when the deficient rat was moribund from acute vitamin deficiency. Most of the rats

TABLE 1

Survival time and changes in weight of rats deprived of thiamine, of pantothenic acid, or of both of these vitamins

EXPERIMENT	NUMBER OF RATS	TYPE OF RATS	DAILY INTAKE		AVERAGE NUMBER OF ACUTE DEFICIENCIES	TOTAL DURATION OF DEFICIENCY		AVERAGE LENGTH OF EACH ACUTE DEFICIENCY	AVERAGE WEIGHT		AVERAGE GAIN IN WEIGHT
			Thiamine	Pantothenic acid		Average	Longest		Initial	At death	
			μg	*μg*		*days*	*days*	*days*	*gm*	*gm*	*gm*
I	10	Deficient	...	200	4.8 (Thiamine)	127	160	26 (Thiamine)	124	132	8
	5	Pair-fed control	100	200	..	127	160	..	118	178	60
	5	Weight-control	100	200	..			..	127	133	6
II	10	Deficient	...	...	4.4 (Thiamine)	117	161	27 (Thiamine)	127	110	— 17
	5	Pair-fed control	100	200	..	117	161	..	140	166	26
	5	Weight-control	100	200	..			..	123	112	— 11
	5	Deficient	100	...	1.2 (Pantothenic acid)	125	134	104 (Pantothenic acid)	121	103	— 18
	5	Stock-diet control	Adequato	Adequate	..	149	161	..	141	355	214

were killed by bleeding from the carotid artery after they had been lightly anesthetized.[2] Two pairs of rats in each experiment were, however, fixed by perfusion to prevent possible post-mortem autolysis in the nervous system. A cannula was passed into the aorta from the left ventricle while the rat was anesthetized. The perfusion was made with fluids

TABLE 2

Methods of histological examination of the nervous system of rats

PART OF NERVOUS SYSTEM	SIDE	FIXATIVE	EMBEDDING MATERIAL	STAIN
Sciatic nerve and posterior tibial nerve	Right	Formalin	Paraffin	Silver
		Osmium tetroxide	Paraffin	Osmium tetroxide
	Left	Formalin	Paraffin	Modified Marchi
		Formalin	Gelatine (frozen section)	Polarized light and Sudan black
Peroneal nerve	Right	Osmium tetroxide	Paraffin	Osmium tetroxide
	Left	Osmium tetroxide	Paraffin	Osmium tetroxide
Lumbar dorsal-root ganglia	Right	Formalin	Paraffin	Methylene blue
Brain	...	Formalin	Paraffin	Methylene blue
Lumbar cord	...	Formalin	Paraffin	Methylene blue

warmed to 37°C., at a pressure of 85 mm of mercury. The vascular system was first washed out with gum-acacia-saline for two to three minutes and then fixed by perfusing a solution of gum-acacia-formol-saline for 30 minutes as recommended by Koenig et al. ('45).

Histological methods indicated in table 2 were used with all rats. The chlorate-osmium-tetroxide modification of the Marchi method was described by Swank and Davenport ('34a, b; '35). Tissues were fixed *in situ* to prevent shrinkage and

[2] Pentobarbitone sodium (Nembutal).

distortion. Portions of the sciatic and posterior tibial nerves were cut longitudinally and those of the peroneal nerve were cut transversely so that myelinated nerve-fibre counts could be obtained.

RESULTS

Repeated acute deficiencies of thiamine; clinical course. The first deficient rat would have died on the 28th day of the experiment had the rats not been injected with thiamine at this time. Details of the number of acute deficiencies of thiamine, the length of survival and the changes in weight are given in table 1. Individual rats showed a wide variation in their requirement of extrinsic thiamine. Considerable inanition occurred secondary to thiamine deficiency and these deficient rats were more than 200 gm lighter at the time they were killed than stock-diet control animals. When severely affected by an acute lack of thiamine most deficient rats developed convulsions. No convulsions occurred among the pair-fed and weight-control animals, although at times some of the weight-control rats lost more weight than their deficient partners. The recovery after an injection of thiamine was rapid and in 4 to 8 hours convulsions could not be induced in rats which, before the injection, suffered from spontaneous convulsive seizures.

Most of the deficient rats showed some ataxia when in the deficient state. Signs of this disorder appeared one to three days before the rats required an injection of thiamine; twenty-four hours after the injection their gait was again normal. Disorders of gait occurred a few hours before convulsions could be induced by handling. There was no alteration in the response to pain stimuli except in the rats that developed spontaneous convulsions. Electrocardiographic records were used to confirm that there was definite bradycardia before injecting thiamine. Figure 1 gives the average heart-rates of the rats, and as can be seen there was a consistent bradycardia before each injection of thiamine. The difference between the average heart-rates of the inanition-

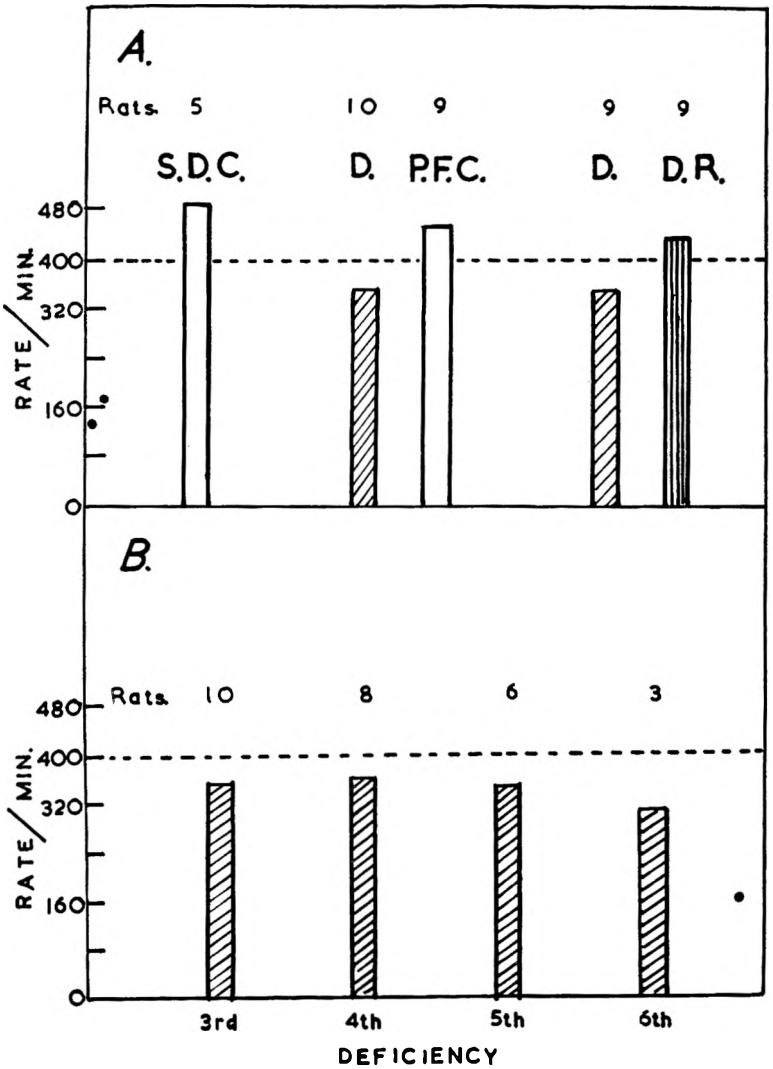


Fig. 1 Average heart-rates of deficient and control rats during repeated acute deficiencies of thiamine.

A Average heart-rates at the time of the third acute deficiency of thiamine.
S.D.C., Stock-diet control rats.
P.F.C., Pair-fed control rats.
D., Deficient rats in deficient condition.
D.R., Deficient rats 7 days after the third injection of thiamine (temporary recovery).

B Average heart-rates of the deficient rats in the third, 4th, 5th and 6th acute deficiencies of thiamine.

control rats (448 per min.) and the deficient rats (347 per min.) was statistically highly significant.

Histological changes. Degenerative changes were not seen either in the myelin sheaths or the axis cylinders of the sciatic and posterior tibial nerves after repeated acute deficiencies of thiamine. Isolated degenerating fibres were occasionally observed in both the deficient and the control rats similar to those reported by Duncan ('30) in the normal rat but no more than one such fibre was seen in any section. The chlorate-osmium-tetroxide modification of the Marchi method tended in both experiments to stain black intact myelin sheaths of both deficient and inanition-control animals. This was a non-specific effect of inanition. Myelinated nerve-fibre counts failed to reveal any significant difference between the deficient and the inanition-control animals (table 3). The neurones of the lumbar dorsal-root ganglia and of the lumbar cord were also intact. Results obtained by perfusion-fixation were slightly better than those obtained when the tissues were fixed by immersion immediately after death. Chromophilic neurones were still present in both the spinal cord and the dorsal-root ganglia which were fixed by perfusion although they were less common than in nervous tissue fixed by immersion. Haemorrhages were not seen in the brain-stem of the rats after repeated acute deficiencies of thiamine.

Combined deficiency of thiamine and of pantothenic acid; clinical course. One of the 5 rats deprived of pantothenic acid alone required an injection of 800 μg of this vitamin after 63 days. All 5 pantothenic-acid-deficient animals developed obvious signs of the deficiency including greying of the hair and porphyrin deposits on the snout; they were killed when moribund 108 to 134 days after the start of the deficiency.

In the combined deficiency signs of pantothenic acid were slower in appearing. Six of the 10 rats deprived of both vitamins survived longer than any rat deprived only of pantothenic acid; however, signs of the latter deficiency had developed in most of the animals before they were killed. Table

1 gives details of the length of survival and changes in weight of the rats in this experiment. Convulsive seizures occurred as frequently and bradycardia was as marked in rats deprived of both vitamins as in rats which lacked only thiamine. In the

TABLE 3

Myelinated nerve-fibre counts of the right peroneal nerve

EXPERIMENT	DEFICIENCY	TYPE OF RATS	NUMBER OF RATS	INDIVIDUAL NERVE-FIBRE COUNTS	MEAN NERVE-FIBRE COUNT	STANDARD DEVIATION
I	Thiamine	Deficient	6	1703 1771 1783 1820 1892 1938	1818	35.4
	Deficiency	Inanition-control	6	1756 1817 1857 1858 1877 1878	1340	46.9
II	Combined deficiency of thiamine and of pantothenic acid	Deficient	6	1675 1679 1739 1763 1839 1891	1773	97.0
		Inanition-control	6	1699 1777 1810 1873 1883 1911	1826	79.3
	Pantothenic acid deficiency	Deficient	3	1594 1797 1835	1742	129.6
		Stock-diet control	5	1737 1786 1837 1859 1927	1829	72.2

third acute deficiency of thiamine the average heart rate of the rats in the combined deficiency was 352 per min. compared with a rate of 347 per min. in the rats deprived of thiamine alone. The pantothenic-acid-deficient rats had an average heart rate of 458 per min., so that this deficiency did not cause bradycardia.

Histological changes. The myelin sheaths and the axis cylinders of the peripheral nerves, as well as the neurones of the lumbar dorsal-root ganglia and lumbar spinal cord were intact after a combined deficiency of thiamine and pantothenic acid. Similarly the nervous system of animals deprived only of pantothenic acid was unaffected by this deficiency. There was no sign of haemorrhage in the brain-stem of any animal; myelinated nerve-fibre counts failed to show a significant difference between the deficient and the inanition-control rats (table 3).

DISCUSSION

Histological observations of the nervous system have shown that repeated acute deprivations of thiamine do not cause peripheral nerve degeneration in the rat, and provide convincing evidence that a single acute thiamine deficiency will not produce pathological changes in these parts of the nervous system in this animal. The findings support the claim of Engel and Phillips ('38) that nerve degeneration does not occur in acute thiamine deficiency. There have been 10 previous studies in which the nervous system has been examined after an acute deficiency of thiamine; in most investigations the deficiency was, in fact, that of the thermolabile fraction of the vitamin B complex. Lee and Sure ('37a, b) and Rodger ('53) were the only workers who found changes in the deficient rats which did not occur in inanition-control animals. The former workers, however, relied entirely on the results of examination of the nerves under polarized light. In the present study examination of the nerves under polarized light was only of doubtful value and Lillie ('48) in a monograph

on histological technique came to the same conclusion. After giving the thiamine analogue "pyrithiamine" to induce an acute deficiency of this vitamin, Rodger ('53) found marked degeneration of myelin with the Marchi method. Two other investigators found no degeneration in acute thiamine deficiency (Stern and Findlay, '29; Engel and Phillips, '38); and Mannell and Rossiter ('54) showed that neither in acute thiamine deficiency, nor in a deficiency of total calories, was there a degeneration of the myelin sheath in a chemical sense. The remaining 5 groups of workers found similar changes in the peripheral nerves of deficient and inanition-control rats (Prickett, '34; Davison and Stone, '37; Vedder and Chinn, '38; Prickett et al., '39; Swenson, '50). All but one of these 5 studies relied entirely on the Marchi stain or the appearance of the myelin under polarized light. Had the Marchi stain been used alone in our experiments the results would have supported their conclusions. The normal appearance in the present study, however, in both the myelin when stained with osmium tetroxide and Sudan black and in the axis cylinders, suggests that blackening of the fibres when stained with the chlorate-osmium-tetroxide modification of the Marchi method represents a change in the myelin other than true degeneration; this change was the result of inanition.

Fixation of the nervous system by perfusion was a doubtful benefit. In general better results were obtained with the neurones in the central nervous system particularly in the lumbar cord but the results of the osmium tetroxide stain for myelin were inferior if the rat was fixed by perfusion. Haemorrhages in the brain-stem in rats deprived of thiamine (thermolabile vitamin B) were first reported by Prickett ('34). He found that 75% of deficient rats were affected, frequently with massive haemorrhages; no pair-fed control rats showed similar lesions. Lee and Sure ('37a, b) and Engel and Phillips ('38) were unable to confirm the findings of Prickett and reported that the brain-stem in acute thiamine deficiency appeared normal. In the present experiment

haemorrhages were not seen in the brain-stem of the deficient rats. Haemorrhages have been reported in the brain-stem of the mouse (Morris, '47) and of the pigeon (Swank and Prados, '42); the latter workers believed that the haemorrhages which were always microscopic were secondary to degenerative changes in the neurones.

Convulsions occurred only in rats suffering from an acute deprivation of thiamine and disappeared within a few hours of injecting thiamine even if all food was removed from the cage. These observations suggest that convulsions were a specific effect of acute thiamine deficiency and not the result of inanition as suggested by Mouriquand and Edel ('48).

Wintrobe and his colleagues have proved that in the pig pantothenic acid deficiency causes definite degenerative lesions in the peripheral nerves (Wintrobe et al., '38; Wintrobe et al., '42; Follis and Wintrobe, '45). In one communication (Wintrobe et al., '44) these workers stated: "autoclaving destroys not only thiamine, but reduces the pantothenic acid content of yeast as well. It is suggested that certain effects heretofore attributed to lack of thiamine may have in reality been due to lack of pantothenic acid." In support of this proposition they showed that dry autoclaving of brewers' yeast for 6 hours reduced the pantothenic acid content from 72 μg to 14 μg per gram. Most previous work on the effect of thiamine deficiency on the nervous system has relied on autoclaved yeast, this being believed to be deficient only in thiamine. It is for this reason that a distinction was made earlier in the paper between thiamine deficiency and deficiency of the thermolabile fraction of the vitamin B complex. The present experiments have not confirmed Wintrobe's suggestion. Not only was there no evidence of degeneration in the peripheral nerves after pantothenic-acid deficiency, but the nervous system remained intact despite a prolonged deficiency of both thiamine and pantothenic acid.

The findings are not at variance with those reported in a previous paper (North and Sinclair, '56) in which definite degeneration was found in the distal segments of the sciatic

and posterior tibial nerves after chronic thiamine deficiency. It is reasonable to assume that repeated acute deficiencies of thiamine, although causing a definite biochemical lesion in the neurone, are not sufficiently prolonged to cause structural damage. It is possible that in chronic thiamine deficiency a concurrent deficiency of pantothenic acid might enhance the tendency to peripheral nerve degeneration. Under the circumstances of the present investigation this did not occur. The difference in strain of rat is a factor which cannot be excluded when considering the varied response to thiamine deficiency. It seems more probable, however, that the type of thiamine deficiency (in the previous report, chronic; in this study, repeated acute) was responsible for the different results obtained. Myelinated nerve-fibre counts in the two strains of rats were consistently different. The mean counts for the albino (North and Sinclair, '56) and hooded rats were respectively 1680 (30 nerves) and 1810 (32 nerves); the difference between these values was highly significant ("t" test: $P < 0.001$).

SUMMARY

In the first experiment the effect of repeated acute deficiencies of thiamine on the nervous system was investigated. Ten deficient and 10 inanition-control rats were employed in the study; the deficient rats survived for an average period of 127 days during which time they underwent three to 6 acute deficiencies of thiamine. The severity of the acute deficiency was in most rats indicated by the onset of convulsive siezures and was confirmed by the presence of bradycardia.

The second experiment was designed to study the effect of a combined deficiency of thiamine and pantothenic acid on the nervous system of the rat. For this purpose 10 deficient and 10 inanition-control animals were employed; there were, in addition, 5 rats deprived of pantothenic acid alone and 5 stock-diet control animals.

In the combined deficiency, deprivation of thiamine which was acute and repeated was superimposed on the longer pantothenic-acid deficiency. The rats survived for an average time of 117 days.

Deficient rats in both studies showed no evidence of degenerative changes in the distal segments of the sciatic and posterior tibial nerves, in the lumbar dorsal-root ganglia or in the lumbar cord. Myelinated nerve-fibre counts of the peroneal nerve failed to demonstrate a significant difference between the deficient and the inanition-control rats. Haemorrhages were not seen in the brain-stem of the deficient rats.

LITERATURE CITED

DAVISON, C., AND L. STONE 1937 Lesions of the nervous system of the rat in vitamin B deficiency. Arch. Path., *23:* 207.

DUNCAN, D. 1930 The incidence of Secondary (Wallerian) degeneration in normal mammals compared to that in certain experimental and diseased conditions. J. Comp. Neur., *51:* 197.

ENGEL, R. W., AND P. H. PHILLIPS 1938 The lack of nerve degeneration in uncomplicated vitamin B_1 deficiency in the chick and the rat. J. Nutrition, *16:* 585.

FOLLIS, R. H. JR., M. H. MILLER, M. M. WINTROBE AND H. J. STEIN 1943 Development of myocardial necrosis and absence of nerve degeneration in thiamine deficiency in pigs. Am. J. Path., *19:* 341.

FOLLIS, R. H. JR., AND M. M. WINTROBE 1945 A comparison of the effects of pyridoxine and pantothenic acid deficiencies on the nervous tissues of swine. J. Exp. Med., *81:* 539.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HUNDLEY, J. M., L. L. ASHBURN AND W. H. SEBRELL 1945 The electrocardiograms in chronic thiamine deficiency in rats. Am. J. Physiol., *144:* 404.

KOENIG, H., R. A. GROAT AND W. F. WINDLE 1945 A physiological approach to perfusion-fixation of tissues with formalin. Stain Technol., *20:* 13.

LEE, J., AND B. SURE 1937a Avitaminosis. xviii. Peripheral nerves in vitamin B_1 deficiency as observed by polarized light. Proc. Soc. Exp. Biol. Med., *35:* 583.

———— 1937b Avitaminosis. xix. Nerve degeneration in albino rats as studied by the freezing-drying method and polarized light with deficiency of vitamin A or of vitamin B_1. Arch. Path., *24:* 430.

LILLIE, R. D. 1948 Histopathologic Technic. Blakiston, Philadelphia.

LIPPINCOTT, S. W., AND H. P. MORRIS 1941 Morphologic changes associated with pantothenic acid deficiency in the mouse. J. Nat. Cancer Inst., *2:* 39.

MANNELL, W. A., AND R. J. ROSSITER 1954 Nutritional deficiency and Wallerian degeneration in the rat. 2. Effect of thiamine deficiency on the concentration of nucleic acid and phospholipid in intact and sectioned nerves. Brit. J. Nutrition, *8:* 56.

MORRIS, H. P. 1947 Vitamin requirements of the mouse. Vitamins and Hormones, *5:* 175.

MOURIQUAND, G., AND V. EDEL 1948 Le spasme de torsion est-il chez le rat caracteristique de l'avitaminose B_1. C. R. Soc. Biol., *142:* 981.

NORTH, J. D. K., AND H. M. SINCLAIR 1956 Nutritional neuropathy. Chronic thiamine deficiency in the rat. Arch. Path., *62:* 341.

PRICKETT, C. O. 1934 The effect of a deficiency of vitamin B upon the central and peripheral nervous systems of the rat. Am. J. Physiol., *107:* 459.

PRICKETT, C. O., W. D. SALMON AND G. A. SCHRADER 1939 Histopathology of the peripheral nerves in acute and chronic vitamin B_1 deficiency in the rat. Am. J. Path., *15:* 251.

RAM, T. 1949 A histopathologic study of chicks deficient in pantothenic acid. Poultry Sci., *28:* 425.

RODGER, F. C. 1953 Experimental thiamin deficiency as a cause of degeneration in the visual pathway of the rat. Brit. J. Ophthal., *37:* 11.

SHAW, J. H., AND P. H. PHILLIPS 1945a Neuropathologic studies of acute and chronic thiamine deficiencies and of inanition. J. Nutrition, *29:* 113.

———— 1945b Neuropathologic studies of pantothenic acid, biotin and folic acid complex deficiencies in the chick. Ibid., *29:* 107.

STERN, R. O., AND G. M. FINDLAY 1929 The nervous system in rats fed on diets deficient in vitamins B_1 and B_2. J. Path. Bact., *32:* 63.

SWANK, R. L. 1940 Avian thiamin deficiency: A correlation of the pathology and clinical behavior. J. Exp. Med., *71:* 683.

SWANK, R. L., AND R. D. ADAMS 1948 Pyridoxine and pantothenic acid deficiency in swine. J. Neuropath. Exp. Neurol., *7:* 274.

SWANK, R. L., AND H. A. DAVENPORT 1934a Marchi's staining method: I. Studies of some of the underlying mechanisms involved. Stain Technol., *9:* 11.

———— 1934b Marchi's staining method: II. Fixation. Stain Technol., *9:* 129.

———— 1935 Marchi's staining method: III. Artefacts and effects of perfusion. Stain Technol., *10:* 45.

SWANK, R. L., AND M. PRADOS 1942 Avian thiamine deficiency. Arch. Neur. Psychiat., *47:* 97.

SWENSON, M. J. 1950 The effect of a thiamine analogue on the histopathology of rat tissues. Am. J. Vet. Research, *11:* 105.

VEDDER, E. B., AND A. B. CHINN 1938 Beriberi or inanition. I. The effect of starvation with and without vitamin B_1. Am. J. Trop. Med., *18:* 469.

WINTROBE, M. M., R. H. FOLLIS JR., S. HUMPHREYS, H. STEIN AND M. LAURITSEN 1944 Absence of nerve degeneration in chronic thiamine deficiency in pigs. J. Nutrition, *28:* 283.

WINTROBE, M. M., M. H. MILLER, R. H. FOLLIS JR., H. J. STEIN, C. MUSHATT AND S. HUMPHREYS 1942 Sensory neuron degeneration in pigs. IV. Protection afforded by calcium pantothenate and pyridoxine. Ibid., *24:* 345.

WINTROBE, M. M., D. M. MITCHELL AND L. C. KLOB 1938 Sensory neuron degeneration in vitamin deficiency. Degeneration of the posterior columns of the spinal cord, peripheral nerves, and dorsal root ganglion cells in young pigs fed a diet containing thiamin (B_1) and riboflavin but otherwise deficient in vitamin B complex. J. Exp. Med., *68:* 207.

NUTRITIONAL STUDIES ON RATS ON DIETS CONTAINING HIGH LEVELS OF PARTIAL ESTER EMULSIFIERS[1]

IV. MORTALITY AND POST-MORTEM PATHOLOGY; GENERAL CONCLUSIONS

BERNARD L. OSER AND MONA OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication March 9, 1956)

In this 4th and final report on the two-year study of rats fed diets containing high levels of partial ester emulsifiers, observations are presented with respect to duration of life span, causes of death, and post-mortem pathological findings in dead or sacrificed animals. Particular attention was directed to the condition of the liver, kidneys, and bladder in view of the questions raised in the report of the Food Protection Committee on the safety of polyoxyethylene stearate as a food additive ('53).

In the concluding section of this paper the results of the several phases of the study are correlated and interpreted in relation to the partial ester emulsifiers both as a class and individually, with particular reference to Myrj 45.

LONGEVITY

Two statistics were employed in the examination of mortality data, (a) percentage survival at stated periods in relation to the group populations at 12 weeks and (b) the

[1] This investigation was supported by a grant from the Atlas Powder Company, Wilmington, Delaware.

median survival time (MST) expressed as the age in weeks when one-half of a group had died. In the computations, non-moribund rats sacrificed for autopsy or rats accidentally killed were not considered as "deaths."

Evidence of the nutritional quality of the basal diet and of the satisfactory maintenance conditions is indicated by the fact that at the end of the first year the control group showed 100% survival, and at two years, about 60% (table 1). Deaths tended to occur earlier in the females than in the males, which was not surprising in view of the added stress of reproduction in this sex and the high incidence of pulmonary disease found in the females who died post-partum. Only occasional deaths occurred among the males in the test groups during the first year and the few deaths among the females occurred during or after parturition. In the second year a somewhat greater proportion of rats died in the emulsifier and Primex groups than in the basal controls.

To assess the effect of the emulsifiers or fats on longevity, mortality rates have been examined in relation to the levels of dietary supplementation. Thus while there were no striking differences among the groups in incidence of death up to one-and-a-half years, during the last quarter of the life span a greater proportion of deaths occurred at the 20% supplementation level than at lower levels. This was especially noted among the females on the diets containing 20% Myrj 52 or the Tweens. These emulsifiers, it will be recalled, induced the greater laxative responses and concomitantly interfered with fertility and maternal interest in the newborn. It is conceivable that the irritation associated with the diarrhea in these groups may have imposed a chronic stress sufficient to affect their longevity.

The 20% Primex group was discontinued at one year but mortality in the 5 and 10% Primex groups over the two-year period was about the same as in the emulsifier groups. An interesting, though fortuitous observation was the survival for two years of all the male rats receiving the mixture of emulsifiers at the 5% level.

Inspection of the data for median survival time in the last column of table 1 reveals that practically all MST values fell within the range 100 ± 10 weeks. Groups in which half the rats had not died at the planned termination period (i.e., when the rats were on test for 104 weeks and hence 108 weeks old) were allowed to continue until half the individuals were dead (except in the two groups specified in footnotes 5 and 7, table 1). The MST was not affected by either the dietary level of any of the emulsifiers, nor was it significantly different in the groups on the emulsifier diets as compared with those on the basal or Primex diets. Regardless of dosage levels and types of supplementation, the variations among the groups could be attributed entirely to chance. The somewhat earlier and greater mortality among the females than among the males likewise failed to meet the test of statistical significance.

PATHOLOGY

Except in a few cases, autopsies were performed on all rats that died during the course of the two-year test or were sacrificed at its termination. Histopathological examinations were conducted on the livers and kidneys of rats at all dosage levels. Other organs were examined microscopically where indicated by their gross appearance and in at least two apparently normal rats of each sex randomly selected from the 20% groups.

Upon gross examination, the lungs appeared to be the most frequently affected organ. However, neither the frequency, character, nor the degree of the pulmonary lesions were any more marked in the test animals than in the controls. The lesions seen included varying degrees of hyperemia with or without hemorrhage, purulent and mucoid congestion, consolidation, superficial fibrinous exudate or adhesions in the pleural cavity. It is of incidental interest to note that although these rats were housed in an air-conditioned room throughout the two-year period, respiratory disease was not noticeably less common than in rats maintained in non-air-conditioned quarters in these Laboratories.

TABLE 1

Percentage survival of F_0 rats at various stages of two-year feeding period

EMULSIFIER OR FAT	NO. OF RATS (AT 12 WEEKS) AND SEX	PERCENTAGE SURVIVAL BY WEEKS ON TEST					MST [1]
		12	26	52 [2]	78	104	
None	12M	100	100	100	83	58	109
	20F	100	100	100	80	60	110
5% Level							
Myrj 45	12M	100	100	83	67	25	91
	20F	100	90	75	60	30	94
Myrj 52	12M	100	100	100	83	58	110
	20F	100	100	90	75	37 [3]	100
Span 60	11M	100	100	100	91	46	109
	20F	100	95	80	80	50	109
Tween 60	12M	100	92	83	59	25	97
	21F	100	95	62	57	43	99
Tween 65	12M	100	100	100	67	42	101
	20F	100	85	80	80	40	100
Tween 80	12M	100	100	100	75	42	98
	20F	100	90	85	75	40	103
Mixture	12M	100	100	100	100	100	> 108 [4]
	20F	100	80	75	55	30	89
Primex	12M	100	100	92	75	33	97
	20F	100	100	95	70	50	107
10% Level							
Myrj 45	12M	100	100	100 [3]	73	55	103
	20F	100	95	90	75	35	106
Myrj 52	12M	100	92	92	67	33	87
	19F	100	95	84	69	53	106
Span 60	10M	100	100	100	100	50	102
	20F	100	100	85	80	45	102
Tween 60	12M	100	100	92	92	67	108 [5]
	20F	100	100	90	75	40	98
Tween 65	12M	100	100	100	67	33	93
	20F	100	95	85	75	50	103
Tween 80	10M	100	100	90	70	30	93
	20F	100	95	90	80	50	109
Mixture	12M	100	100	100	83 [3]	45	103
	20F	100	90	75	75	50	109
Primex	12M	100	100	100	83	33	98
	20F	100	95	85	75	55	109
20% Level							
Myrj 45	12M	100	100 [6]	91	82	55	100
	20F	100	95	75	70	55	111
Myrj 52	12M	100	92 [6]	91	82	55	107
	21F	100	95	81	81	29	98
Span 60	12M	100	83	75	67	50	110
	20F	100	95	85	75	60	108 [7]
Tween 60	11M	100	100	91	70	60	89
	20F	100	95	80	70	25	92
Tween 65	12M	100	92	91	80	40	90
	21F	100	100	91	86	33	101
Tween 80	12M	100	83	83	70	60	90
	20F	100	95	70	65	30	92
Mixture	12M	100	100	100	90	60	104
	20F	100	95	80	70	50	105
Primex	12M	100	100	100 [8]	..	..	..
	20F	100	100	100 [5]	..	..	..

[1] MST = mean survival time expressed as age in weeks when one-half of the group had died.

[2] Some animals were sacrificed for autopsy at this period.

[3] One accidental death.

[4] These rats were sacrificed between the 108th and 120th weeks.

[5] Eleven of these rats were sacrificed between the 108th and 121st weeks.

[6] One rat sacrificed because of malocclusion.

[7] Twelve of these rats were sacrificed between the 108th and 117th weeks.

[8] Entire group terminated.

A few mammary fibroadenomas were found among the 20 or so females of each group in the F_0 generation (table 2). It will be recalled that these rats were allowed to reproduce continuously throughout the two-year test period. The number of such tumors seen during the latter half of the life cycle in the various test and control groups varied from one to 4 in most groups with as many as 5 or 6 in a few. In some of the emulsifier series (e.g. Myrj 45, Tween 65) these mammary growths appeared to increase in incidence with increasing dosage level; however, the opposite effect was noted in

TABLE 2

Number of mammary fibroadenomas during two-year period in F_0 generation

(About 20 female rats per group initially)

EMULSIFIER OR FAT	LEVEL, PER CENT			
	0	5	10	20
None	3			
Myrj 45		1	2	4
Myrj 52		2	2	1
Span 60		4	4	2
Tween 60		3	2	2
Tween 65		0	3	6
Tween 80		5	5	1
Mixture		1	2	3
Primex		2	5	..[1]

[1] Discontinued at one year; no mammary tumors noted up to that time.

one series (Tween 80) and no directional tendency was apparent in the remainder. The more frequent incidence of mammary fibromas in the 5% Tween 80 group was probably without significance in view of the lower incidence at the 20% level; moreover, these tumors were just as common in the 10% Primex group. It is even unlikely that the 6 mammary growths observed among the 21 females in the 20% Tween 65 group can be attributed to dosage inasmuch as this percentage incidence approximates the upper limit of the fiducial range, all groups considered.

The nature and extent of liver changes seen post mortem are shown in table 3. Livers described as showing mild to

TABLE 3

Liver pathology

Each "x" indicates one observation (see text)

EMULSIFIER OR FAT	LEVEL	NUMBER OF RATS AUTOPSIED	NUMBER OF LIVERS EXAMINED HISTOPATHOLOGICALLY	LIVER CORD CELL REGENERATION	ROUND CELL INFILTRATION	FOCAL ACIDOPHILIC OR FATTY DEGENERATION	LOBULAR MALFORMATION	HEPATITIS	NECROSIS (FOCAL)	ENLARGED	INFARCTION	MISCELLANEOUS
	%											
None	0	31	9	x		x *		x		xx		
Myrj 45	5	28	8		x(L)			x	x	x		
	10	30	12		x			xx	x *	xxx	x	Leukemic infiltration
	20	31	10	xx		x			xx	x		Metastatic lung cancer
Myrj 52	5	32	10		x	x		x		x		
	10	31	13		x			x	xx			
	20	32	8	x	x				xx	x		
Span 60	5	32	8							xx		Hepatosis
	10	31	8	xx					x	xxxx		Hepatoma
	20	31	9			xx			xxx	xxxx		
Tween 60	5	33	10	x			xx	x				
	10	31	6						x	xxx		Small cyst
	20	30	9						x			
Tween 65	5	32	9	x			x					
	10	32	10	x		x						
	20	33	10	xx	x	x				x	x	Metastatic lung cancer
Tween 80	5	31	6	x	x							
	10	31	9		x		x			xxxx		Bile duct cystadenoma
	20	32	10	x	x	x		x		xxx		Cystic hepatosis
Mixture	5	32	8				x			x		
	10	32	11	x						xx		Bile duct cystadenoma
	20	32	8	x	xx	x				x		
Primex	5	32	10						x			
	10	31	10		x	x	x		x	xxxx		Leukemic infiltration
	20	32	7	xx	xxx				x			Small bile duct cyst

(L) = Lymphoma.
* = Focal except where marked with asterisk, then diffuse.

moderate pathology usually presented a granular or speckled surface, pale color, with occasional consolidated areas or superficial fibrinous adhesions. Except for those animals with hypertrophied livers (table 4) the mean weights of the livers of the rats sacrificed at two years fell within the range 4.0 ± 0.5% of body weight. The scattered cases where individual liver weights were considered to deviate excessively from the means for their respective groups varied from 5.1 to 8.7% of body weight except for one female on the 10% Tween 80 diet with a cystadenoma of the biliary duct, whose liver weight was 13.6%. The only emulsifier series in which a trend toward enlarged livers was noted, albeit only at the higher dosage levels, was Span 60; histopathologically these livers showed moderate central or lobular necrosis of no strikingly greater degree than was occasionally seen in other groups. Only two of the 20% Myrj 45 rats that died during the two-year test showed more than a mild degree of liver damage, namely, moderate focal interlobular necrosis; however one control rat that died showed severe diffuse fatty degeneration, two in the 5% Primex group showed reactive hepatitis and diffuse necrosis, respectively, and three in the 10% Primex groups showed similar necrotic or degenerative lesions.

From the large volume of pathological data recorded, those for the liver, kidneys, and bladder have been summarized in tables 3 and 5. Each item checked in the table does not necessarily indicate a different rat inasmuch as in some cases two, or occasionally more, findings were checked for a single liver. However all except very mild or questionable pathology is recorded in the tables. The most commonly observed hepatic changes observed histopathologically were mild to moderate congestion, round cell infiltration and regeneration of liver cells, with occasional foci of cloudy swelling, hematopoiesis, acidophilic or fatty degeneration, or disturbance of the lobular cell architecture. More severe pathology, principally focal or diffuse necrosis, was seen in a few instances, but the limited and sporadic incidence of these cases among the emulsifier, control and Primex groups suggests no pattern relating the

TABLE 4

Mean liver and kidney weights and deviations of rats sacrificed at two years[1]

EMULSIFIER OR FAT	LEVEL	LIVER			KIDNEYS		
		No. of rats included in average	Average weight	Weight of deviates[2]	No. of rats included in average	Average weight	Weight of deviates[2]
	%		gm	gm		gm	gm
None	0	16	3.74	5.73, 5.14	16	0.79	1.47
Myrj 45	5	8	3.78	8.26	8	0.77	1.92
	10	13	3.78	8.65, 6.21, 5.27	13	0.83	1.38, 1.18, 1.13
	20	22	3.81	6.52	15	0.73	...
Myrj 52	5	12	3.84	6.42	10	0.78	1.09, 1.21
	10	14	3.94	...	12	0.90	1.92, 1.67
	20	9	3.72	5.88	7	0.82	1.34, 1.61
Span 60	5	17	3.54	6.42, 5.35	13	0.86	1.32, 1.15, 1.16, 1.24
	10	10	3.98	6.02, 8.50, 5.75, 5.64	7	0.95	1.33, 1.13, 1.11, 1.19, 1.23, 1.12, 1.10
	20	11	3.57	6.20, 7.18, 5.59, 5.37	21	1.32[3]	[3]
Tween 60	5	11	3.64	...	10	0.78	1.26
	10	13	3.84	7.35, 5.98, 5.28	14	0.85	1.67, 1.11
	20	12	3.59	...	11	0.84	1.78
Tween 65	5	15	3.82	...	13	0.82	1.15, 1.25
	10	13	3.55	...	13	0.81	...
	20	9	3.89	5.20	9	0.78	1.10
Tween 80	5	13	3.54	...	12	0.73	1.16
	10	9	3.99	13.60, 5.87, 6.20, 5.42	9	0.86	1.12, 1.28, 1.34, 1.46
	20	8	3.55	7.28, 5.66, 5.50	12	0.88	1.13
Mixture	5	19	3.56	5.48	20	0.80	...
	10	14	3.53	5.61, 6.20	15	0.79	1.18
	20	14	3.87	5.31	11	0.92	1.35, 1.12, 1.15, 1.10
Primex	5	16	3.53	...	15	0.81	1.36
	10	14	3.69	6.27, 5.63, 5.44, 5.28	17	0.82	...

[1] Expressed as per cent of body weight.
[2] Based on average ± 2 SD of organ weights of the control rats.
[3] Eighteen out of 21 rats exceed maximum limit of controls.

TABLE 5

Kidney and bladder pathology

Each "x" indicates one observation (see text)

EMULSIFIER OR FAT	LEVEL	NUMBER OF RATS AUTOPSIED	NUMBER OF KIDNEYS EXAMINED HISTOPATHOLOGICALLY	KIDNEY: CASTS IN TUBULES	KIDNEY: CLOUDY SWELLING	KIDNEY: CHRONIC INTERSTITIAL NEPHRITIS[1]	KIDNEY: NEPHROSIS	KIDNEY: CYSTS OR ABSCESSES	KIDNEY: PYELONEPHRITIS	KIDNEY: MILD FOCAL NEPHRITIS	KIDNEY: FOCAL TUBULAR DEGENERATION	KIDNEY: FOCAL ROUND CELL INFILTRATION	KIDNEY: KIDNEYS ENLARGED	KIDNEY: CALCULI	KIDNEY: TUMORS	BLADDER: CALCULI	BLADDER: CYSTITIS	BLADDER: CYST
	%																	
None	0	31	9	xx xxx	xxx			xx										
Myrj 45	5	28	8	xx	x	x	x						x					
	10	30	11	xx	xx			xxx	x	x		x	xx		x(M)		x	
	20	31	10		xxx	x	x	x	x			x	x	xxx		x(H)	x	
Myrj 52	5	32	9	xxx	x		x	xx										
	10	31	14	xxxx	x			x	xxxx	x	xx							
	20	32	9		xx	xx				x			xx	xx				x
Span 60	5	32	8	xx										x	x			
	10	31	8	xx	xx	xx*x*							xxx xxx				x	
	20	32	10	x	xx	x*x*x*		x			xx		18 x's					
Tween 60	5	33	9	xx	x	x			x									
	10	31	6	xxx				x				x						
	20	30	9		x	x	x		x			x	xxxx	x		x		
Tween 65	5	32	9	xx			xx		x					x				
	10	32	10	xxx	xxxx					x			x					
	20	33	10			x	x	x					x	x				
Tween 80	5	31	8	xx	xx	x		xx				x	x					
	10	31	9	xxx	x			x			x		x					
	20	30	10		x	xx	x		x		x		xxx	xx		xx		
Mixture	5	32	7	xx		x*x*			x			x	xx	xx		x		
	10	32	11						x		xx			x		x †		
	20	32	8		x				x				xxx	x		xx		
Primex	5	32	10	xxx xx	xx	x	x	x	xxx							x		
	10	30	9	x	x	x				x	x	x		xx				
	20	32	7		x													

* Except where marked with asterisk, then glomerular.
† Large calculus seen in kidney.
M = metastatic.
H = with papillary hyperplasia.

observed liver pathology to either the emulsifiers *per se* or to the levels at which they were fed.

Abnormally high kidney weights were seldom found at the termination of the two-year period. The individual cases which deviated significantly from the normal limits established by the basal control group, are shown in the last column of table 4. Only in the 10 and 20% Span 60 series were the kidney weights for the groups as a whole notably increased. However as may be seen in table 5 no organic changes were seen microscopically in the kidneys of these rats which would distinguish them from the other groups. Except for this evidence of hypertrophic change, the miscellaneous renal changes recorded in the table for both test and control groups appeared to be of the type associated with senility in laboratory rats. There was no connection between the increased incidence of any form of renal pathology and the dosage levels; for example in the Myrj 52 series, pyelonephritis was noted in 4 of the 14 kidneys in the 10% group but in none of the 9 kidneys in the corresponding 20% group. Similarly the incidence of tubular casts and cloudy swelling was, if anything, lower at the 20% level of emulsifiers and Primex than at the 5 or 10% levels.

Three of the 20% Myrj 45 rats that died before the termination of the two-year test showed kidney damage, namely, mild or moderate cloudy swelling in three cases, and one medullary abscess with diffuse cortical hydronephrosis. Evidence that such renal pathology was not specific for Myrj 45 may be seen in the fact that cloudy swelling and occasionally focal abscesses or necrotic lesions were found among the basal control or Primex rats that died during the test.

Of the 228 kidneys examined histopathologically in this study, 16 revealed the presence of calcareous deposits or calcified casts. All except 4 were visible only upon microscopic examination. One of the exceptions was a rat in the 5% mixed emulsifier group which developed a suppurative nephritis with a phosphatic renal calculus weighing nearly 8 gm; stones were also seen in the bladder. Another was in a rat

in the 20% Tween 80 group that had mild chronic interstitial nephritis. Two instances of renal calculus formation seen macroscopically were in the 20% Myrj 45 group but none were found at the lower levels of this emulsifier; oxalate stones were found in the bladder of one of these rats. One of the control rats revealed calcified tubular casts. In the Primex group one rat had vesicular calculi, 4 others showed either calcified casts or deposits in the kidneys, and an additional rat had a bladder stone. Even the occurrence of bladder stones in a single rat at each of the three dose levels of the emulsifier mixture may be coincidental in view of the paucity of these deposits among the groups receiving the individual emulsifiers.

The scattered and sporadic incidence of the calcareous deposits seen microscopically in the kidneys or macroscopically in the bladders contraindicates the existence of a causal connection between any particular emulsifier and calculus formation.

Other organs examined microscopically included the stomach, gastrointestinal tract, heart, spleen, pancreas, adrenals, thyroid, gonads, lymph nodes, bone marrow and spinal cord. A detailed summary of the positive findings failed to disclose any consistent type or frequency of pathology. It might have been expected that the Myrj 52 or Tween groups, in which the laxative responses were noted at the higher dosages (Oser and Oser, '57), would have shown some evidence of organic injury in the gastrointestinal tract but such was not the case. At autopsy indications of gastric hyperplasia were found in only one rat in each of the following groups (except as noted): controls, 5% Tween 80, 10% Myrj 45, Myrj 52, 20% Myrj (two cases), Tween 65, Tween 80 (two cases), and mixture of emulsifiers. Hemosiderosis was not noted in any of the tissues examined. A few rats among both the emulsifier and control groups showed signs of splenic involvement either at death or termination, and pancreatic tumors were seen in one rat in each of 4 groups: 5% Myrj 45, 10% Tween 60 and 5 and 10% Tween 80. A few isolated instances of adrenal lesions, including two metastatic?) tumors were

observed. Neoplasms were also found at or before termination of the two-year period on various areas of the skin (besides the mammary region) or in the uterine or gonadal tissues in about 2 or 3% of the 800 rats comprising the F_0 generation. However, the varied nature and distribution of these lesions suggests that they were incidental rather than ascribable to the experimental diets.

GENERAL CONCLUSIONS

In interpreting the results of this two-year feeding study, emphasis has been laid on the comparison of emulsifier group responses not only with those of rats on the basal, unsupplemented diet, but with those on the hydrogenated vegetable fat (Primex) diets which were likewise presumed to be normal. Before proceeding with an over-all evaluation of the findings, several points appear to be worth noting. First, more rats were employed at each dosage level than in any previously reported study of long-time feeding of the partial ester emulsifiers; second, the rats were derived from a single strain and colony and had a known nutritional history; third, the animals were assigned to groups according to a balanced litter distribution; finally, precautions were taken to maintain environmental, sanitary, and operating conditions as nearly uniform and as close to the optimum as possible during the entire course of the experiment. Nevertheless, individual variations were observed within sexes within groups, which occasionally appeared to exceed normal limits. Statistical probability requires however that in a normal distribution at the $p = 0.05$ level, one deviate may be expected in every 20 cases. Consideration has been given to the frequency and degree of deviations from the norm, especially with regard to trends related to dietary supplementation levels in order to distinguish causal responses from such incidental aberrancies.

Also worthy of emphasis in assessing these results is the fact that the dosage levels of the emulsifiers were selected in accordance with the principle recommended by the Food and

Drug Administration, in that the lowest (5%) level represented a substantial multiple of the maximum conceivable human dietary level consistent with the use of these emulsifiers as functional additives in foods, whereas the highest (20%) level represented a dosage which was expected to induce an adverse response in the experimental subjects.

On the basis of the criteria of rate of growth to maturity, economy of food utilization, reproductive and lactating efficiency, hematological responses, longevity, and intercurrent pathology, it can be categorically stated that at the 5% level none of the partial ester emulsifiers induced any significant effect on the physiological responses. With certain reservations, the same general conclusion might be drawn with respect to the 10% emulsifier levels. At this dosage the Myrj 45 groups in the successive generations showed some lowering in survival rate of the newborn as was the case also in one or more generations of rats on other emulsifier diets, excluding Myrj 52. At the 10% level the more hydrophilic emulsifiers showed a moderate laxative effect which became intensified with further increase in the feeding level. At the 20% dosage level, a slight but not statistically significant depression in growth, together with a reduction in food intake, occurred in all the emulsifier groups. Taking the 4 successive generations into account, only the Tween 60 series revealed a consistent trend in the direction of reduced efficiency of utilization of the food calories as the dietary level increased from 10 to 20%. This observation could not be correlated with laxative response inasmuch as the latter was also seen at the high levels of Myrj 52 and Tweens 65 and 80 where there was no significant diminution in caloric utilization.

Feeding experiments with stoichiometrically equivalent dietary levels of the polyols of these emulsifiers showed that laxation may be evoked by the non-surface-active, long-chain polyoxyethylene alcohols as readily as by the intact, surface-active, hydrophilic esters. It is significant that neither the short chain (polyoxyethylene (8) or sorbitan) polyols nor their stearate esters (Myrj 45 and Span 60) affected lax-

ation even at the highest level. Studies with Myrj 45 and 52 on human subjects (Oler and Cramer, '55) showed no effect on gastric motility or on the mouth-to-cecum transit time. The longer chain polyol moieties have been shown to be more poorly absorbed from the intestinal tract (Shaffer and Critchfield, '47) and are probably responsible for the laxative effect at the higher concentrations.

The laxative response in its more exaggerated form was accompanied by denudation and inflammation (without suppuration) around the perianal region. This would appear to be the only physiological disturbance observed in these animals which might account for the failure of many litters to survive beyond 4 days. Two factors, however tend to refute the existence of such a causal relation; first, that diminution in viability was noted at the highest levels of Myrj 45 and Span 60 where no laxative effect was observed, and second, the absence of an adverse influence on viability of the young in the 20% Tween 80 series where the laxative response was quite common. The addition of neutral fat to the diet of these groups where the high levels of emulsifier alone was associated with reduced viability of the young, resulted in some improvement.

It should be pointed out that the effects on laxation and survival of the newborn described in the foregoing paragraphs were not observed in every animal nor to the same degree within each of the groups concerned.

The behavior of the three succeeding generations of rats with respect to growth, food utilization, reproduction, etc. followed the pattern set by the parent generation, except for a slight but persistently lower efficiency of food (and caloric) utilization. A similar effect was observed in the Primex series as well as the emulsifier series. In view of the fact that the food efficiency remained essentially constant in the three descendent generations, this phenomenon can be explained by the fact that the nutritional reserves of the initial generation of rats (bred on the laboratory stock diet) prior to being placed on the test diets differed in kind and degree from those

of their progeny, in whom the reserves remained uniform throughout the successive generations. In any case it is clear that no evidences of cumulative toxicity or of progressively changing phylsiological response, adverse or otherwise, were seen in the 4 consecutive generations despite the life-time restriction of their food intake to diets containing up to 20% of the partial ester emulsifiers.

Hematological observations made at several stages throughout the two-year period revealed no significant departures from the normal ranges of red and white cell counts, differtial leukocyte counts or in the blood sugar, non-protein nitrogen or cholesterol values.

Mortality statistics indicated no evidence of untoward effect on longevity in any of the 5 or 10% emulsifier groups nor, indeed, at the 20% level of Myrj 45 or Span 60. During the final quarter of the two-year test more deaths occurred among the females on the 20% diets containing Myrj 52 or the three Tweens than in the other groups. It is suggested that the responses with respect to laxation, survival of newborn, and mortality at two-years in the groups receiving the highest level of the latter four emulsifiers, may have a common basis. Whether this is the cumulative stress of the laxative effect or a more basic but obscure manifestation of the long-chain polyoxyethylene esters remains to be determined.

Post-mortem examinations revealed that most of the deaths during this long term feeding study were attributable to respiratory disease. Many of the deaths among the females occurred during or after parturition and even in these cases the lungs were usually involved. Mammary fibroadenomas were found in from one to 6 animals in the emulsifier, fat and control groups (each comprising initially 20 or 21 females). Although a few tumors of the skin, gonadal organs, or occasionally other sites, were found, there was no association between the incidence, character, or severity of these lesions and any particular dosage level or test material. Even in the series (Myrj 45 and Tween 65) where the number of mammary tumors appeared to increase with the dietary levels of

emulsifier, the maximum incidence was within the statistical limits established by all groups, including the control and Primex animals.

Some liver pathology was found in all groups, both test and control, at the conclusion of the study. These included mild to moderate cellular regeneration or infiltration, focal degenerative or necrotic lesions, and a few cases of hepatitis, bile duct cystadenomas, etc. However the incidence and variety of these changes was widely scattered and, with the sole exception of Span 60, no specific emulsifier was involved. Most of the liver pathology was observed when the rats were sacrificed after two years and seldom was the death of a rat associated with, much less ascribable to, hepatic injury. In clinical studies with human subjects, both normal and patients with non-hepatic disorders, Waldstein et al. ('54) observed no deleterious effects from the oral administration of 6 gm of Span 60 or Tween 60 daily for 28 days as judged by liver function and blood chemical criteria. Kreusi and Van Itallie ('56) conducted tests on 10 patients receiving 3 to 6 gm of Myrj 45 daily while convalescing from hepatic disease and likewise found no adverse effects either clinically or in a series of liver function tests.

In the Span 60 groups, particulary at the 20% level, a marked increase in liver weight was noted; nevertheless these livers for the most part appeared to be histologically normal, focal necrotic lesions being found in only a few.

Similarly, the frequency, distribution, and severity of renal pathology observed upon gross and microscopic examination, were such as could be considered expected changes in senile rats. No special significance is attached to the finding of casts in the urinary tubules or to cloudy swelling, and the incidence of chronic interstitial nephritis, nephrosis, cysts and abscesses, was unassociated with any particular diet. The only striking observation in respect to renal changes was the predominantly enlarged kidneys seen in the 20% (and to a lesser extent in the 10%) Span 60 group. The kidneys of these animals, which also had enlarged livers, likewise showed no microscopic

pathology sufficient to account for the hypertrophy. It would appear therefore that the ingestion of high dosages of this emulsifier imposes a physiological stress on the rat which results in hypertrophy of both the liver and kidneys without specific injury to these organs.

It is of interest in this connection to refer to the report of Krantz et al. ('52) to the effect that Myrj 52 and various Tweens did not significantly alter the oxygen uptake of sliced kidney tissue.

There was a sporadic incidence of microscopic calcareous deposits in the kidneys of both control and test animals, but in only 4 of the 228 kidneys examined were the calculi grossly visible, two being in the 20% Myrj 45 group.

The cumulative incidence of bladder stones in the F_0 generation animals involved one rat in each of the following groups: 5% mixed emulsifiers and Primex, 10% mixed emulsifiers, 20% Myrj 45, Tween 60, and mixed emulsifiers; and two rats in the 20% Tween 80 group. In view of the fact that there were about 30 rats per group, this incidence can not be regarded as significantly related to the ingestion of any of the emulsifiers.

Histopathological examination of the major organs and tissues examined in rats at the termination of the two-year feeding test, including tumors, mammary or otherwise, showed no lesions which by their frequency or nature could be said to differentiate the emulsifier groups, irrespective of dosage level, from the control or Primex groups.

The findings here reported support the conclusions of Graham and co-workers ('54, '55) with respect to the innocuous effect of the chronic ingestion by rats of bread containing 50 times the normal concentration of Myrj 45 (as well as other additives). Similarly, the negative responses observed by Krehl et al. ('55) in 500-day feeding tests with rats on diets containing 6% of the various emulsifiers, including Myrj 45, are corroborated.

The present investigations are in essence interpreted, as providing ample justification for the use of the partial ester

emulsifiers (Myrj 45, Myrj 52, Span 60, and Tweens 60, 65, and 80) as food additives in concentrations cumulatively totaling a few tenths of 1% of the average American diet, with reasonable assurance that the chronic consumption of such a diet would entail no hazard to human health.

LITERATURE CITED

GRAHAM, W. D., AND H. C. GRICE 1955 Chronic toxicity of bread additives to rats. J. Pharm. Pharmacol., *7:* 126.

GRAHAM, W. D., H. TEED AND H. C. GRICE 1954 Chronic toxicity of bread additives to rats. Ibid., *6:* 534.

KRANTZ, J. C., JR., R. D. MUSSER AND N. J. KNAPP 1952 Sugar Alcohols. XXIX. Sorbitan polyoxyethylene fatty acid esters and polyoxyethylene monostearate and respiration of kidney. Proc. Soc. Exp. Biol. Med., *81:* 640.

KREHL, W. A., G. R. COWGILL AND A. D. WHEDON 1955 Non-deleterious effects of polyoxyethylene esters in the nutrition of rats and cats. J. Nutrition, *55:* 35.

KREUSI, O. R., AND T. B. VAN ITALLIE 1956 Effect of polyoxyethylene (8) monostearate (Myrj 45) on liver function in patients convalescing from hepatic disorders. Food Res., *21:* 565.

REPORT OF FOOD PROTECTION COMMITTEE, FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL 1953 The safety of polyoxyethylene stearates for use as intentional additives in foods. Publication 280 (July).

OLER, W. M., AND V. C. CRAMER 1955 Effects of two commercial emulsifiers, Myrj 45 and Myrj 52, on gastric acidity and gastrointestinal motility of human subjects. Gastroenterology, *28:* 281.

OSER, B. L., AND M. OSER 1957 Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. III. Clinical and metabolic observations. J. Nutrition, *61:* 149.

SHAFFER, C. B., AND F. H. CRITCHFIELD 1947 The absorption and excretion of the solid polyethylene glycols. J. Am. Pharm. Assn., Sci. Ed., *36:* 152.

WALDSTEIN, S. S., H. M. SCHOOLMAN AND H. POPPER 1954 The effect of feeding large amounts of emulsifiers polyoxyethylene (20) sorbitan monostearate (Tween 60) and sorbitan monostearate (Span 60) to humans. Am. J. Digestive Diseases, *21:* 181.

COMPARISON OF THE METABOLIC ENERGY CONTRIBUTIONS OF FOODS BY GROWTH UNDER CONDITIONS OF ENERGY RESTRICTION[1]

E. E. RICE, W. D. WARNER, P. E. MONE AND C. E. POLING
Research Laboratories, Swift & Company, Chicago, Ill.

(Received for publication August 2, 1956)

INTRODUCTION

It is frequently desirable to measure the relative nutritional values of similar foods or of a single food before and after certain processing operations. A number of methods can be used for this purpose, including studies of protein quality, caloric values, digestibility, growth under conditions of ad libitum or restricted feedings, reproductive efficiency, lactation, blood regeneration rates or gross observations of hair coat or other physical characteristics.

Each of these methods has certain advantages and limitations, but the data obtained frequently are difficult to interpret because of variable food intake and the excretion of endogenous materials with food residues. Most of them require an extended period of rather exacting research. Such detailed methods are essential for complete elucidation of the metabolic functions of nutrients but they are time consuming when many food items are to be compared.

It is one of the concepts of nutrition that energy requirements are paramount — that food, regardless of its nature, will be used for energy to the greatest extent possible until minimum energy requirements are met, irrespective of other

[1] This paper was presented before the American Institute of Nutrition, Federation Meetings, Atlantic City, April 1956.

needs. This being true, it would seem that young growing animals which were offered fully adequate amounts of all nutrient essentials except calories should gain in proportion to some function of the number of calories provided and that this growth should be constant for any given intake of available calories irrespective of their origin. Growth attained when feeding different foods under such a program should vary in proportion to the metabolically available energy of the respective foods. Presumably amino acids, fats, or carbohydrates which are altered or bound in such a manner that they cannot be used for calories under these conditions would not be available for other functions. Growth should thus be a direct measurement of maximum, comparative food values. Obviously, changes might occur, which would interfere with the utilization of a nutrient, such as an amino acid, for growth or other specific metabolic function but which would not destroy it as a source of calories. Destruction of calorie potential, however, would inevitably indicate nutrient destruction.

A procedure relating growth to the calorie supply would measure metabolically available energy and would circumvent many problems relating to the digestibility, body storage, and excretion of endogenous materials. Furthermore, the constant need for energy should assure an immediate reflection of the dietary treatment without the necessity for prolonged preliminary periods of equilibration. Since only energy utilization is involved, and this for a short period, growth responses should be more consistent than in studies where long-term deficiencies might result in hormone or enzyme disturbances which would complicate interpretation of the data.

If materials of a similar composition were used as supplements to a basal diet restricted in calories but otherwise adequate, growth proportional only to the added energy would be expected. However, if several types of foodstuffs were to be compared the relative effects of the various nutrients on caloric efficiency might alter growth responses slightly, despite the adequacy of the basal portion of the diet. For example,

Forbes, Swift, James, Bratzler and Black ('46a) have suggested that the amount of fat in the diet will affect the relative efficiency of the utilization of the total food of the diet. Such an effect might complicate interpretation of data for different types of foods but would not be a handicap in considering products of similar composition.

Experiments to test such a procedure [2] have been designed and are reported herewith.

EXPERIMENTAL PROCEDURE

Since the purpose of the experiment was to measure the gains induced by the feeding of known amounts of calories as food in addition to a basal ration limiting only in calories, a highly nutritious basal diet was formulated. The percentage composition of this, initially, was: casein (vitamin free), 43.82; sucrose, 13.14; celluflour, 3.29; salt mix,[3] 6.57; cornstarch, 30.44; vitamin E feed supplement,[4] 1.42; vitamin mixture, 1.32.

The vitamin mixture supplied 1.25 mg thiamine hydrochloride, 1.25 mg riboflavin, 1.25 mg pyridoxine hydrochloride, 7.0 mg calcium pantothenate, 6.35 mg niacin, 1.60 mg vitamin K, 40.0 μg folic acid, 16.0 μg biotin, 16.0 mg *p*-amino benzoic acid, 16.0 mg i-inositol, 160 mg choline chloride, and 1.11 gm liver powder (1:20) per 100 gm diet. In addition to this diet, each rat was fed one drop per week of oleum percomorphum containing 1250 U.S.P. units vitamin A and 180 U.S.P. units vitamin D per drop. After several tests the ration was modified by the addition of 4 parts of destearinized cottonseed oil to 96 parts of the above ingredients.

[2] Since submitting this paper for publication our attention has been called to a similar procedure reported in an abstract of a paper presented before the American Chemical Society Meeting in 1947 (Abstracts, 111th meeting ACS, Atlantic City, April 14 to 18, 1947, A rat growth assay for the physiological availability of potential calories in foods, by B. L. Oser and D. L. Melnick).

[3] Jones and Foster ('42).

[4] Myvamix, manufactured by Distillation Products Industries. It contains 44 I.U. of vitamin E per gram as *d*-*α*-tocopherol acetate.

Proximate analyses of typical samples of the initial formulation indicated that it contained approximately 92% solids, including 5.9% ash, 6.8% nitrogen, and 0.7% fat. The calculated gross calorie content (using the factors 4, 4 and 9) is 3.5 Cal. per gram. When fed to weanling rats, 5 gm per day of this diet was sufficient to permit slow gain (2 to 8 gm per week). This quantity of ration contained sufficient protein, vitamins and minerals to permit optimum growth when sufficient calories were supplied. The inclusion of cottonseed oil increased the fat content by 4.0% and improved growth by 2 to 5 gm per week.

Inasmuch as this technique, if successful, was to be used first to check the relative biological availability of several fatty materials, prime steam lard was selected as a source of additional calories in the comparison of growth at several levels of energy intake. Male albino rats [5] were weighed, individually caged, and fed 5 ± 0.1 gm of the basal diet daily for 7 days. Water was supplied ad libitum. At the end of this period the animals were weighed and arranged into uniform groups of 4 each (still individually caged) and either continued on the basal regimen or changed to diets composed of the basal mixed with exactly weighed quantities of lard or test material. Each experiment was replicated at least once.

Initially, animals were weighed again after 3, 7, 14 and 21 days of supplementation. Responses to the various supplements or test materials were so uniform, however, that 7-day weights reflected the increases in calorie intake as definitively as weights for longer periods. Table 1 presents data for three replications of tests with 4 rats per group (i.e., a total of 12 per treatment) and indicates the good reproducibility experienced. In most cases subsequent tests were continued for only one week of supplemented feeding.

It is obvious from table 1 that increases in the amount of lard eaten resulted in increased growth. When the gains are plotted against the amount of supplement, a smooth curve is obtained. That these gains were primarily the result of

[5] Holtzman Rat Co., Madison, Wisconsin.

TABLE 1

Growth responses of rats fed calorie-restricted diets

SUPPLEMENT TO 5 GM BASAL DIET	AVERAGE GAINS FOR REPLICATES OF 4 RATS									
	1-week period					3-week period				
	Rep. 1	Rep. 2	Rep. 3	Av. 12 rats	Coefficient of variation [1]	Rep. 1	Rep. 2	Rep. 3	Av. 12 rats	Coefficient of variation [1]
gm	*gm*	*gm*	*gm*	*gm*	%	*gm*	*gm*	*gm*	*gm*	%
0	7.3	6.8	5.0	6.3	28.1	19.7	17.5	14.0	17.1	24.0
Lard, 0.5	18.0	17.8	18.3	18.0	8.2	42 7	40.7	39.0	40.8	5.3
ard, 1.0	26.8	26.8	26.0	26.5	5.9	60.2	64.7	55.7	60.2	7.9
ard, 1.5	33.3	30.0	34.0	32.4	8.3	78.0	73.3	77.5	76.2	5.3
Lard, 2.0	38.5	37.7	37.0	37.8	4.6	89.5	89.5	86.5	88.5	4.9
Lard, 2.5	42.0	42.5	43.3	42.6	4.2	99.1	98.2	97.0	98.3	3.7
Lard, 3.0	45.5	48.0	44.5	46.0	4.6	103.0	107.2	99.0	103.8	4.5
Tallow, 2.0	33.3	33.5	35.0	33.9	3.9	83.3	82.5	74.2	80.0	5.9
60-titer tallow, 2.0	12.2	14.7	12.5	13.2	13.6	31.5	27.5	30.2	29.8	10.5
Sucrose, 1.1	16.5	20.8	16.5	17.9	20.3	37.2	36.8	35.2	36.4	8.3
Sucrose, 4.5	33.3	36.0	35.8	35.0	6.7	78.9	75.0	76.7	76.8	4.8
Average					9.1					7.7

[1] Calculated on the basis of the ind' idual data for each animal.

increased calorie intake rather than a specific effect of lard was demonstrated by measuring the gains following supplementation with casein, glucose, sucrose, celluflour, hydrogenated tallow, and several fatty acids. In each case the gains observed were interpreted by reference to a curve prepared by plotting gains of similar groups of rats fed the standard levels of prime steam lard at the same time. See table 2. Since the supplements were fed at several different times

TABLE 2

Relative growth promoting effects of calories from various sources

DAILY SUPPLEMENT	NUMBER ANIMALS USED	AV. GAIN IN 1 WK.	PSL [1] VALUE	CALORIE EQUIVALENT	THEORETICAL CALORIE CONTENT
gm		*gm*	*gm*	*Cal.*	
Sucrose, 1.13	12	17.9	0.50	4.5	4.5 [2]
Sucrose, 4.50	12	35.0	1.75	15.7	17.7 [2]
NBS sucrose, 2.0	12	21.8	0.85	7.7	7.9 [2]
Commercial sucrose, 2.0	12	22.1	0.90	8.1	7.9
Glucose·H_2O (commercial), 4.90	12	33.4	1.65	14.8	16.6 [3]
Casein, 3.00	8	29.3	1.42	12.8	12.7 [4]
PSL, 1.0 + casein, 1.1	12	32.7	1.50	13.5	13.6 [5]
Hamburger, 6.0	8	38.4	2.15	19.3	17.8 [6]
Celluflour, 3.0	8	6.4	0.04	0.4	0
Lard, 2.0	8	36.5	2.0		
Lard, 1.6 + hardened tallow, 0.4	8	35.5	1.84		
Lard, 1.2 + hardened tallow, 0.8	8	33.9	1.68		
Lard, 0.8 + hardened tallow, 1.2	8	29.9	1.33		
Hardened tallow,[7] 2.0	8	13.9	0.32		
None	8	5.5	0		

[1] "Prime steam lard" values; the weight of prime steam lard which would give growth equivalent to that promoted by the supplement, as estimated from the reference curve.

[2] National Bureau of Standards reference value supplied = 3.94 calories gm.

[3] Calculated from sucrose value.

[4] Weight × 4.75 Cal./gm × 90% solids. Kriss and Voris, '37; Metta, Chalam and Mitchell, '54.

[5] (1.0 × 9.0) + (1.1 × 4.7 × 0.90).

[6] [(16.7% protein × 4.25 Cal.) + (25.0% fat × 9.0 Cal.)] × 6 = 17.8.

[7] 60-titer tallow.

the gains for test substances could not be compared directly to each other but have been interpreted by comparison to the appropriate reference curve. The reference curves were very similar but it is believed that more accuracy can be attained by comparing test groups to a reference curve determined simultaneously than to one previously established or to one which uses a composite of the values obtained in several experiments. A composite curve might be accepted for less critical evaluations.

The growth observed can be expressed either in terms of "prime steam lard" values (PSL values) or estimated as calories. The PSL value for a supplement is the weight of prime steam lard which would give growth equivalent to that promoted by the supplement, as estimated from the reference curve. If lard has a heat of combustion of 9.5 Cal. per gram as reported by Atwater (1899) and a digestibility of approximately 95%, Crockett and Deuel ('47), the metabolically available energy should be very close to the 9.0 Cal. per gram which is conventionally used. This factor has been accepted in converting prime steam lard values to calories. The close agreement of the calorie values determined experimentally for the various types of supplement with the theoretical values supports the fundamental premise underlying this method — that energy, irrespective of origin, is the critical factor in experiments of this nature.

The feces from each of the rats fed prime steam lard in securing the data shown in table 1 were collected separately during the third week and analyzed for fat in order to determine absorption of the fats at the various levels. No preliminary collection period was used. Instead, the value for fat appearing in the feces of the rats fed only the basal was used as the control. The difference between the fat excreted by the various reference groups and the control group was assumed to be extra fecal fat due to ingestion of the fat supplements. The percentages of the extra dietary fat absorbed are in the range reported for lard by most investigators, i.e., 93 to 98%, and tend to validate the use of an average value of 95%

absorption in calculating calorie contributions (table 3). The gradual decline in fat absorption with increasing amounts of fat may be a reflection of the relative decrease in protein level reported by Barnes, Primrose and Burr ('44) to affect fat absorption, or it may be due to progressively greater saturation of the fat absorption mechanisms.

Similar data were obtained for rats fed various fatty acids except that feces for each group were pooled and analyzed for a 5-day period during the week of supplementation. The digestibilities calculated from the dietary fat intakes and fecal excretions agree well with availability values estimated from the growth data. These latter values were calculated by referring growth obtained when the basal diet was supplemented with the various fatty acids to that shown in a prime steam lard curve representing reference groups raised at the same time. (These reference groups are not the same ones listed in the first part of the table.) The value obtained from the curve indicates the amount of prime steam lard which would have given the same growth. "Metabolic Availability" of the calories in the test substance may be estimated by dividing the calories determined experimentally (PSL value $\times$ 9 Cal. per gram) by the theoretically available calorie content of the test substance.

During the development of this procedure two modifications were introduced. It had been assumed when formulating the basal diet that essential fatty acids would not be critical components because of the long depletion periods reportedly needed to develop symptoms of deficiency, even with special defatted rations. However, since fats to be studied by this technique might vary in their content of unsaturated fatty acids, experiments were carried out to determine the effect of essential fatty acids on the one week gains. Groups fed rations containing approximately 100 mg of unsaturated fatty acids gained more than would be expected on the basis of additional calories alone. Thus in one test the average one week gains for 12 rats for several conditions were: 2 gm hydrogenated tallow, 14.1 gm; 2 gm hydrogenated tallow + 0.15 gm

TABLE 3

SUPPLEMENT AND AMOUNT PER RAT PER PERIOD	NO. RATS FED	FECAL FAT PER RAT PER PERIOD	ADDITIONAL FAT EXCRETED DUE TO SUPPLEMENT	APPARENT FAT ABSORPTION FROM SUPPLEMENT	DIGESTIBILITY	PSL VALUE	ESTIMATED AVAILABILITY [1]
gm	*gm*	*gm*	*gm*	*gm*	%		%
0	12	0.15	. . .	. . .	. .		
Lard, 3.5	12	0.24	0.09	3.41	98		
Lard, 7.0	12	0.41	0.26	6.74	96		
Lard, 10.5	12	0.58	0.43	10.07	96		
Lard, 14.0	12	0.82	0.67	13.33	95		
Lard, 17.5	12	1.33	1.18	16.32	94		
Lard, 21.0	12	1.45	1.30	19.70	94		
0	12	0.19	. . .	. . .	. .		
Elaidic acid,[2] 5.0	8	3.30	3.11	1.89	38	0.43	41
Oleic acid,[3] 5.0	8	0.79	0.60	4.40	88	0.92	87
Oleic acid, 10.0	12	1.50	1.31	8.69	86	0.84	80
Myristic acid, 10.0	12	4.57	4.38	5.62	56	0.43	41 [4]
Palmitic acid, 10.0	12	7.78	7.59	2.41	24	0.23	22
Stearic acid, 10.0	12	9.08	8.89	1.11	11	0.13	12

[1] Since 95% of the lard consumed is absorbed, PSL values must be multiplied by 0.95 to give an estimate of the "available lard values." These may be compared directly to other fatty materials of similar caloric content.
[2] Trans octadecanoic acids prepared with a selenium catalyst.
[3] Technical grade.
[4] Estimated 14 gm of food refused and growth corrected upward to compensate for this percentage of uneaten food.

partially hydrogenated soybean oil, 16.8 gm; 2 gm hydrogenated tallow + 0.15 gm cottonseed oil, 19.6 gm. The increase in the gains when soybean oil (partially hydrogenated to less than 1% of linoleic acid) was added to the hydrogenated tallow amounted to 2.7 gm. This is equivalent to 130 mg of lard and is approximately the amount expected from any digestible fat. The increased gain with the cottonseed oil, 5.5 gm, is equivalent to 0.28 gm of lard. Similar comparisons involving a total of 72 rats showed better gains when cottonseed oil was fed at a low level than when slightly hydrogenated soybean oil was fed in the absence of fats containing unsaturated fatty acids. However, when the additions were made to lard or shortenings containing unsaturated fatty acids, the soybean oil was fully as effective as the cottonseed oil. Since both the partially hydrogenated soybean oil and the cottonseed oil should be utilized well, the unsaturated fatty acids must have accounted for the slight stimulation. These observations suggest that when calorie intake is restricted essential fatty acids may influence growth more readily and earlier than heretofore reported. For this reason in subsequent tests the basal ration was modified to include 4% of destearinized cottonseed oil.

Four tests (including replicates 1, 2 and 3 in table 1) were conducted in an animal room maintained at a temperature of 80 $\pm$ 2°F. Other experiments were run in new quarters maintained at a temperature of 76 $\pm$ 1°F. and a relative humidity of 45 to 50%. After the change had been made it was noted that a slightly lower growth was attained in the cooler room. Thus the average gain for 4 control group replications was 6.2 gm as compared with 2.6 gm for three control group replicates in the new quarters. Three supplements fed under the two conditions promoted gains of 13.7 and 9.4, 16.4 and 14.6, and 19.6 and 16.8 respectively, for the old and new quarters.

It is not known whether or not the slightly lower environmental temperature was solely responsible for the decreased

gains but the observation suggested that temperatures might be a critical factor in tests of this nature and that temperature differentials (about 4°F.) between the top and bottom rows of cages on racks might be important. All incoming animals are randomly distributed as they are taken from the shipping containers into individual cages on racks with 5 tiers of 6 cages each. In one test each animal was fed a commercial laboratory food for two days, weighed and then fed 5.0 gm of basal ration for 7 days and reweighed. Uniform groups for continuation of the experiment with the various test and reference supplements were assembled on the basis of these final weights and weight gains, discarding animals which

TABLE 4

Effect of environmental temperature upon weight gains of individually caged rats

POSITION	NO. RATS	AV. GAIN IN 1 WK.	STAND. DEV.	AV. TEMPERATURE
		gm.		*F.*
Top row	35	7.8	2.3	77.9
2nd row	35	7.3	2.4	...
3rd row	36	7.0	3.1	76.0
4th row	22	5.8	2.3	...
5th row	32	5.9	2.6	73.3

either did not gain or gained exceptionally well, or which were outside an acceptable weight range. The average gains during the 7-day basal period for animals in all top rows of cages, all second rows, etc. are shown in table 4. The gains in the 4th and 5th rows were significantly lower than those in the other rows.

Other less extensive checks have confirmed this demonstration that the animals at the higher and warmer levels gained more. These differences, though slight, are indications of the care which must be taken in the conduct of critical animal experiments. In our calorie restricted feeding experiments each replicate contained one animal at each of the top 4 cage levels, thereby equalizing temperature effects.

DISCUSSION

The procedure described gives an indirect measure of the energy available under normal conditions of usage and eliminates need for extensive, exact analyses of food, excrement and body tissues and for the involved assumptions and calculations which are necessary when endogenous and exogenous sources of energy are interrelated in balance experiments. The close agreement of experimental and theoretical calorie values for materials of several food types, as shown in table 2, justifies the assumption that the procedure measures available energy and that it is not markedly influenced by changes in the proportion of the nutrients supplied. These findings differ from those of other investigators such as Forbes et al. ('46b) and Meng and Youmans ('55) who found that increasing percentages of fat in the diet resulted in improved economy of energy utilization. However, the conditions of those experiments were such that the adult animals could deposit fat in their tissues, whereas, in these tests, the energy was used more completely for growth.

Correlation between the growth data and the absorption measurements is as good as can be expected for two independent biological measurements, and both the growth and balance data agree with published figures except in the case of elaidic acid, which has been reported by Paul and McCay ('42) to be well absorbed. The present findings indicate only approximately 40% absorption of transoctadecanoic acids, including elaidic acid, which is more in line with the 56% absorption reported by Paul and McCay for guinea pigs.

Several investigators have suggested that the presence of one fat may influence the utilization of others. Thus Hoagland and Snider ('43) showed that olive oil improved the utilization of trilaurin, trimyristin, and tripalmitin but not of tristearin. Mattil and Higgins ('45) also found that the mixing of unsaturated fats with saturated fats increased the availability of the saturated products.

The improvements, although slight, led to the conclusion that higher saturated fatty acids are poorly absorbed even

when fed as a mixture with unsaturated fats. The data in table 2 confirm this conclusion. If one considers the several tests where lard and hardened tallow have been fed it may be seen that as much as 60% of the hardened tallow was utilized when fed with lard (assuming full utilization of lard), but that only 16% was utilized when it was fed alone.

These initial tests suggest that this method may be applicable in many types of research, where it is necessary to detect changes in nutritive value relating to fat, protein, or carbohydrate. The prompt growth response is positive evidence of a net utilization of energy, and the method bypasses the need to determine absorption, tissue deposition, extra excretion or other factors required in the more refined techniques. This method is particularly applicable where rapid, inexpensive surveys of a considerable number of samples is involved. Preliminary tests with chickens suggest that they may also be satisfactory as test subjects.

SUMMARY

A method has been devised for measuring the available energy of foodstuffs in terms of one-week gains in the weight of young rats fed calorically restricted diets under carefully controlled conditions.

When tested with lard, tallow, hardened tallow, glucose, sucrose, casein, and celluflour the theoretical values for available energy were obtained.

The method permits approximation of the energy contributions of foodstuffs without resort to complex and laborious balance studies.

LITERATURE CITED

ATWATER, W. O. 1899 12th Annual Report of Storrs Agricultural Experiment Station.

BARNES, R. H., M F. PRIMROSE AND G. O. BURR 1944 The influence of the protein content of the diet upon fat digestibility. J. Nutrition, *27:* 179.

CROCKETT, M. E , AND H. J. DEUEL, JR. 1947 A comparison of the coefficient of digestibility and the rate of absorption of several natural and artificial fats as influenced by melting point. Ibid., *33:* 187.

FORBES, E. B., R. W. SWIFT, W. H. JAMES, J. W. BRATZLER AND A. BLACK 1946a Further experiments on the relation of fat to economy of food utilization. I. By the growing albino rat. Ibid., *32:* 387.

FORBES, E. B., R. W. SWIFT, E. J. THACKER, V. F. SMITH AND C. E. FRENCH 1946b Further experiments on the relation of fat to economy of food utilization. II. By the mature albino rat. Ibid, *32:* 397.

HOAGLAND, R., AND G. G. SNIDER 1943 Digestibility of certain higher saturated fatty acids and triglycerides. Ibid., *26:* 219.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. Ibid., *24:* 245.

KRISS, M., AND L. VORIS 1937 A further contribution to the derivation of factors for computing the gaseous exchange and the heat production in the metabolism of proteins. Ibid., *14:* 215.

MATTIL, K. F., AND J. W. HIGGINS 1945 The relationship of glyceride structure to fat digestibility. I. Synthetic glycerides of stearic and oleic acids. Ibid., *29:* 255.

MENG, H. C., AND J. B. YOUMANS 1955 Utilization of a "synthetic" triglyceride preparation by weanling rats. Ibid., *55:* 527.

METTA, V. C., AND H. H. MITCHELL 1954 Determination of the metabolizable energy of organic nutrients for the rat. Ibid., *52:* 601.

PAUL, H., AND C. M. MCCAY 1942 The utilization of fats by herbivora. Arch. Biochem., *1:* 247.

PLASMA LIPID AND ORGAN CHANGES ASSOCIATED WITH THE FEEDING OF ANIMAL FAT TO LAYING CHICKENS[1]

HAROLD S. WEISS AND HANS FISHER
Department of Poultry Science, Rutgers University, New Brunswick, New Jersey

(Received for publication August 16, 1956)

In the course of an experiment to determine the value of an animal fat-supplemented, high-energy diet in stimulating early sexual maturity in the pullet, observations were made on performance, plasma lipids, and gross post mortem organ appearance that suggested undesirable effects related to the added animal fat. Although laying rations containing added animal fat have been shown to increase feed efficiency (Hill et al., '56; Lillie et al., '52), little has been reported concerning any biochemical or physiological changes resulting therefrom. The studies of Lorenz et al. ('38) and Walker et al. ('51) indicate that levels of fat as high as 18% of the diet of the laying bird have little effect on the blood lipids, but these results were based on added fats of *vegetable* origin. Recent mammalian studies suggest that a differential lipemic response may be expected from fats of vegetable and animal origin (Beveridge et al., '56; Aftergood et al., '56).

PROCEDURE

Diets. The all-mash diets used are listed in table 1. Diet A is a standard starting ration and diet B a standard laying

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Poultry Science, New Brunswick. Supported in part by a grant-in-aid from the Central Jersey Farmers Cooperative Association, Hightstown, N. J.

ration in use at the University farm. Neither diet contains added fat, but the meat scrap of diet B contains 6% fat. Diet C is an experimental high energy ration containing 10%

TABLE 1

Experimental diets

INGREDIENT	DIETS				
	A	B	C	D	E
	%	%	%	%	%
Yellow corn meal	53.6	33.5	38.5	52.2	57.2
Ground wheat		20.0		10.0	10.0
Ground oats		20.0			
Ground barley				5.0	5.0
Alfalfa (dehydrated)	3.0	6.0	3.0	3.75	3.75
Soybean meal (50% protein)	34.0		30.0		
Soybean meal (44% protein)		5.0		13.1	13.1
Meat scrap (50% protein)		7.5		2.5	2.5
Fish meal (60% protein)			10.0	1.25	1.25
Corn distillers solubles	4.0		2.0		
Butyl fermentation solubles	1.0		2.0	2.0	2.0
Dried whey			2.0		
Vitamin B_{12}-antibiotic supplement	0.5	0.25			
Dicalcium phosphate	2.2		1.0	1.25	1.25
Steamed bone meal		2.0			
Limestone				3.0	3.0
Salt	0.5	0.35	0.3	0.25	0.25
Trace mineral mix	1.0 [1]	3.0 [1]	1.0 [1]	0.05 [2]	0.05 [2]
Choline chloride (25%)	96 gm		100 gm		
Niacin	1 gm				
Vitamin A and D oil	0.2 [3]	0.4 [4]	0.2 [3]		
Vitamin premix [5]				0.63	0.63
Fat [6]			10.0	5.0	

[1] Mico concentrate, Limestone Corp. of America, Newton, N. J.

[2] Delamix, Limestone Corp. of America, Newton, N. J.

[3] 300 D_3 and 1500 A.

[4] 400 D_3 and 1000 A.

[5] Furnished per 100 lbs: 50,000 I.U. Vitamin A
50,000 I.C.U. Vitamin D_3
1 gm Niacin
5 gm Choline Cl
0.15 mg Vitamin B_{12}.

[6] Yellow grease, Standard colors of the Fat Analysis Committee of the American Oil Chemists' Society (FAC color), 37 to 39; free fatty acids (FFA), 15 to 20%; moisture and unsaponifiable material (MIU), maximum 2.0; activated oxygen method (AOM), value not less than 20 hours.

added fat, with the protein concentration increased to 26% in anticipation of decreased feed consumption resulting from the higher energy level (Hill et al., '56). Diet D is a commercial laying ration which normally contains 3% fat but for the purpose of this experiment was increased to 5%. In ration E, the 5% fat of diet D was replaced with 5% corn meal. The meat scrap of both diets D and E contains approximately 12% fat in contrast to the meat scrap used in diet B.

Plasma lipids. Plasma cholesterol was determined by the method of Zlatkis et al. ('53), except that 5.0 ml of glacial acetic acid was added to 0.1 ml plasma, followed at once with 3.5 ml of the color reagent and immediate mixing. Recovery tests have been uniformly good by this technique.

Total plasma lipid was measured by the turbidimetric method of Huerga et al. ('53) with the following modifications: (a) 0.1 ml of plasma was added to 2 ml of Bloor's mixture, and brought up to 2.0 ml after incubation; (b) 0.5 ml of dioxan and 1.65 ml of the sulfuric acid solution were added in turn to a 12 × 75 mm cuvette containing the dried, extracted lipid and read in a Coleman Jr. spectrophotometer. A standard curve was determined as described by Huerga, using samples of chicken plasma selected to cover a wide range of lipid values. A straight line relationship was found to hold between 500 and 4000 mg %, the limits of the samples. The optical density for these limits was 0.07 and 0.38, respectively, giving a slope that indicated considerably less sensitivity than that obtained for human serum.

Post mortem data. Liver, gall bladder, spleen, and kidneys of all birds killed were inspected visually for abnormalities, including fatty infiltration. Body fat was estimated by use of a gross scoring technique in which 1 = normal, 2 = slight excess fat, and 3 = definite excess fat. Aortic atherosclerosis was estimated according to the method of Katz and Stamler ('53, p. 137). Histological studies were made on tissues preserved in 10% formalin and stained with H and E.

Experiment 1. A July hatch of White Leghorns was reared on diet A until 8 weeks, at which time 127 pullets were placed

on the high-energy diet C (10% fat). One hundred thirty-five birds, serving as controls, were placed on diet B simultaneously. Birds were on the floor throughout the experiment. A random selection of these birds was used for the determination of plasma cholesterol levels, by taking the average of three values per bird over a 6-week interval between the 29th and 35th week of age. Ten treated and 4 controls from among the sampled population were sacrificed at the 35th week and gross organ appearance, including aortic atherosclerosis, noted. Histological studies were not made. The birds killed were selected to include equal numbers with high and low cholesterol levels within each group. The remainder of the fat-fed birds were subsequently placed back on diet B and general performance noted for an additional three months.

Experiment 2. Twenty-five White Leghorn pullets, April hatched, were placed on diet D (5% fat) when one year old, and 25 others, serving as controls were placed on ration E. All birds had been on diet B for 9 months prior to the test. As noted earlier, diet B did contain some animal fat through the use of meat scrap. Thus, all birds on experiment 2 had received some animal fat in their diet for the 9 month pre-test. All birds were floor-housed. A single blood sample from 10 randomly selected birds within each group was taken after 8 weeks of treatment and used to determine plasma cholesterol and total plasma lipids. These 20 birds were subsequently sacrificed and gross organ appearance noted. Representative blocks of liver and kidney tissue were preserved for histological examination from 5 birds in each group.

RESULTS

Experiment 1. The treated birds gained weight rapidly after being placed on the 10% fat feed, and within two weeks weighed a highly significant 72 gm more than the controls. Thereafter the treated birds maintained a weight advantage of approximately 100 gm over the controls (table 2). Production began during the 18th week in the treated group, two weeks before the controls and on a weekly percentage

production basis remained higher than the control until the 27th week (table 2). However, from the 27th week on the controls forged ahead of the treated birds in production. The treated group, despite their earlier start, never attained the rate of lay of the controls. Mortality was almost twice as high in the treated birds as in the controls. Following return of the treated birds to the control diet (diet B) egg production returned to that of the control group.

TABLE 2

Body weight, egg production, and mortality of White Leghorn pullets (135 controls and 127 treated) placed on a 10% fat diet at 8 weeks of age (exp. 1)

AGE	BODY WEIGHT			EGG PRODUCTION	
	Control	Treated	Pooled S. E.	Control	Treated
weeks	*gm*	*gm*	*gm*	*%*	*%*
8	575.2	584.3	18.8	..	..
12	900	990 [1]	11.0	..	..
18	....	....	....	0.0	0.1
20	....	....	....	0.2	4.0
22	....	....	....	2.0	9.5
24	1530	1643 [1]	23.7	7.8	17.2
26	....	....	....	33.3	47.2
28	....	....	....	56.8	47.1
30	....	....	....	64.0	49.5
32	....	....	....	67.1	53.0

Mortality between the 8th and 35th wk: Controls, 8.4%.
Treated, 15.0%.

[1] Significant at 1% level.

Plasma cholesterol determined on samples of the two groups, was significantly elevated (by 58.3 mg %) in the high fat birds (table 3). Of perhaps more interest than the average plasma cholesterol is the decided unequal distribution of values, despite almost equal ranges, in the two groups. Thus, where there were only two controls above 250 mg %, 7 of the treated group were above this level. Body weights of these sampled birds (table 3) measured 5 weeks prior to the first cholesterol determination show an even greater weight advantage of the treated group than was seen in the larger population (table 2). However, in contrast to data on humans

(Anderson et al., '52) body weight was not correlated with plasma cholesterol in either the control or treated birds ($r = +0.05$).

Egg production was approximately equal in the two groups of birds used for cholesterol determinations, between the 30th and 34th week (table 3), in contrast to the lower production of the entire treated group during this period (table

TABLE 3

Plasma cholesterol and post mortem data on White Leghorn pullets fed a 10% fat diet for 27 weeks (exp. 1)

	CONTROLS	TREATED	POOLED S. E.
No. birds	14	16	...
Plasma cholesterol, mg %[1]	172.9	231.2[2]	17.4
Body weight, gm[3]	1496.4	1741.9[4]	47.1
Production[5]	14.0	14.6	1.32
Correlation of production and plasma cholesterol	+ 0.4	— 0.6[2]	...
	POST MORTEM		
No. birds	4	10	...
Fatty livers, %	50	50	...
Fatty kidneys, %	25	90	...
Atherosclerosis score	0.26	0.51	0.13
Excess abdom. fat, %	0	0	...
Active ovaries, %	75	100	...

[1] Average of three values between 29th and 35th week.
[2] Significant at 5% level.
[3] One measurement at 24th week.
[4] Significant at 1% level.
[5] Eggs/bird between the 30th and 34th week.

2). The correlation of egg production to cholesterol was positive, but not significant ($r = +0.4$) in the controls, and negative and significant at the 5% level ($r = -0.6$) in the treated group. Presumably, then, an even greater difference in plasma cholesterol between treated and controls would have been found if the sampled groups had production levels the same as the larger population.

Post mortem findings alone showed distinct differences between control and treated birds with respect to kidney fat

and aortic atherosclerosis (table 3). A ring of fat, or fatty capsule, was noted along the lateral edge of the kidney in 4 of the fat-fed birds. At least minimal aortic atherosclerosis was observed in all birds, but the score was twice as high for the treated as the control birds. Within the treated group

TABLE 4

Performance, plasma lipids and post mortem data on White Leghorn hens fed a 5% fat diet for 8 weeks (exp. 2)

		CONTROLS	TREATED	POOLED S. E.
		Performance		
No. birds		25	25	...
Production, %		58.7	56.6	...
Mortality, %		0	0	...
		Plasma lipids and post mortem data		
No. birds		10	10	
Body weight, lb.		4.03	4.16	0.17
Plasma cholesterol, mg %		234.2	264.5	26.5
Total plasma lipids, gm %		1.37	1.68	0.28
Abdominal fat score [1]		1.20	2.35 [2]	0.21
Liver	Friable, %	30	70	...
	Petechial, %	20	10	...
	Fatty	30	60	...
	Total abnormal, %	40	70	
Kidney	Fatty infiltration, %	20	20	
	Fatty capsule, %	0	80	
	Off color	30	80	...
	Total abnormal, %	40	100	...

[1] Fat score based on 1 = normal, 2 = slight excess, 3 = definite excess.
[2] Significant at 1% level.

there appeared to be little differentiation between those with high and low plasma cholesterol levels. No incidence of excess abdominal fat was encountered in any of the birds examined.

Experiment 2. Egg production was approximately the same, and mortality zero in the control and treated groups for the 8 weeks of the trial (table 4). Body weight, determined on a sample of the larger population, was slightly higher in the

fat-fed group, but not significantly so. The plasma cholesterol levels of the controls was much higher than in experiment 1, possibly due to the small but appreciable animal fat contributed by the meat scrap throughout the 9 months pre-test period as well as the experimental period. The fat-fed group showed an additional mean elevation in cholesterol over controls of 32.3 mg % but the difference was not significant. However, as in experiment 1, the distribution of cholesterol values was unbalanced, with 5 of the treated birds having levels above 300 mg %, compared to only two of the controls. Mean total plasma lipid values were higher in the treated group by 310 mg %, but this elevation also was not significant. A highly significant correlation coefficient of + 0.8 was found between plasma cholesterol and total plasma lipid, in both groups.

The incidence of abnormal appearing livers and kidneys, as determined by gross observation at autopsy, was approximately twice as high in treated as the control birds (table 4). Particular attention is drawn to the fatty capsule around the kidney observed in 80% of the high-fat group and in none of the controls. The abdominal fat scores indicated a significant deposition of excess body fat in the treated birds. All the birds examined had active ovaries. The correlation between body weight and body fat was low (+ 0.4), and not quite statistically significant. Neither body weight nor gross abdominal fat was correlated with total plasma lipid.

Histological examination of livers and kidneys from some of the treated and control birds revealed evidence of fatty metamorphosis in both organs. However, the incidence and severity seemed about the same in the liver of the two groups and only slightly greater in the kidneys of the treated birds.

DISCUSSION

The greater gain in weight of the growing pullets on the 10% animal fat diet (exp. 1) is in accordance with the results of Lillie et al. ('52) and Hill et al. ('56) obtained with animal-fat-supplemented, high-energy diets fed to laying birds. Un-

doubtedly the earlier start of production in these birds is related to their increased weight. However, the failure of the treated birds to maintain a satisfactory rate of lay after only 10 weeks of production was entirely unexpected. This was especially so since the decline in production occurred during the winter months, when the data of Hill et al. ('56) show that high-energy rations exert their greatest beneficial effects. The general appearance of the birds, both ante and post mortem, as well as their performance following return to the control diet, makes it unlikely that any infectious agent was responsible for their poorer production. Without attaching excessive importance to this one trial in which adverse effects on production and mortality were observed, it would nevertheless appear that a deranged fat metabolism was involved, as suggested by: the significant elevation of plasma cholesterol in the treated birds; the post mortem indications of increased fat deposits in and around the kidney; and the greater severity of aortic atherosclerosis.

The second experiment, utilizing a 5% fat level, tends to bear out the thought that fat metabolism but not necessarily production or mortality, may be adversely affected by feeding animal fat for a short 8-week experimental period. Both plasma cholesterol and total plasma lipid were elevated though not significantly; a higher level of body fat was encountered; and the incidence of gross liver and kidney abnormalities, particularly fatty infiltration in and around the kidney, was greater.

Two experiments cannot be thought of as replicates in view of the different ages of birds, levels of fat used, duration of the feeding trial, protein and energy levels of the experimental diets, and even the composition of the control and pre-experimental diets. Nevertheless, by considering these variables, some of the differences between the experiments may be clarified. Thus, the failure of the elevation in plasma cholesterol and total lipid in experiment 2 to be significant might be explained by the shorter feeding trial and the lower fat level in the diet. It might also be due to the 9-month

consumption of the small amount of animal fat contained in the pre-test rations, since Beveridge et al. ('56) indicated that the composition of the diet preceding trials with various fats may modify the lipemic response. The animal fat in the control and pre-test diet may also account for the higher plasma cholesterol values of the controls in experiment 2, inasmuch as differences in normal plasma cholesterol in mature hens are not likely due to age (Kaishio, '33). On the other hand, the age of the bird may modify its response to added dietary lipids since Rodbard et al. ('51) have shown an altered hypercholesterolemic response to cholesterol feeding in the cockerel at 8 and 20 weeks of age, and Lorenz et al. ('38) suggest an interaction between dietary fat and the ovarian factors that influences fat metabolism and elevates plasma lipids with the advent of egg formation.

The age of the birds apparently was the variable which influenced the body fat response to the fat diets. No excess deposits were encountered in the 35-week-old pullets even after 27 weeks on the 10% level, but a significantly elevated deposition occurred in the 14-month-old hens after only 8 weeks on a 5% level. Similarly, the incidence of liver and kidney abnormalities appeared to be higher in the older birds despite the shorter feeding period and lower fat level. Perhaps here the high protein content of the 10% fat diet was a factor, since Donovan and Balloun ('55) report an inverse relationship between dietary protein level and the fat content of the chick's liver. A seasonal effect may also have been involved in these responses since the birds in experiment 1 were sacrificed in March and those in experiment 2 in June. Hill et al. ('56) have shown that the production stimulus and increased efficiency of high energy diets is limited to the winter months, suggesting the extra energy in these diets is not consumed in normal metabolism during warm weather and may thus be deposited in the body and viscera as fat.

It does not seem likely that the high protein content of the 10% fat diet was directly connected with the hypercholesterolemia. The data of Mann et al. ('53) indicate that

the dietary-stimulated increase in plasma cholesterol of monkeys is diminished by high-protein intake. In the rabbit, too, high-dietary protein levels apparently reduced the hyperlipemia resulting from cholesterol supplementation (Loewe et al., '54).

The decreased egg production and increased mortality noted in experiment 1, but not in experiment 2, might also be ascribed to the higher fat level and prolonged feeding of the first test, and the associated significant elevation in plasma cholesterol. However, Lillie et al. ('52) fed a diet containing 8% lard to laying birds for two years, and Hill et al. ('56) as high as 5% tallow for 9 months without any failure in performance. Neither of these workers report any studies on plasma lipid levels or organ appearance with their diets. A unique feature of experiment 1 was the feeding of the high-fat diet for several months before egg production commenced. Since on normal, low-fat rations the fat metabolism of the pullet undergoes a major change at the time egg production begins, it is not unlikely that the adverse effects observed can be traced back to a derangement during this initial, egg formative period. Levels of fat up to 18% of the diet have been fed to layers without any apparent hyperlipemia or hypercholesterolemia (Lorenz et al., '38; Walker et al., '51). However, these fats were of vegetable origin, and the data of Beveridge et al. ('55, '56) in man, and Aftergood et al. ('56) in the rat show that fats from animal and plant sources may differ markedly in their effect on plasma lipids. Hypercholesterolemia has been produced in hens by feeding vegetable oils plus cholesterol (Stamler et al., '54) but performance was not given.

That the higher plasma cholesterol levels were associated with other physiological effects may be seen in the greater severity of atherosclerosis in the treated birds of experiment 1. Experimentally induced hypercholesterolemia has been shown to be closely associated with the incidence and severity of atherosclerosis in the bird (reviewed by Katz and Stamler, '53). Atherosclerosis is said to increase with age in the bird

and the incidence of the spontaneous form reportedly ranges as high as 70% (Dauber, '44), but the relationship of this pathological condition to performance has not been clarified. It is equally difficult to specifically relate any of the other abnormal findings to performance in these birds. Logically one would assume that the liver and kidney conditions encountered would adversely affect production, but information is not available that permits direct association of the gross and microscopic pathology with function of the organs or performance of the bird.

The significant correlation of + 0.8 between plasma cholesterol and total plasma lipid suggests that one of these measurements alone, preferably the cholesterol, since it is technically the quicker and easier, might be sufficient to characterize the degree of lipemia. Similar high correlations (+ 0.63 and + 0.77) have been described by others between free cholesterol and neutral fat (Lorenz et al., '38; Walker et al., '51).

It may be worth noting that two field conditions have been called to our attention with symptoms similar to those described here. One was in a New Jersey flock of White Leghorn pullets maintained in cages and fed a 2.5 % animal-fat ration. After 6 months of laying a decrease in production began. The birds appeared normal, and on gross autopsy showed no signs of infection, but did demonstrate extremely fatty livers and an excess of abdominal fat. No biochemical or histological studies were possible to delineate the condition further. The other is reported in a communication from Grumbles ('56), who indicates that pathologists at the Texas Experiment Station are finding a fatty liver condition quite frequently in caged layers, mostly associated with rations containing added animal fat. Perhaps the caged layers, since they require less energy for general metabolism because of restricted activity, also tend to dispose of more of the dietary energy in the form of body fat as do the floor birds in the summer. Human studies lend support to the concept of an inverse relationship between

exercise or energy expenditure and plasma lipid levels (Mann et al., '55).

These results suggest that further studies of the fat metabolism of the hen are in order before making any increases in the animal fat content of laying rations.

SUMMARY

Depending on the age of birds and the duration of feeding trial, 5 to 10% of added animal fat to laying rations resulted in the development of an apparent derangement in lipid metabolism characterized by one or more of the following symptoms: elevated plasma cholesterol and total plasma lipid; excess deposits of body fat; friable and fatty livers; fatty deposits in and around the kidney; and greater severity of aortic atherosclerosis.

ACKNOWLEDGMENT

The authors are grateful for the histological studies made by Dr. D. C. Tudor and for the technical assistance of Mr. S. Ferdo. The authors gratefully acknowledge material assistance rendered by Commercial Solvents Corp., New York, N. Y.

LITERATURE CITED

AFTERGOOD, L., R. B. ALFIN-SLATER AND H. J. DEUEL, JR. 1956 Comparative effect of cottonseed oil and lard on cholesterol metabolism in the rat. Fed. Proc., *15:* 541.

ANDERSEN, J. T., A. LAWLER, M. LOWEN AND A. KEYS 1952 Changes in blood cholesterol and lipoproteins during experimental overnutrition. *Ibid.*, *11:* 181.

BEVERIDGE, J. M. R., W. F. CONNELL AND G. A. MAYER 1956 Dietary factors affecting the level of plasma cholesterol in humans: The role of fat. Canadian J. Biochem., *34:* 441.

BEVERIDGE, J. M. R., W. F. CONNELL, G. A. MAYER, J. B. FIRSTBROOK AND M. S. DEWOLFE 1955 The effects of certain vegetable and animal fats on the plasma lipids of humans. J. Nutrition, *56:* 311.

DAUBER, D. V. 1944 Spontaneous arteriosclerosis in chickens. Arch. Path., *38:* 46.

DONOVAN, G. A., AND S. L. BALLOUN 1955 Effect of dietary protein and energy on chick liver fat accumulation. Proc. Soc. Exp. Biol. Med., *90:* 692.

GRUMBLES, L. C. 1956 Personal communication. Poultry Pathologist, Dept. of Veterinary Research, Texas Agric. Exp. Station, College Station, Texas.

HILL, F. W., D. L. ANDERSON AND L. M. DANSKY 1956 Studies of the energy requirements of chickens. 3. The effect of dietary energy level on the rate and gross efficiency of egg production. Poultry Sci., *35:* 54.

HUERGA, J. DE LA, C. YERINICK AND H. POPPER 1953 Estimation of total serum lipids by a turbidimetric method. Am. J. Clin. Path., *23:* 1163.

KAISHIO, Y. 1933 The cholesterol content in the blood of the hen. Proc. 5th World's Poultry Congress, *2:* 620.

KATZ, L. N., AND J. STAMLER 1953 Experimental atherosclerosis. Charles C. Thomas, Springfield, Ill.

LILLIE, R. J., J. R. SIZEMORE, J. L. MILLIGAN AND H. R. BIRD 1952 Thyroprotein and fat in laying diets. Poultry Sci., *31:* 1037.

LOEWE, L., M. G. GOLDNER, S. M. RAPOPORT AND I. STERN 1954 Failure of protein to protect against cholesterol atherogenesis in underfed rabbits. Proc. Soc. Exp. Biol. Med., *87:* 360.

LORENZ, F. W., C. ENTENMAN AND I. L. CHAIKOFF 1938 The influence of age, sex and ovarian activity on the blood lipids of the domestic fowl. J. Biol. Chem., *122:* 619.

MANN, G. V., S. B. ANDRUS, A. MCNALLY AND F. J. STARE 1953 Experimental atherosclerosis in Cebus monkeys. J. Exp. Med., *98:* 195.

MANN, G. V., K. TEEL, O. HAYES, A. MCNALLY AND D. BRUNO 1955 Exercise in the disposition of dietary calories: Regulation of serum lipoproteins and cholesterol levels in human subjects. New England J. of Med., *253:* 349.

RODBARD, S., L. N. KATZ, C. BOLENE, R. PICK, M. LOWENTHAL AND G. GROS 1951 The age factor in hypercholesteremia and atheromatosis in the chick. Circulation, *3:* 867.

STAMLER, J., R. PICK AND L. N. KATZ 1954 Inhibition of cholesterol-induced coronary atherogenesis in the egg producing hen. Ibid., *10:* 251.

WALKER, H. A., M. W. TAYLOR AND W. C. RUSSELL 1951 The level and interrelationship of the plasma lipids of the laying hen. Poultry Sci., *30:* 525.

ZLATKIS, A., Z. BENNIE AND A. J. BOYLE 1953 A new method for the direct determination of serum cholesterol. J. Lab. and Clin. Med., *41:* 486.

THE EFFECT OF POWDERED MILK ON DENTAL CARIES IN THE RAT

JOSEPH C. MUHLER
Department of Chemistry and School of Dentistry, Indiana University, Bloomington

(Received for publication August 10, 1956)

McClure ('55) has described the development of smooth-surface dental caries in rats by feeding a diet in which the milk powder component of the ration was heat processed. The diet used by McClure was only moderately high in refined carbohydrate and contained no coarse corn particles. Its percentage composition was: heat-processed whole-milk powder, 35; glucose, 18; cornstarch, 45 and dehydrated liver, 2. In addition, an oral supplement composed of vitamins A, D and E was given weekly. The exact reason for the high incidence of caries through the use of this diet is not known, but McClure feels that one important factor is the loss of lysine as a result of heating the milk powder.

Several observations may be made as a result of these interesting studies, the solution of which may provide important information concerning the role of nutritional factors in the etiology of human dental caries. For example, practically speaking, only smooth-surface caries resulted from the ingestion of the heat-processed milk diets used by McClure, and with the highest incidence of dental caries occurring in the mandibular arch. A very low incidence of occlusal caries developed as did few cavities in the maxillary arch. Since human dental caries occurs with a high frequency in the maxillary arch, it seems necessary to eventually develop a diet which will simulate this condition. There was also a pronounced failure of the experimental animals to gain weight

normally when fed this diet. What effect this chronic inanition has on the caries process is not known. Stephan and Harris ('55) have reported that the greatest amount of smooth-surface dental caries was generally found in those rats which gained the least weight. Muhler ('55a) and others have shown that the total amount of dental caries occurring in the rat was in large measure dependent upon the amount of food eaten. The purpose of this study then, was twofold; first, to determine the dental caries experience in rats which received a coarse corn diet containing as one of its components heat-treated whole-milk powder. This diet without heat-processed milk powder has been shown to result in the production of predominately occlusal caries (Muhler, '55b) with only a moderately high incidence. Secondly, since a marked failure to gain weight occurred in the animals receiving the heat-processed milk diets, it was considered important to determine the dental caries incidence in rats receiving the heat-processed milk diet but in which the same rate of growth is maintained in respect to a control group receiving the non-heat-processed whole-milk diet. This seemed to be best accomplished by feeding whole-milk powder by stomach tube to rats which received the heat-processed milk diet. Such a technique would provide information as to whether the caries rate could be returned to that of the control by restoring that component of the diet destroyed during the heat processing treatment. Furthermore, by using stomach-tube techniques any topical effect of milk powder itself on the teeth would be ruled out.

EXPERIMENTAL

This study was divided into two series. Series I was composed of 80 weanling rats of the Sprague-Dawley strain. The animals were divided into two groups of 40 rats, each of which was further subdivided according to sex and initial body weight. Group I received a diet composed of yellow corn grits, 64; whole-milk powder, 30; alfalfa meal, 3; irradiated yeast,

2 and sodium chloride, 1%. Group II received the same diet except that the whole-milk powder was previously heated in a pyrex glass tray at 100°F. until brown. About two pounds of the whole-milk powder was placed in a pyrex dish two inches deep and placed in an oven maintained at a temperature of 100°F. At periodic intervals of 15 to 30 minutes it was stirred. Upon complete browning of the milk powder (which requires from 4 to 6 hours) it was allowed to cool, and was then pulverized to a fine powder and added to the diet as required.

In series II, 160 weanling animals were divided as in series I into 4 groups. Groups I and II, respectively, received diets identical to those fed to the animals in series I. Group III received the same diet as group II, namely, the corn diet containing the heat-processed whole-milk powder, but in addition received by stomach tube twice daily supplements of whole-milk powder slurried in distilled water. The amount of whole-milk powder received varied with age, beginning with 3 gm of solid milk powder per day at weaning and increasing to 10 gm per animal per day after 65 days on the experiment. In general, an attempt was made to provide by stomach tube the same amount of whole-milk powder the animals were ingesting ad libitum in the form of heat-processed milk. Group IV received a diet essentially different from that received by group I in that it contained casein substituted for the whole-milk powder. Its composition was: vitamin test casein, 29; yellow corn grits, 64; alfalfa meal, 3; irradiated yeast, 2; sodium chloride, 1 and vitamin mixture, 1%. The composition of the vitamin mixture has been described previously (Muhler, '54).

All of the animals were housed in pairs in raised metal cages in an air-conditioned room. They received their diets and distilled water ad libitum. The duration of the experiment was 100 days after which the rats were sacrificed by ether and the heads were removed for dental caries evaluation as previously described (Muhler, Nebergall and Day, '54).

DATA AND DISCUSSION

The dental caries experience of the animals in this study is shown in table 1. The effect of heat processing the powdered whole milk on the dental caries experience in the animals is readily noted in both experiments. In the series I animals, those receiving the stock corn diet had a incidence of dental caries of 5.4 lesions (sexes combined), while those which received an identical diet except that the whole-milk powder

TABLE 1

The dental caries experience in rats receiving the different experimental diets

GROUP	NO. OF ANIMALS	SEX	WEIGHT GAIN	MOLARS AFFECTED	MEAN NO. OF LESIONS		PROBABILITY [1]	
			gm					
Series I								
Stock corn diet (I)[2]	20	M	251	3.6	5.7	5.4		
	16	F	148	2.9	5.1			
Stock corn diet with heat-processed milk (II)	17	M	33	5.4	9.8	9.3	0.01	
	15	F	29	4.3	8.8			
Series II								
Stock corn diet (I)	16	M	246	3.8	6.5	6.1		0.01
	19	F	136	3.3	5.7			
Stock corn diet with heat-processed milk (II)	16	M	23	5.6	10.3	9.4	0.01	
	15	F	36	4.2	8.5			
Stock corn diet with heat-processed milk + unheated whole powdered milk by stomach tube (III)	14	M	224	3.5	5.6	4.5	> 0.06	0.001
	14	F	140	2.6	3.4			
Stock corn diet with vitamin mixture and casein in place of milk (IV)	18	M	205	3.1	7.5	6.7	< 0.5	0.01
	19	F	124	3.0	5.9			

[1] Probability calculated from the mean number of lesions, sexes combined. Probability calculated on the results obtained from the "t" test (Snedecor, '46).

[2] The number in parentheses refers to the various groups as described in the experimental section.

was heat processed had 9.3 lesions. This difference was significant at the 0.01 level of confidence. Similarly, in the series II animals the same marked significant increase in dental caries is observed. These data corroborate the conclusions reached by McClure ('55) in which a low sugar non-coarse corn diet containing heat-processed milk powder was used. However, instead of developing predominately smooth-surface caries, as found with the diet used by McClure, the predominate lesion was the occlusal type. Apparently, the heat-processed whole-milk powder is not entirely the reason for the development of smooth-surface caries, but a combination of the refined carbohydrate and the heat-processed whole-milk powder. It is possible that the use of heat-processed whole-milk powder in these studies did predispose to smooth-surface caries, but due to the presence of the coarse-corn-particle diet the lesions progressed so rapidly that the surface was lost before the termination of the experiment. Additional study is needed to clarify the exact role on caries initiation of each component of these diets.

One of the most interesting observations obtained from this study is in the group III animals which were fed the heat-processed whole-milk-powder diet but received in addition whole-milk powder by stomach tube. By such a technique it was possible to completely eliminate the increased cariogenic effect of the heat processing treatment, for the animals which received the heat-processed milk had 9.4 lesions while those which received the same diet plus the whole-milk powder by stomach tube had only 4.5 lesions. This difference is significant at the 0.001 level of confidence. This observation seems of importance since it suggests that the milk powder contains some as yet unknown heat-labile factor(s) which when ingested passes to the teeth by way of the systemic circulation and confers a marked degree of protection against the damaging effect resulting from the ingestion of the heat-processed whole-milk powder. The work of McClure would suggest that this heat-labile anticariogenic factor is lysine.

The effect of the feeding of the whole-milk powder by stomach tube to the series III animals resulted in more significant effects than on the dental caries experience, since this procedure returned the weight gain increment to that of the control animals receiving the whole-milk powder ad libitum. In both series I and II the average weight gain for the entire 100 day experimental period for the group receiving the heat-processed whole milk (group II) was approximately 30 gm (sexes combined), while the group which received the same diet plus the whole-milk powder by stomach tube (group III) gained at a normal rate and at the termination of the experimental period weighed essentially the same as the control group (group I). This suggests that the milk factor does not have a local effect. Purely from a dental caries viewpoint, much additional work seems indicated concerning the relationship of chronic inanition as produced by feeding the heat-processed milk diets and its effect upon the endocrine system and the effect that this has upon the incidence of dental caries. The effects on dental caries produced by the heat-processed whole-milk powder might have resulted from a direct effect upon the endocrine system as a result of the pronounced failure to gain weight. These effects might be mediated by way of the salivary glands since previous work has indicated a direct relationship between the endocrine glands, the salivary glands and the dental caries experience in rats (Muhler and Shafer, '54; Shafer and Muhler, '55). It is entirely possible that the results of these experiments are explainable solely on the basis that the milk powder provided by stomach tube contained a sufficient amount of the heat-labile milk factor to cause the dental caries incidence to decrease; additional studies are needed to clarify this.

SUMMARY

The ingestion by growing rats of a coarse corn diet containing 30% of heat-processed whole-milk powder resulted in a significant increase in the incidence of occlusal dental

caries when compared to results obtained with rats receiving an identical diet containing non-heat-treated whole-milk powder. When the rats received the heat-processed milk diet and in addition received daily supplements of whole-milk powder by stomach tube the dental caries increment as well as the pronounced failure to gain weight in these animals was returned to the level of the control. It is suggested that milk powder contains a heat-labile factor which confers some pronounced degree of anticariogenicity mediated by way of the systemic circulation.

ACKNOWLEDGMENT

The author wishes to acknowledge the technical assistance of Louis B. Spear, Jr., and Robert Radcliff throughout this study.

LITERATURE CITED

McCLURE, F. J. 1955 Dietary Factors in Experimental Rat Caries, pps. 107-124, *Advances in Experimental Caries Research,* R. F. Sognnaes, Editor, A.A.A.S. Publication.

MUHLER, J. C. 1954 Retention of fluorine in the skeleton of the rat receiving different levels of fluorine in the diet. J. Nutrition, *54:* 481.

———— 1955a The effect of chronic inanition and frequency of food ingestion on the dental caries experience in the rat. J. Nutrition, *57:* 441.

———— 1955b The cariogenicity of various corn diets in the rat. J. Dent. Res., *34:* 364.

MUHLER, J. C., W. H. NEBERGALL AND H. G. DAY 1954 Studies on stannous fluoride and other fluorides in relation to the solubility of enamel in acid and the prevention of experimental dental caries. J. Dent. Res., *33:* 33.

MUHLER, J. C., AND W. G. SHAFER 1954 Experimental dental caries. IX. The effect of feeding desiccated thyroid and thiouracil on dental caries in rats. Science, *119:* 687.

SHAFER, W. G., AND J. C. MUHLER 1955 Experimental dental caries. VI. The effects of hypophysectomy on dental caries and the salivary glands of the rat. J. Dent. Res., *34:* 531.

SNEDECOR, G. W. 1946 Statistical Methods Applied to Experiments in Agriculture and Biology. Iowa State College Press, Ames.

STEPHAN, R. M., AND M. R. HARRIS 1955 Location of Experimental Caries on Different Tooth Surfaces in the Norway Rat, in *Advances in Experimental Caries Research,* 47-65, American Association for Advancement of Science Publication.

THE ENERGY VALUE OF SELF-SELECTED DIETS CONSUMED BY YOUNG COLLEGE WOMEN [1]

A. NELL DAVIS AND FLORENCE I. SCOULAR
WITH THE TECHNICAL ASSISTANCE OF PATTI WILKERSON AND KAREN ROHNE
School of Home Economics, North Texas State College, Denton

(Received for publication August 23, 1956)

As a routine procedure in the elementary nutrition course at North Texas State College, individual dietary records are kept and their nutritive value estimated. The low caloric intake of these college women was evident from the first. Since the caloric value of self-selected diets is of importance in assessing the adequacy of other nutrients, it seemed wise to determine quantitatively the caloric value of college women's diets. Consequently, the determination of the caloric value of the foods consumed was made a part of the present long-time metabolism study of young college women consuming self-selected diets.

PROCEDURE

The subjects participating in this study were young college women between the ages of 17 and 27 years who were living in the Home Management House Duplex at the time of this study. They engaged in the usual home activities in addition to their regular class work during the 5-day collection periods reported. They were responsible for planning the menus and preparing the food to satisfy the appetites of the individual members of the group. The technique used in collecting the food for analysis in the present study was previously reported by Holt and Scoular ('48). In brief, this involved

[1] Financed mainly by a Faculty Research Fund and augmented by Williams-Waterman Grant.

the placing of a serving of each food from each meal, similar in all respects to that eaten by the college women, in weighed glass jars at the same time that the subjects were served at the table. This food is referred to as the composite food samples. After weighing each day's composite food, it was thoroughly mixed in a Waring Blendor before aliquots were removed for drying. The drying was accomplished in a hot-air oven at a temperature below 100°C. Moisture determinations were made before the samples were ground in a mortar. One-gram duplicate samples were burned in a Parr Oxygen Bomb Calorimeter and their caloric values calculated.

Each of the 89 girls kept a daily record of the amount of fluid milk, sugar as such, and carbonated beverages [2] consumed during the 5-day collection period. These records were charted and used in making suitable additions to the calorie value of the composite foods to obtain each subject's total caloric intake. The reason for the separate charting of these foods was that they were permitted ad libitum so that each girl could satisfy appetite and continue her usual dietary practices since her total composite food intake was limited to the servings collected for analysis.

DISCUSSION OF RESULTS

The caloric values of the composite self-selected food samples collected for 445 days during 1953 to 1956 ranged from 871 to 2478 with an average of 1748 Cal./day. All of the 89 subjects had some fluid milk during the 5-day collection period but not all of them had fluid milk each day. The daily calorie value of this milk ranged from zero to 606 with an average of 282 Cal. Furthermore, all but three of the subjects had sugar as such or carbonated beverages or both during the 5-day period. The sugar and carbonated beverages contributed from zero to 210 with an average of 93 Cal/.day. The total caloric intake (composite food, fluid milk, sugar and carbonated beverages) ranged from 1534 to 3000 with an

[2] Only one brand of bottled cola beverage permitted in order to decrease calorie variations.

average of 2174/day. The National Research Council's ('53) recommended daily allowances were used in evaluating the energy provided by these self-selected diets for comparison purposes although no recommendation is given for the 20 to 25 age group of the present study. While the ages ranged from 17 to 27 years, there was only one subject in each of the age levels, 17, 25 and 27, and two in the 23 age level. Since 44 of the subjects were under 20 years of age (group I) and 45 were over 20 (group II), the NRC caloric recommendation for girls 16 to 20 years old is used for group I and the value for women 25 years and over for group II. This arbitrary use of the value for women in group II seemed justified by the need to guard against over-eating in the early adult years. A 5% reduction in the normal values because of the warmer climate of the Southwest where this study was undertaken gave values of 2280 and 2185 Cal./day for groups I and II respectively, for comparison purposes.

In table 1 the average 5-day caloric values of the self-selected diets of group I and group II are distributed according to the height and weight of the subjects. For reference their weights for height are compared with the NRC's desirable weight for height which disregards age after the ages 25 to 30 years. While this comparison may not do justice to either group it does give an indication of weight trends, especially to excessive underweight and overweight. Sixty-six or approximately 74% of these young women were within the weight range for height. Of the 11 in the underweight classification (—) 4 had extremely small bones and narrow frames and probably should not be in this classification. Only two of the 12 overweight (+) subjects could be called obese, the others exceeded the range by only a few pounds and had large bones and body frames.

It is to be noted that 31 of the 44 subjects in group I as compared with 18 of the 45 in group II fall in the 50 to 60-kg (includes NRC-G and NRC-W weights with a 10% range) grouping. The fact that group II has 21 (47%) subjects who

TABLE 1

The average 5-day caloric values of self-selected diets distributed according to age, height, and weight

HEIGHT	NRC WEIGHT FOR HEIGHT			CALORIC INTAKE						WEIGHT	CALORIC INTAKE					
				Under 20 yr. (Group I)			Over 20 yr. (Group II)				Under 20 yr. (Group I)			Over 20 yr. (Group II)		
cm	(−)	*av.*	(+)	*no.*	*av.*	*/cm*	*no.*	*av.*	*/cm*	*kg*	*no.*	*av.*	*/kg*	*no.*	*av.*	*/kg*
152.0–154.8 (60–61)[1]		3		1	2101 ± 0	14.6 ± 0.0	2	2080 ± 135	13.8 ± 0.9	40–45 (88–99)[2]	1	2237 ± 0	50 ± 0.0	3	2094 ± 307	47 ± 6.6
154.9–157.4 (61–62)	1	3	1	3	2021 ± 163	13.2 ± 1.5	2	1991 ± 20	13.0 ± 0.2	45–50 (99–110)	2	2296 ± 46	51 ± 3.2	3	2032 ± 319	42 ± 6.5
W[3] 157.5–159.9 (62–63)	4	5		4	2384 ± 256	15.3 ± 1.6	5	2009 ± 325	10.5 ± 2.7	G 50–55 (110–121)	18	2135 ± 285	41 ± 5.6	6	2255 ± 154	42 ± 4.1
160.0–162.5 (63–64)	3	9		5	2282 ± 220	14.4 ± 0.8	7	2199 ± 217	13.8 ± 1.3	W 55–60 (121–132)	13	1796 ± 179	34 ± 3.1	12	2293 ± 285	42 ± 4.8
G[4] 162.6–165.0 (64–65)	2	12	4	11	1945 ± 217	12.1 ± 1.4	7	2231 ± 316	13.8 ± 1.8	60–65 (132–143)	6	2118 ± 179	21 ± 2.0	11	2230 ± 222	38 ± 5.1
165.1–167.5 (65–66)		14	4	8	2101 ± 277	13.0 ± 0.4	10	2198 ± 179	13.3 ± 1.0	65–70 (143–154)	3	2125 ± 224	32 ± 2.9	4	2175 ± 231	37 ± 6.1
167.6–170.1 (66–67)		10		7	2049 ± 169	12.3 ± 1.0	3	2213 ± 191	13.3 ± 1.2	70–75 (154–165)	1	2000 ± 0	29 ± 0.0	3	2071 ± 104	29 ± 1.5
170.2–172.6 (67–68)		6	1	4	2040 ± 178	12.3 ± 0.9	3	2062 ± 177	12.1 ± 0.7	75–80 (165–176)				2	2039 ± 30	26 ± 0.9
172.7–175.2 (68–69)		4	1	1	2000 ± 0	11.7 ± 0.0	4	2252 ± 165	13.2 ± 1.0	80–85 (176–187)				1	1870 ± 0	24 ± 0.0
175.3–177.7 (69–70)	1		1				2	2304 ± 434	13.2 ± 2.4							
Summary 163.9 (64.5)	11	66	12	44	2103 ± 211	13.1 ± 1.0	45	2158 ± 216	13.0 ± 1.3	56.7 (124.7)	44	2101 ± 183	38 ± 3.6	45	2141 ± 206	36 ± 4.4

[1] Height in inches in parentheses.
[2] Weight in pounds in parentheses.
[3] W — NRC height and weight for the average woman.
[4] G — NRC height and weight for 16–20 year old girls.

exceed this weight range justifies the comparison of this group with the recommended allowances for women 25 years and older. The summary (table 1) shows that these Texas college women were taller and heavier than the average 16- to 20-year-old girl and the average 25-year-old woman, namely, 163.9 cm and 56.7 kg as compared with 162 cm and 54 kg for girls, and 157 cm and 55 kg for women. A previous study of 106 Texas college students by Scoular and Foster ('46) also found these women to be taller than the average. They were lighter in weight, however, than the college women of other states.

The daily caloric intakes of group I averaged 2103 $\pm$ 211 and 2101 $\pm$ 183 which were lower than the average calories consumed by the girls in group II, 2158 $\pm$ 216 and 2141 $\pm$ 206, based on height and on weight of the subjects. The heavier weight of group II together with the higher energy intake suggests the need for calorie consciousness and a gradual decrease in calorie-rich food by Texas college women who are 20 years of age or older. Both groups could use the 13.0 Cal./cm as a basis for determining total energy intake for maintenance of the desired weight range for height. Actually 33 of group I and 22 of group II (60% of 89) consumed this amount of energy and maintained their weight on such an intake. The same thing is not true when the fuel needs are based on calories per kilogram. The total daily average energy intakes are lower than either the 2280 or 2185 Cal./day, the NRC values adjusted for a warm climate. An earlier report on the caloric intake of Texas women was made by Lamb and McPherson ('48) of students living in a cooperative house at Texas Technological College. They estimated that these women consumed 0.4% less than the daily recommended allowances. Odland et al. ('55), using daily dietary record books, reported that 14 of 100 Montana freshman college women consumed less than 2/3 with 60 consuming between 2/3 and 3/3 of the NRC's recommended allowance. Only Frank and Johnston ('55) of Cornell University determined the total

energy needs of their 8 subjects. They found that the determined values agreed well with the predicted ones. Their three subjects in the twenties (22, 23 and 27 years) actually consumed an average of 13.5 ± 0.5 Cal./cm[3] and 39.4 ± 0.9 Cal./kg [3] as compared to 13.0 ± 1.3 and 36.0 ± 4.4 for the 45 subjects in group II of the present study. The Texas women are taller and lighter than those of the Cornell study. Yet the Texas women consume fewer calories to maintain their weight range. It is recognized that 5-day collection periods cannot be used to formulate standards since short periods may exclude variations which would be evident in longer periods. The shorter time, however, provided more subjects since the week-ends were not interrupted. Perhaps consideration should also be given to the suggestion of Passmore and Durnin ('55) that fuel needs should be re-evaluated in the light of labor saving devices and more leisure time. The young women of the present study were engaged in their usual college activities in addition to managing a home which contains an abundance of labor saving devices.

SUMMARY

The average height, 163.9 cm, and the average weight, 56.7 kg, of 89 Texas college women are greater than those given by the National Research Council for 16- to 20-year-old girls and for 25-year-old women.

Sixty-six of the women were within the weight range for height, 11 were underweight and 12 overweight.

The average daily caloric intake was 2103 ± 211 and 2101 ± 183 for group I (under 20 years) and 2158 ± 216 and 2141 ± 206 for group II (over 20 years) when distributed on the basis of the height and the weight of the individuals.

Thirty-three of group I and 22 of group II (60% of 89) consuming 13 Cal./cm maintained their weights on such an intake.

[3] Calculated from the published data.

LITERATURE CITED

FRANK, R. M., AND F. A. JOHNSTON 1955 Total energy needs of women 22 to 36 years old. J. Am. Diet. Assn., *31:* 1007–1009.

HOLT, F., AND F. I. SCOULAR 1948 Iron and copper metabolism of young women on self-selected diets. J. Nutrition, *35:* 717–723.

LAMB, M. W., AND C. M. MCPHERSON 1948 Trends in dietary practices of college women. J. Home Econ., *40:* 19–21.

NATIONAL RESEARCH COUNCIL 1953 Reprint and Circular Series no. 302, Washington.

ODLAND, L. M., L. PAGE AND L. P. GUILD 1955 Nutrient intakes and food habits of Montana students. J. Am. Diet. Assn., *31:* 1134–1142.

PASSMORE, R., AND J. V. G. A. DURNIN 1955 Human energy expenditure. Physiol. Rev., *35:* 801–840.

SCOULAR, F. I., AND L. FOSTER 1946 Food intake of college women. J. Am. Diet. Assn., *22:* 401–403.

THE PROTEIN METABOLISM OF YOUNG COLLEGE WOMEN CONSUMING SELF-SELECTED DIETS [1]

FLORENCE I. SCOULAR, A. NELL DAVIS, JUNE K. PACE,[2]
ADA B. RANKIN [3] AND GAYLE J. BOSHART [4]
WITH TECHNICAL ASSISTANCE OF BETTY WHITE, LUCY S. CROW AND PAT PURCELL
School of Home Economics, North Texas State College, Denton

(Received for publication August 23, 1956)

From a study of the food consumption of 18 Oklahoma college women, Coons and Schiefelbusch ('32) concluded that not only was it lower than that of a generation ago but that these women consumed less than those reported for other sections of the United States. Also, that the protein content of the self-selected diets analyzed (range of 41 to 71 with an average of 56 gm) was more deficient than the calorie content. This statement was based on a comparison of Oklahoma women with chiefly men students from different sections of the country since the earlier studies were made with men subjects. A later cooperative study, McKay et al. ('42), determined the protein intake and retention of 124 young college women of the central states and obtained a range of 5.55 to 17.58 gm of nitrogen with a mean of 10.10 gm (35 to 110 gm of protein with an average of 63 gm) consumed daily. These authors concluded on the basis of the mean intake that the protein of the diets varied only slightly from the 60 gm of protein recommended by the National Research Council's Committee on Food and Nutrition in 1941. Is there a difference

[1] Financed mainly by Faculty Research Funds and augmented by Williams-Waterman Grant, during 1954–1956.

[2] Present address, Bureau of Home Nutrition, United States Department of Agriculture, Washington.

[3] Present address, County Home Demonstration Office, Palo Pinto, Texas.

[4] Present address, Dietary Department, St. Joseph Hospital, Memphis, Tennessee.

in the food habits of college women from different geographic locations? In order to answer this question a long-time metabolism study of young Texas college women on self-selected diets was initiated in 1946. The purpose of the present paper is to report on the protein metabolism of the subjects participating during the first 10 years.

PROCEDURE

The subjects participating in the present study were 171 young college women between the ages of 17 and 27 years who lived in the Home Management House Duplex at the time the collections were being made. They engaged in the usual home activities in addition to their regular class work during the 5-day periods reported. They were responsible for planning the menus and preparing the food to satisfy the appetites of the individual members of the group. The technique used in collecting the food and excreta was reported by Holt and Scoular ('48). In brief, this involved the placing of a serving of each food from each meal, similar in all respects to that eaten by the college women, in weighed glass jars at the same time that the subjects were served at the table. This food is referred to as the composite food samples. Milk was analyzed separately in order to permit each girl to consume the amount desired, by removing daily aliquots to give one homogeneous sample for analysis. A record was kept of the amount of fluid milk consumed by each girl each day, and suitable additions were made to each individual's record of food intake, to include the milk protein.

Carmine was used as the fecal marker for the 5-day metabolism periods. Aliquots of both food and feces were obtained for analysis after being weighed and macerated in a Waring Blendor. Aliquots were removed from the 24-hour urine collections which had been made directly into the amber gallon bottles.

The nitrogen content of foods, feces and urine was determined by the macro Kjeldahl method (Association of Official Agricultural Chemists, '50) using the factor 6.25 to convert

to total grams of protein for comparison with the NRC's ('53) recommended daily allowances.

RESULTS AND DISCUSSION

The protein content of the food selected by 171 young college women for 5 successive days (total 855 days) was determined. The daily urinary output of nitrogen was also determined for each of these subjects while the fecal output was determined for the 5-day period of 145 (85%) of the women and computed to daily "fecal protein." For the 26 (15%) individuals for whom the "fecal protein" was not determined, the average fecal protein (12%) of the 145 women was used in obtaining the retentions recorded. This determined average is slightly higher than the 10% used by Kramer et al. ('34) in the calculation of protein intakes based upon output. The percentage of the protein intake absorbed [5] by the 171 subjects of the present study ranged from 65 to 98 with an average of 85%. The lowest absorption values, 65 to 78%, were associated with the diets consumed by one group of subjects. The water content of the feces did not differ from that obtained with other diets. The weights of the feces were greater however, suggesting more undigested substance from the higher vegetable intake. The foods included during this 5-day period were lower in total protein and particularly high in vegetable proteins as compared with the foods usually selected during the 5-day period. Excluding the absorption values of this group, the average absorption becomes 88%. This figure is lower than the generally accepted 92% for the protein of a mixed diet, but similar to the 87.5% reported by Hegsted et al. ('46) for an all-vegetable diet. With the exception mentioned these self-selected diets (including fluid milk) of the present study were composed of approximately equal amounts of vegetable and animal protein, even when the total protein intake was low.

[5] Calculated as $\frac{\text{protein intake minus fecal protein}}{\text{protein intake}} \times 100 = \text{per cent absorbed.}$

In table 1 the average daily total protein intake for the 5-day period is given together with the number of subjects in positive and in negative balance. The retentions given in this study were based solely upon the difference between intake and urinary and fecal excretions. For reference the weight for height of these subjects is compared with the NRC's ('53) desirable weight for height which disregards age after

TABLE 1

Total daily protein intake and balance of 171 college women

INTAKE			BALANCE				NRC WEIGHT FOR HEIGHT		
			Positive		Negative				
gm	*gm*	*no.*	*no.*	*%*	*no.*	*%*	(+)	*av.*	(−)
19–25	(3–4)[1]	3	1	33	2	67		3	
25–31	(4–5)	8	3	38	5	62	1	5	2
31–38	(5–6)	22	2	9	20	91	5	15	2
38–44	(6–7)	24	12	50	12	50	2	12	10
44–50	(7–8)	26	21	76	5	24	3	17	6
50–56	(8–9)	19	14	74	5	26	1	12	6
56–63	(9–10)	37	29	88	8	22	5	27	5
63–69	(10–11)	14	13	93	1	7	5	4	5
69–75	(11–12)	12	11	92	1	8	3	8	1
75–81	(12–13)	1	1	100					1
81–88	(13–14)								
88–94	(14–15)								
94–100	(15–16)	1	1	100				1	
100–106	(16–17)	3	3	100				3	
106–113	(17–18)	1	1	100					1
Summary		171	112		59		25	107	39

[1] Expressed as grams of nitrogen.

the ages of 25 to 30. The arbitrary use of the scale for the younger subjects of the present study seems justified in obtaining excessive underweight and overweight trends at the time the collections of food and excreta were made. The average daily protein intake ranged from 19 to 113 gm with an average of 52 gm. If the NRC's recommended daily allowance of 75 gm protein for girls 16 to 20 years of age and 55 gm for those over 20 years is used in evaluating the protein intake of the young women of the present study, we find only

6 of the 171 consuming 75 gm or more of protein. Sixty-three consumed from 55 to 75 gm of protein and the 102 women remaining consumed less than 55 gm of protein/day. Only the Missouri group of young women, Mertz et al. ('52), consumed as little protein (21 gm) as the lowest in the present study. Reynolds et al. ('53) report a low of 28 gm of protein for older women while McKay et al. ('42) found 35 gm of protein to be the smallest amount consumed by college women.

There were twice as many underweights as overweights (26 to 12) among the women of the present study consuming less than 55 gm of protein/day while the number in each category was similar for those with intakes of 55 to 75 gm protein/day. The number of underweight women on the lower protein intakes may account for some of the positive retentions at the lower levels. Allison ('51) reports from his work with dogs that with sufficient depletion of protein stores, a positive retention can occur on very low levels of protein intake. There was a wide range in the daily protein intake of the young women of the present study. On a few occasions the day to day variations reached 100%. Only a few of the women consumed a uniform amount of fluid milk each day. This and the fact that errors in collection techniques are ruled out, further supports the idea that some of these young women had very poor protein stores. In addition, the 6 women receiving more than 75 gm of protein/day were in positive balance which suggests depleted stores; Allison ('51) also found that when the stores were filled high intakes of protein produced negative balances because the extra protein was not needed. Furthermore, there were 5 times as many positive as negative balances (53 to 10) among the women consuming 55 to 75 gm of protein. One hundred and twelve (66%) of the 171 women were in positive protein balance, although the average daily intake of protein for the entire group of women was only 52 gm. According to Ohlson et al. ('52) negative nitrogen balances occurred at any level of intake when the caloric intake was less than 1500. Assuming the caloric intake of the 171 women of the present study to

TABLE 2

The average 5-day protein intake of self-selected diets distributed according to age, height and weight

HEIGHT	AVERAGE PROTEIN INTAKE (Group I)		Retention (+)	Retention (−)	AVERAGE PROTEIN INTAKE (Group II)		Retention (+)	Retention (−)
cm	*gm*	*gm/cm*			*gm*	*gm/cm*		
147.3–149.8 (58–59)[1]	65.63 ± 0.00	0.45 ± 0.00	1					
149.9–152.3 (59–60)								
152.4–154.8 (60–61)	63.40 ± 24.40	0.42 ± 0.16	2	1	45.65 ± 7.23	0.31 ± 0.05	1	2
154.9–157.4 (61–62)	56.46 ± 10.17	0.37 ± 0.06	2	3	47.05 ± 2.72	0.31 ± 0.04	2	1
W[3] 157.5–159.9 (62–63)	52.49 ± 11.07	0.33 ± 0.07	7	4	49.78 ± 10.35	0.33 ± 0.06	4	3
160.0–162.5 (63–64)	53.03 ± 10.27	0.33 ± 0.11	9	9	54.80 ± 15.18	0.33 ± 0.10	6	3
G[4] 162.6–165.0 (64–65)	47.75 ± 9.61	0.29 ± 0.06	14	5	56.77 ± 13.81	0.35 ± 0.08	10	3
165.1–167.5 (65–66)	49.01 ± 9.58	0.29 ± 0.06	15	3	48.69 ± 9.50	0.36 ± 0.11	7	7
167.6–170.1 (66–67)	49.06 ± 9.76	0.29 ± 0.06	11	4	52.33 ± 12.15	0.31 ± 0.05	3	6
170.2–172.6 (67–68)	41.19 ± 17.49	0.25 ± 0.11	4	2	64.99 ± 0.52	0.38 ± 0.01	1	1
172.7–175.2 (68–69)	48.31 ± 2.65	0.28 ± 0.02	3		53.88 ± 8.88	0.31 ± 0.05	3	1
175.3–177.7 (69–70)	60.51 ± 8.52	0.35 ± 0.10	2		61.96 ± 4.24	0.36 ± 0.03	3	1
177.8–180.3 (70–71)	43.19 ± 0.00	0.25 ± 0.00	1					
180.4–182.8 (71–72)	63.94 ± 0.00	0.36 ± 0.00	1					
Summary 164.4 (64.7)	53.37 ± 11.25	0.33 ± 0.08	72	31	56.59 ± 8.46	0.34 ± 0.06	40	28

[1] Height in inches in parentheses.
[2] Weight in pounds in parentheses.
[3] W — NRC height and weight for the average woman.
[4] G — NRC height and weight for the 16 to 20 yr. old girl.

TABLE 2 *(continued)*

The average 5-day protein intake of self-selected diets distributed according to age, height and weight

WEIGHT	AVERAGE PROTEIN INTAKE (Group I)				AVERAGE PROTEIN INTAKE (Group II)			
kg	*gm*	*gm/kg*	*Retention* (+)	(−)	*gm*	*gm/kg*	*Retention* (+)	(−)
40–45 (88–99)[2]					51.95 ± 9.13	1.06 ± 0.17	4	
45–50 (99–110)	55.86 ± 11.30	1.11 ± 0.20	6	1	46.30 ± 7.48	0.98 ± 0.18	6	2
G[4] 50–55 (110–121)	51.01 ± 12.64	0.95 ± 0.21	23	12	50.00 ± 14.26	0.97 ± 0.28	12	5
W[3] 55–60 (121–132)	50.47 ± 8.61	0.84 ± 0.17	18	3	59.51 ± 11.14	1.03 ± 0.20	6	4
60–65 (132–143)	49.49 ± 14.80	0.71 ± 0.24	15	9	48.50 ± 9.25	0.78 ± 0.16	7	7
65–70 (143–154)	51.26 ± 11.01	0.74 ± 0.17	4	2	55.32 ± 11.06	0.82 ± 0.16	3	5
70–75 (154–165)	52.56 ± 8.55	0.73 ± 0.16	3	1	55.71 ± 10.78	0.79 ± 0.15	2	2
75–80 (165–176)	54.59 ± 11.57	0.74 ± 0.18	1	2	64.42 ± 0.00	0.81 ± 0.00		1
80–85 (176–187)	61.56 ± 0.00	0.77 ± 0.00	1		59.90 ± 0.00	0.75 ± 0.00		1
85–90 (187–198)	50.84 ± 12.26	0.57 ± 0.14	1	1	72.81 ± 0.00	0.85 ± 0.00		1
58.7 (129.1)	54.18 ± 11.34	0.80 ± 0.18	72	31	56.44 ± 10.44	0.88 ± 0.19	40	28

[1] Height in inches in parentheses.
[2] Weight in pounds in parentheses.
[3] W — NRC height and weight for the average woman.
[4] G — NRC height and weight for the 16 to 20 yr. old girl.

be similar to that of the 89 studied (Davis and Scoular, '57) no negative balances due to low caloric intakes should result since the lowest determined value was 1534 Cal. However, the work of Leverton et al. ('51) implies that the young women of the present study who consume both low protein and low calories should select high-quality protein at each meal if the protein is to be well utilized.

When the average daily protein intakes are distributed according to age, weight and height, table 2, the very low and the very high protein ingestion levels of a few are no longer evident. As in the report of the caloric intake of 89 of these women (Davis and Scoular, '57) it was considered expedient to separate them into two groups, group I (under 20 years) and group II (over 20 years). Unlike the equal distribution of the subjects of the former study into these two age groupings, there are more in group I (103 or 60%). According to the NRC's daily recommended allowances group I should have 75 gm and group II 55 gm of protein/day, whereas the averages are 53.37 $\pm$ 11.25 and 54.18 $\pm$ 11.34 for group I and 56.59 $\pm$ 8.46 and 56.44 $\pm$ 10.44 for group II, based on height and weight, respectively. Seventy per cent of group I and 60% of group II were in positive balance on these intakes. Yet these young women are taller (average 164.4 cm) and heavier (average 58.7 kg) than either of NRC's averages (16 to 20 years, 162 cm and 54 kg; 25 years, 157 cm and 55 kg). Group I varied more in the average daily intake of protein (41.19 to 65.63 gm) as well as in height (147.3 to 182.8 cm) than group II. The latter's protein intake varied from 45.65 to 64.99 gm and their height from 152.4 to 177.7 cm. However, the average protein intake based on height is similar for the two age groups, 0.33 $\pm$ 0.08 and 0.34 $\pm$ 0.06 gm/cm for groups I and II, respectively. This is not true when the protein intakes are distributed according to body weight of the subjects. The greater daily variation in protein intake (46.30 to 72.81 gm) occurs in group II with group I varying from 49.49 to 61.56 gm/kg. The range in body weight is also slightly greater for group II, 40 to 90 kg as compared to 45 to 90 kg

for group I. Group I (the younger) consumed an average of 0.80 ± 0.18 gm of protein/kg in contrast to the 0.88 ± 0.19 gm/kg of group II. From the present study as in the previous one of caloric intakes (Davis and Scoular, '57) height rather than weight seems to give a more consistent means of evaluating the dietary practices of these young college women.

SUMMARY

The average daily protein intake of 171 young college women on self-selected diets ranged from 19 to 113 gm protein with an average of 52 gm/day.

The subjects absorbed from 65 to 98% of the ingested protein with an average absorption value of 85%.

Six of the young women consumed 75 or more grams of protein daily, 66 consumed from 55 to 75 gm and the remainder (102) less than 55 gm/day.

Sixty-six per cent (112) of the women were in positive protein balance on these intakes.

Group I (under 20 years) ingested an average of 0.80 ± 0.18 gm protein/kg while group II (over 20 years) consumed 0.88 ± 0.19 gm/kg of protein.

When the ingested protein was distributed according to the height of the subjects, the average protein intake was 0.33 ± 0.08 gm/cm for group I and 0.34 ± 0.06 gm/cm for group II.

LITERATURE CITED

ALLISON, J. B. 1951 Interpretation of nitrogen balance data. Fed. Proc., *10:* 676–683.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official and tentative methods of analysis. The Association, Washington, D. C., p. 12.

COONS, C. M. AND A. T. SCHIEFELBUSCH 1932 The diets of college women in relation to their basal metabolism. J. Nutrition, *5:* 459–465.

DAVIS, A. N., AND F. I. SCOULAR 1957 The energy value of self-selected diets consumed by college women. Ibid., *61:*

HEGSTED, D. M., A. G. TSONGAS, D. B. ABBOTT AND F. J. STARE 1946 Protein requirement of adults. J. Lab. Clin. Med., *31:* 261–266.

HOLT, F., AND F. I. SCOULAR 1948 Iron and copper metabolism of young women on self-selected diets. J. Nutrition, *35:* 717–723.

KRAMER, M. M., H. F. EVERS, M. G. FLETCHER AND D. I. GALLEMORE 1934 Protein, calcium and phosphorus intakes of college women as indicated by nitrogen, calcium and phosphorus outputs. Ibid., *7:* 89–96.

LEVERTON, R. M., M. R. GRAM AND M. CHALOUPKA 1951 Effect of the time factor and calorie level on nitrogen utilization of young women. Ibid., *44:* 537–546.

MCKAY, H., H. B. PATTON, M. A. OHLSON, M. S. PITTMAN, R. M. LEVERTON, A. G. MARSH, G. STEARNS AND G. COX 1942 Calcium, phosphorus and nitrogen metabolism of young college women. Ibid., *24:* 367–384.

MERTZ, E. T., E. J. BAXTER, L. E. JACKSON, C. E. RODERUCK AND A. WEIS 1952 Essential amino acids in self-selected diets of older women. Ibid., *46:* 313–322.

NATIONAL RESEARCH COUNCIL 1953 Recommended dietary allowances. National Academy of Sciences, Washington, D. C., p. 22.

OHLSON, M. A., W. D. BREWER, L. JACKSON, P. P. SWANSON, P. H. ROBERTS, M. MANGEL, R. M. LEVERTON, M. CHALOUPKA, M. R. GRAM, M. S. REYNOLDS, R. LUTZ AND E. L. BATCHELDER 1952 Intake and retention of nitrogen, calcium and phosphorus by 136 women between the ages of 30–85 years. Fed. Proc., *11:* 775.

REYNOLDS, M. S., M. F. FUTRELL AND C. A. BAUMANN 1953 Nitrogen balance and amino acid content of self-selected diets of women. J. Nutrition, *29:* 359–364.

GROWTH RESPONSE AND LIVER FAT DEPOSITION IN RATS FED BREAD MIXTURES WITH VARYING LEVELS OF NON-FAT MILK SOLIDS AND LYSINE

JANE K. JAHNKE AND CECILIA SCHUCK
Department of Foods and Nutrition, Purdue University, Lafayette, Indiana

(Received for publication July 30, 1956)

It is well known that the cereal grains are deficient in the amino acid, lysine. The addition of lysine to low protein rations with a cereal as the sole or principal source of protein has been shown to improve the growth rate of young experimental rats (Osborne and Mendel, '14; Harris, et al., '43; Pecora and Hundley, '51; Sure, '52; Rosenberg and Rohdenburg, '52). The above observation is largely responsible for the suggestion that lysine be added to bread formulas as an adjunct to the present enrichment ingredients.

It would seem that the addition of lysine to cereal products might prove to be of nutritional benefit in areas of the world where the protein supply is limited and sources of high quality protein are unavailable in adequate quantities. Although this is not the situation for the population as a whole in this country (Phillips, '51; Block and Bolling, '51; Mertz, et al., '51), some individuals may not have an adequate intake of high quality protein. This may be especially true of low income groups whose calorie needs are largely met by cereal products. Fortification of bread with lysine might be expected to benefit these groups.

The use of non-fat milk solids in commercial bread formulas has increased the lysine content of bread, but for a loaf of

acceptable quality only a limited amount can be used. Approximately three to 6 parts of non-fat milk solids per 100 parts of flour have been used. When bread containing three parts non-fat milk solids was supplemented with varying levels of lysine, Rosenberg and Rohdenburg ('52) found that the growth of rats was markedly improved with each increment of lysine used. As a result of this study it was suggested that bread be fortified with 0.25 parts lysine per 100 parts of flour.

The present study was undertaken for the purpose of observing certain physiological responses of young rats fed bread mixtures containing varying levels of non-fat milk solids with and without lysine supplementation.

Some investigators have found that at certain high levels of lysine supplementation growth rate is depressed (Pecora and Hundley, '51; Russell et al., '52; Miner et al., '55). This may be related to secondary deficiencies of other amino acids created by the administration of an excess of a single amino acid (Mitchell and Smuts, '32; Pecora and Hundley, '51; Sure, '52; Harper et al., '55). Furthermore an imbalance of amino acids has been implicated in the deposition of excess liver fat (Litwack et al., '52; Harper et al., '53; Winje et al., '54; Elvehjem, '56). Our present background of information on amino acid supplementation suggests a need for further investigation in this field before advocating the addition of lysine to bread or other cereal products.

EXPERIMENTAL

Three series of weanling albino rats consisting of 28 rats each were observed for 4-week periods. The rats were of the Wistar Strain from the Purdue University colony with a similar genetic and nutritional background. The 28 rats of each series were divided into 7 groups of 4 rats each, two males and two females. One group was placed on what was considered an optimal diet and the other 6 groups were placed on experimental diets consisting of bread formulas

varied with respect to non-fat milk solids and lysine supplementation.

The optimal diet contained 20% protein in the form of casein and was assumed to be liberal in all other required nutrients.

The general formula for the 6 experimental diets is given in table 1. The formula with three parts of non-fat milk solids corresponds to a commonly used commercial bread formula with brewers' yeast substituted for compressed yeast. The amount of brewers' yeast used was estimated to contain the same amount of protein as 2.5 gm of compressed yeast which is the level of the latter per 100 gm flour in commercial bread formulas.

TABLE 1

General composition of the experimental diets

INGREDIENT	AMOUNT	INGREDIENT	AMOUNT
	gm		*gm*
Flour	100	NaCl	2
Sucrose	6	Non-fat milk solids	3, 6, 12
Hydrogenated fat [1]	3	Salt mixture [2]	3
Brewers' yeast	0.75	Cod liver oil	3
		Vitamin concentrates	0.16

[1] Crisco.

[2] Wesson, L. G., Science, *75:* 339 ('32).

The salt mixture (Wesson modification of O. and M.), the cod liver oil and vitamin concentrate mixture were added in order to insure a diet that was adequate in all respects with the exception of total protein and the amino acid, lysine. The vitamin mixture consisted of the following in milligrams: thiamine hydrochloride, 0.4; pyridoxine hydrochloride, 0.4; nicotinic acid, 5.0; riboflavin, 0.7; calcium pantothenate, 3.0; folic acid, 0.2; menadione, 0.5; inositol, 15.0; *p*-aminobenzoic acid, 10.0; ascorbic acid, 10.0; vitamin B_{12}, .003; biotin, 0.2; α-tocopherol, 10.0; choline chloride, 100.0.

The flour used was all-purpose enriched flour. The non-fat milk solids which were prepared by a spray process were similar to the product supplied to the baking industry.

The variations among the 6 experimental diets are shown in table 2.

The nitrogen content of the 6 diets was determined by the macro-Kjeldahl method and converted into protein using the factor 6.25. The total lysine and tryptophan content of the diets and the lysine to tryptophan ratios were calculated from data published by Block and Bolling ('51). The importance of the ratio of the two amino acids has been emphasized by some of the investigators.

At the beginning of the experiment with each series an effort was made to distribute litters as evenly as feasible among the diets. Also weights were as evenly distributed as

TABLE 2

Non fat milk solids and lysine content of experimental diets
(Grams per 100 gm flour)

DIETS	NFMS	ADDED LYSINE
1	3	...
2	6	...
3	12	...
4	3	0.25
5	6	0.25
6	12	0.25

possible. Growth increments and food consumption were recorded weekly.

At the end of the experimental period the rats were sacrificed and the livers were prepared for fat analysis. They were macerated and dried under a fan at room temperature for 24 hours. Thereafter they were left in the open air for 4 days to dry more thoroughly. The dried livers were then ground in a mortar to a fine powder and stored in the refrigerator in corked glass vials for subsequent analysis. Moisture determinations were made on 10 samples selected at random from the three series of animals. Since strict uniformity was observed in drying procedures and the standard deviation of the mean moisture content was found to be only $\pm$ 0.33% for these samples, it seemed reasonable to assume

a similar moisture content for all of the liver powders. The fat was extracted from approximately 0.5 gm samples with ethyl ether for 4 hours using a battery of Goldfisch extractors. The ether was evaporated with steam and the extraction beakers placed in a vacuum oven for three hours at 50°C. after which they were placed in desiccators and allowed to return to room temperature before weighing.

RESULTS AND DISCUSSION

The protein content of the experimental diets based on nitrogen analyses and the calculated lysine and tryptophan content are given in table 3. Table 4 compares the mean

TABLE 3

Protein, lysine and tryptophan content of experimental diets

DIETS	PROTEIN	LYSINE	TRYPTOPHAN
	%	%	%
1	10.78	0.241	0.076
2	11.55	0.311	0.081
3	12.76	0.493	0.089
4	11.36	0.449	0.076
5	12.01	0.512	0.081
6	13.28	0.632	0.089
7	23.59	1.908	0.288

weight increments and the mean food and nitrogen efficiency with the lysine content and the lysine to tryptophan ratio of the 6 experimental diets. The results of the liver fat analyses are recorded in table 5. The optimal diet (VII) has been included for reference in observing the effects of the experimental diets on liver fat deposition.

With the level of non-fat milk solids used in the bread formulas (3, 6 and 12 parts per 100 parts flour) each increase resulted in improved growth. The addition of 0.25 parts of lysine to the formulas with the above levels of non-fat milk solids stimulated growth in every case. This suggests that even with 12 parts of non-fat milk solids the need for lysine was not met. Applying the Newman-Keuls (Duncan, '55)

sequential range test the differences in mean weight gains on diets I to VI inclusive were found to be statistically significant with the exception of diets III (12 parts non-fat milk solids) and IV (3 parts non-fat milk solids plus 0.25 parts lysine). The rats on diet IV did not show a statistically significant higher mean weight gain than the rats on diet III.

Reference to table 4 shows a direct relationship between the order of growth response and the total lysine content of the diet. Growth rate was observed to increase with increased

TABLE 4

Mean weight gains compared with lysine content and lysine/tryptophan ratio of the experimental diets[1]

DIET	LYSINE	L/T	WEIGHT GAIN		
	%		*Total*[2] *gm*	*gm/100 gm food intake*	*gm/gm nitrogen*[3] *intake*
I	0.241	3.17	22	11.23	6.88
II	0.311	3.88	33	17.93	9.88
III	0.493	5.44	68	27.35	13.14
IV	0.449	5.91	66	26.14	14.40
V	0.512	6.33	89	30.41	15.85
VI	0.632	7.10	122	35.83	16.90

[1] Twelve rats in each group (4 from each of the three series).
[2] Standard error = ± 3.55.
[3] Standard error = ± 0.5356.

lysine content of the diet. However, as previously noted, the difference in growth rate between diets III and IV was not statistically significant. The rate of growth likewise appeared to be directly related to the lysine and tryptophan ratio with the exception of diets III and IV. An approximately 5:1 lysine/tryptophan ratio has been considered as desirable for the rat.

Data recorded in table 4 indicate that food efficiency was increased with increased lysine content of the diet. The same relationship was observed between the lysine content of the diet and nitrogen efficiency with the exception of diets III and IV. The Newman-Keuls sequential range test showed that

the nitrogen efficiency of diets III and IV did not differ significantly. Also diets IV and V and diets V and VI did not differ significantly in nitrogen efficiency.

The mean liver fat content of the rats on the optimal diet (VII) was significantly lower than that of rats on the 6 experimental diets with the exception of diet III (Newman-Keuls test). Although the mean liver fat in the rats on diet III was less than that of rats on the other experimental diets, the difference was not statistically significant. The rate of growth did not appear to exert a consistent influence on

TABLE 5

Mean weekly gain and mean percentage liver fat

DIET	GAIN/WK.	LIVER FAT [1]	
		Dry wt. basis	Wet wt. basis
	gm	%	%
I	5.5	12.5	3.4
II	8.2	12.8	3.6
III	17.0	11.1	3.1
IV	16.5	14.5	4.3
V	22.2	13.6	3.9
VI	30.5	12.3	3.7
VII	38.7	8.6	2.5

[1] Diets I–VII — Standard error = ± 0.904.
Diet VII — Standard error = ± 0.994.

liver fat deposition. This is in agreement with the observations of Harper et al. ('53).

If the liver fat content of rats on the optimal diet is considered normal for the rats observed in this study, the elevation of liver fat levels on the experimental diets does not appear to be large. The low level of protein in the diets (approximately 11 to 13%) may have been a contributing factor in liver fat deposition. Also it is possible that the increased amount of liver fat present in the rats on the experimental diets was a temporary condition. This is suggested by the observation of Singal et al. ('53).

It is hoped that the results of this study may contribute information helpful in judging the desirability of fortifying bread and other cereal products with lysine.

SUMMARY

The growth response and liver fat deposition of rats fed diets consisting of bread mixtures containing three levels of non-fat milk solids (3, 6 and 12 parts per 100 parts flour) and these same levels of milk solids plus 0.25 parts of lysine have been reported. Twelve rats were placed on each of the diets numbering I to VI.

Improved growth resulted with increasing milk levels. The addition of lysine stimulated growth at each milk level. The difference in mean growth rate was statistically significant for all diets except III (12 parts non-fat milk solids) and IV (3 parts non-fat milk solids + lysine).

Food efficiency was increased with increased total lysine content of the diet. Nitrogen efficiency was likewise increased with the exception of diets III and IV which did not differ significantly with respect to their effect on nitrogen efficiency.

A comparison of the mean liver fat content of the rats on the 6 experimental diets with that of rats on what was considered an optimal diet showed the latter to be significantly lower than the mean liver fat content of rats on all experimental diets except diet III. The elevation of liver fat levels was small and there was no statistically significant difference among the experimental diets.

In this study 3 parts of non-fat milk solids per 100 parts flour plus 0.25 parts lysine gave results comparable in beneficial effects with 12 parts non-fat milk solids in bread mixtures. Since the latter is not likely to be used by commercial bakers because of cost and unacceptability of the resulting product, it would seem that the former might be given consideration for raising the lysine content of diets where this appears to be desirable. Further studies in this field are to be recommended, however.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. W. B. Bradley, Scientific and Research Director of the American Institute of Baking for information on commercial bread formulas; to Dr. B. W. Fairbanks, Director Dry Milk Institute, Inc., for the non-fat dry milk solids used in the study; to Dr. F. W. Quackenbush, Head, Department of Biochemistry, Purdue University, for advice on liver fat analysis; to Mrs. Judith Davey for technical assistance with the fat analyses; and to Dr. V. L. Anderson for the statistical treatment of the data.

LITERATURE CITED

BLOCK, R. J., AND D. BOLLING 1951 Amino Acid Composition of Proteins and Foods. Charles C Thomas, Springfield, Ill.

DUNCAN, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 26.

ELVEHJEM, C. A. 1956 Amino acid balance in nutrition. J. Am. Dietet. Assn., *32:* 305.

HARPER, A. E., D. A. BENTON AND C. A. ELVEHJEM 1955 L-leucine, an isoleucine antagonist in rats. Arch. Biochem. Biophys., *57:* 1.

HARPER, A. E., W. J. MONSON, D. A. BENTON AND C. A. ELVEHJEM 1953 The influence of protein and certain amino acids, particularly threonine, on the deposition of fat in the liver of the rat. J. Nutrition, *50:* 383.

HARRIS, H. A., A. NEWBERGER AND F. SANGER 1943 Lysine deficiency in young rats. Biochem. J., *37:* 508.

LITWACK, J. L., L. V. HANKES AND C. A. ELVEHJEM 1952 Effect of factors other than choline on liver fat deposition. Proc. Soc. Exp. Biol. Med., *81:* 441.

MERTZ, E. T., E. J. BAXTER, L. E. JACKSON, C. E. RODERUCK AND A. WEIS 1951 Essential amino acids in self-selected diets of older women. J. Nutrition, *46:* 313.

MINER, J. J., W. B. CLOWER, P. R. NOLAND AND E. L. STEVENSON 1955 Amino acid supplementation of corn-cottonseed meal diet for growing and fattening swine. J. Animal Sci., *14:* 24.

MITCHELL, H. H., AND D. B. SMUTS 1932 Amino acid deficiencies of beef, wheat, corn, oats and soybean for growth in white rats. J. Biol. Chem., *95:* 363.

OSBORNE, T. B., AND L. B. MENDEL 1914 Amino acids in nutrition and growth. J. Biol. chem., *17:* 325.

PECORA, L. J., AND J. M. HUNDLEY 1951 Nutritional improvement of white polished rice by addition of lysine and threonine. J. Nutrition, *44:* 101.

PHILLIPS, R. W. 1951 Expansion of live stock production in relation to human needs. Nutr. Abst. Revs., *21:* 241.

ROSENBERG, H. R., AND E. L. ROHDENBURG 1952 Fortification of bread with lysine. III. Nutritional value of fortified bread. Arch. Biochem. Biophys., *37:* 461.

RUSSELL, W. C., M. W. TAYLOR AND J. M. HOGAN 1952 Effect of excess amino acids on growth of white rat. Ibid., *39:* 249.

SINGAL, S. A., S. J. HOGAN, V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1953 Production of fatty livers in rats on threonine and lysine-deficient rations. J. Biol. Chem., *200:* 867.

SURE, B. 1952 Influence of lysine, valine and threonine additions on efficiency of proteins of whole wheat. Arch. Biochem. Biophys., *39:* 463.

WINJE, M. E., A. E. HARPER, D. A. BENTON, R. E. BOLDT AND C. A. ELVEHJEM 1954 Effect of dietary amino acid balance on fat deposition in livers of rats fed low protein diets. J. Nutrition, *54:* 155.